Interaction of Hsp70 chaperones with substrates

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Determination of the structure of the substrate binding domain of the *Escherichia coli* Hsp70 chaperone, DnaK, and the biochemical characterisation of the motif it recognizes within substrates provide insights into the principles governing Hsp70 interaction with polypeptide chains. DnaK recognizes extended peptide strands composed of up to five consecutive hydrophobic residues within and positively charged residues outside the substrate binding cavity.

Many *in vivo* protein folding processes are not spontaneous, rather they require the assistance of so-called molecular chaperones[1–3]. All cells contain members of distinct families of evolutionary conserved chaperones that share the ability to transiently associate with folding substrates. The activities of chaperones include the prevention of protein aggregation, the refolding of kinetically trapped intermediates, the translocation of proteins across membranes, and the assembly and disassembly of protein complexes. Members of the different chaperone families can cooperate in protein folding reactions[4–7], suggesting that assisted folding processes *in vivo* are promoted by a rather flexible network of chaperones[7,8].

A major component of this network is the Hsp70 chaperone system, which is present in multiple compartments of most cells[3,9] except specific archaebacteria[10] (K. Willison and A. Horwich, personal communication). The prokaryotic Hsp70 system is composed of the DnaK chaperone (Hsp70 homologue) and the DnaJ (Hsp40 homologue) and GrpE co-chaperones, and that of eukaryotes is composed of Hsp70, the co-chaperones Hsp40 and HiP and perhaps additional factors[2,3,11]. The number of Hsp70 family members expressed in eukaryotic cells is high, for example, 14 in *Saccharomyces cerevisiae*[12], which reflects the diversity of cellular functions of this system.

Hsp70 chaperones are involved in: (i) the import of proteins into cellular compartments including mitochondria, the endoplasmic reticulum and the *Escherichia coli* periplasm[13]; (ii) the folding of proteins in mitochondria and of subsets of proteins in the endoplasmic reticulum and the cytosol[1,2,8,13]; (iii) the disassembly of protein complexes such as the nucleoprotein complex which initiates replication of bacteriophage λ DNA[14] and the clathrin baskets of clathrin coated vesicles[15]; (iv) the degradation of unstable proteins and the bacterial heat shock transcription factor σ^{32} (refs 16–18); and (v) the control of the activity of regulatory proteins such as heat shock transcription factors and the initiator protein of plasmid replication, RepA[19–22]. In addition, Hsp70 chaperones assist in the refolding of misfolded proteins[23,24], a function that is of particular importance for the metabolism of cells subjected to stress, such as heat shock. This function probably accounts for the fact that the synthesis of many, though not all, Hsp70 proteins is induced by stress[19,25].

Hsp70 proteins transiently associate with short linear peptide segments of folding intermediates[26,27]. Such an association can prevent aggregation of substrates, by shielding exposed hydrophobic patches, and assist refolding, probably by decreasing the concentration of free, aggregation-prone folding intermediates. The affinity of Hsp70 for substrates is tightly regulated by ATP[26] and by co-chaperones which control the key points of the ATPase cycle[28–33]. Furthermore, the DnaJ (Hsp40) co-chaperones themselves associate with protein substrates and sites of potential Hsp70 action (for example, protein translocation sites and clathrin coated vesicles) and, through high affinity interaction with Hsp70, target Hsp70 chaperones to substrates[22,23].

The substrate recognition properties of Hsp70 proteins, together with those of other chaperone systems, thus provide a cellular definition for native and non-native protein conformations, the molecular basis of which is now being elucidated for DnaK. This review discusses the emerging principles of Hsp70–substrate interactions and summarizes current knowledge of the still elusive mechanism of coupling of these interactions with the nucleotide status of the chaperone.

Substrate binding domain

Hsp70 proteins consists of an N-terminal ATPase domain of 45,000 M_r and a C-terminal domain which is further divided into a substrate binding subdomain of ~15,000 M_r[34] and a C-terminal subdomain of 10,000 M_r with unknown function (Fig. 1). High resolution structural information on Hsp70 proteins exists for the ATPase domain of bovine brain cognate (Hsc70)[35], the ATPase domain of DnaK in complex with its dimeric cofactor GrpE[74] and Hendrickson and coworkers have recently succeeded in the crystallization and structure determination of an internal fragment of DnaK with substrate binding activity (Val 389–Ala 607) at a resolution of 2.0 Å (Fig. 2)[36]. The notorious

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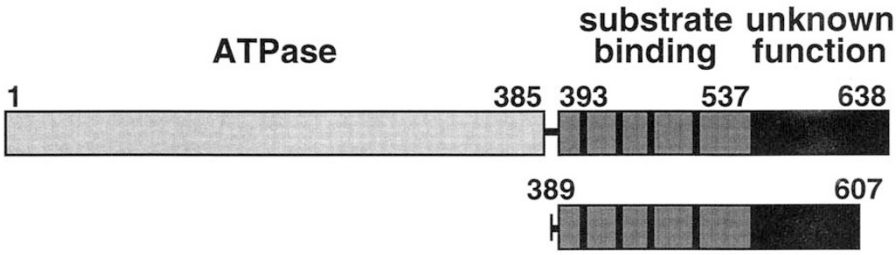

Fig. 1 Domain structure of DnaK. DnaK (top) and the crystallized DnaK fragment (bottom) are depicted as a three-domain protein with assigned functions. Filled bars indicate the $L_{1,2}$, $L_{3,4}$, $L_{4,5}$, $L_{5,6}$ loops. Numbers indicate first or last residues of the domain boundaries and the DnaK fragment. Val 386–Leu 392 constitutes the linker between the ATPase and the substrate binding domain.

oligomerization behaviour of this fragment was overcome by addition of a peptide substrate (NRLLLTG) that binds to the substrate binding pocket of DnaK with an equilibrium dissociation constant (K_d) of 11 μM[37] thereby competing out the oligomerization reaction.

The Val 389–Ala 607 fragment forms a rather flat structure composed of two subdomains connected by a helical linking region (Fig. 2). The N-terminal segment (Asp 393–Lys 502) forms a compact β-sandwich composed of eight antiparallel β-strands. The four lower strands have a regular twist commonly seen in β-sheets and are connected by short loops. The four upper strands form an unusual β-sheet with the β1 and β2 strands tilted upward by 20° relative to the neighbouring β4 and β5 strands. The upper strands are connected by two outer ($L_{3,4}$; $L_{5,6}$) and two inner ($L_{1,2}$; $L_{4,5}$) loops protruding upwards from the β-sandwich. The N-terminal segment is followed by α-helices A–E (Glu 509–Gln 605). Helices A and B are packed onto the β-sandwich through hydrophobic and ionic interactions, stabilizing the β-sandwich and contributing to substrate binding. They form a linking region to the C-terminal helices C–E which, together with the C-terminal end of helix B, constitute a separate subdomain (Gln 538–Gln 605) with unknown function.

Substrate binding cavity

The peptide NRLLLTG is bound to a hydrophobic cavity formed by the $L_{3,4}$ and $L_{1,2}$ loops of the upper part of the β-sandwich (Figs 2, 3). This cavity is stabilized by interactions with the $L_{4,5}$ and $L_{5,6}$ loops and helix B. This helix acts as a lid for the substrate binding cavity by positioning the outer $L_{3,4}$ loop through ionic interactions and thus entirely enclosing the cavity (Fig. 2*c*). The bound peptide is in an extended conformation, in pleasing congruence with the conclusions of a previous NMR study, using a different peptide[38]. Extended conformations are probably the rule for peptide segments associated with DnaK given the structural constraints of the cavity.

Several types of interactions are involved in binding of the peptide to DnaK (Fig. 3). First, hydrogen bonds between the peptide backbone and the cavity-forming loops (four bonds to $L_{3,4}$; one to $L_{1,2}$) mediate recognition of the extended peptide conformation. Second, van der Waals interactions of peptide side chains with both loops as well as several β-strands confer amino-acid sequence specificity to the DnaK–peptide interaction (Fig. 3). Hydrophobic contacts exist to the five central residues of the peptide and are strongest for Leu_4 which points down into a deep hydrophobic pocket (Fig. 3*c*). This central position within the substrate binding cavity of DnaK is defined as 'position 0'. Zhu *et al.* predict that this pocket, while being tailored to bind Leu, would also bind Ile and Met. Smaller residues may also bind but would pay an enthalpic penalty for not filling the pocket and Phe, being slightly too large, would only fit with an adjustment of the pocket. Tyr and Trp appear to be too bulky for binding and charged residues are unlikely to be tolerated.

It should be emphasized that contacts within the entire cavity, and not just within position 0, are involved in peptide binding to DnaK. This is supported by the finding that single amino acids are poor substrates for DnaK[39]. Positions +1 and –1 have a hydrophobic character with a negative potential at further distance from peptide backbone. Leu_3 (–1) and Leu_5 (+1) are bound with their side chains orientated upwards (Fig. 3*b*) forming van der Waals contacts with Met 404 and Ala 429 and, for Leu_3, Ser 427. At positions +2 and –2, Arg_2 and Thr_6 make van der Waals contacts to four and two residues of DnaK respectively (Fig. 3). At positions +1, +2 and –2 the peptide side chains are only partly buried and, compared to positions 0 and –1, have fewer contacts to the chaperone, making it difficult to predict the substrate side-chain specificities of these positions. Furthermore, it can not be excluded that the binding of anions in the cavity, such as phosphate and sulphate may change the specificity determinants of some positions.

A third contribution to peptide binding is provided by an arch formed by residues Met 404 and Ala 429 of the substrate binding loops (Fig. 3*a*,*b*). The arch completely encloses the peptide backbone at Leu_4, without contacting it, and thereby may provide a physical barrier to peptide dissociation. The flexibility of the arch-forming Met 404 side chain is restricted by van der Waals contacts to Leu_3 and Leu_5 which may provide additional entropic contributions to peptide binding. It will be interesting to see whether other peptides lacking Leu in positions –1 and +1 also lead to arch formation in DnaK.

Fourth, Zhu *et al.* note that the protein surface outside the binding cavity has a negative electrostatic potential which may influence substrate association in a less specific fashion. This is now supported experimentally[43].

Substrate binding motif

Two approaches have been used to identify the motif in substrates recognized by DnaK. In the first approach, phage display libraries were panned to select peptides with affinity for DnaK[41]. This study identified 44 hexameric sequences that mediate DnaK binding to the phages, including the sequence of the peptide used for crystallization of the DnaK substrate-binding domain. A comparison with 48 random sequences displayed on phages revealed an enrichment of Leu, Ile and Lys and a disfavouring of Phe, Glu and His. The number of binding peptides identified in this study was, however, too small to deduce the substrate recognition principles of DnaK.

In a recent study a library of 4360 cellulose-bound 13-mer peptides[42] representing 37 complete sequences of proteins was screened for DnaK binding[43]. The peptides overlapped by 10 residues and thus presented all potential binding sites to DnaK. This approach avoids precipitation of hydrophobic peptides and allowed identification of DnaK binding sites within protein sequences and, by sequence alignment of neighbouring binding peptides, the recognition motif. The binding motif is composed of a hydrophobic core of 4–5 residues, consistent with the size of

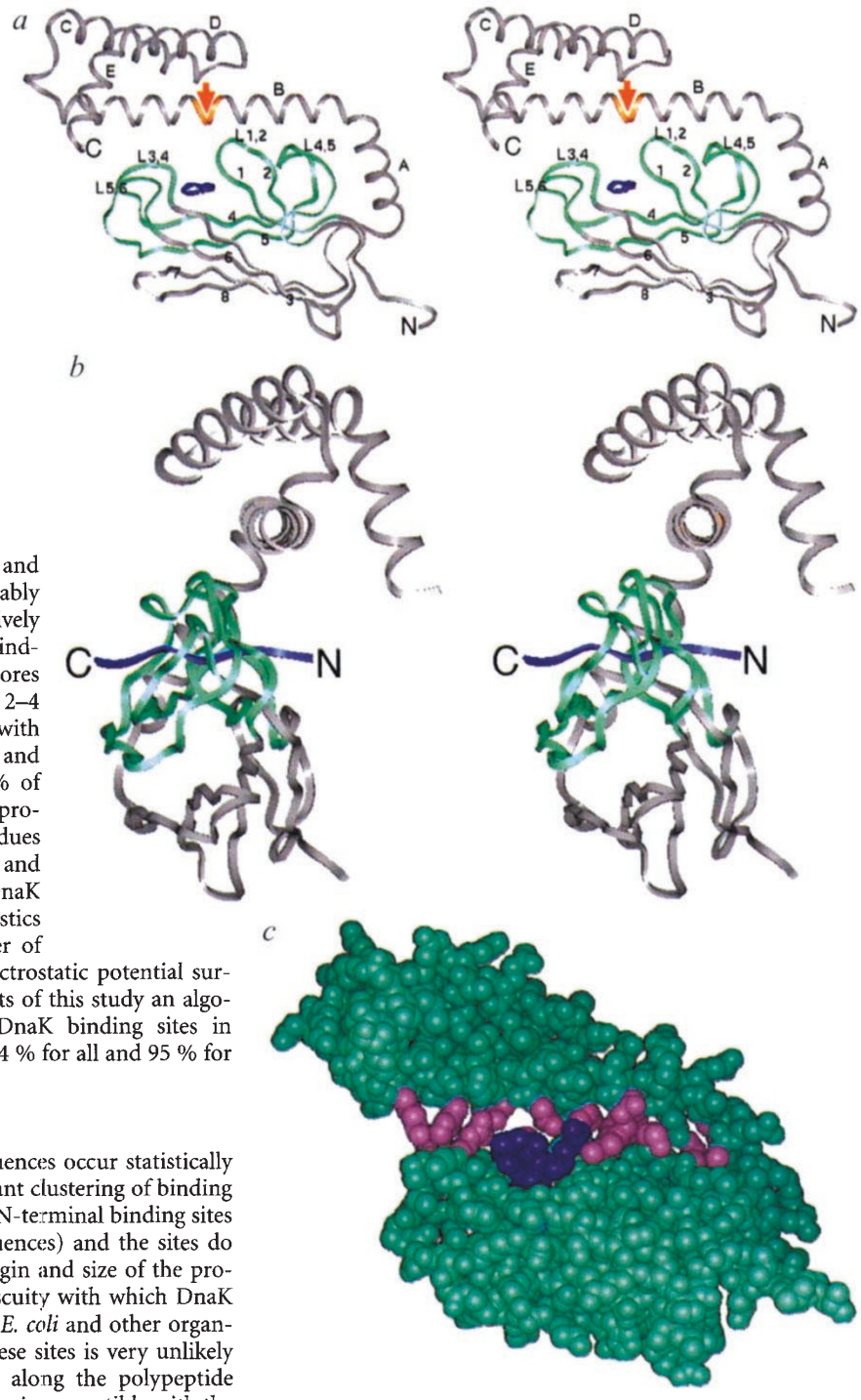

Fig. 2 Structure of the substrate binding domain of DnaK in complex with a peptide substrate. *a*, Ribbon diagram (in stereo) of the standard view of DnaK substrate binding domain and *b*, a view rotated by 90° counterclockwise around the vertical axis of the standard view. The peptide substrate is shown in blue, the strands (β1, β2, β4, β5) and loops ($L_{1,2}$, $L_{3,4}$, $L_{4,5}$, $L_{5,6}$) of DnaK constituting the upper β-sheet are shown in green, the hinge-forming residues Arg 536 to Gln 538 (arrow) are shown in orange. Helices, strands and loops are labelled, N and C indicate the N and C termini of, in (*a*), the DnaK fragment and, in (*b*), the substrate peptide. *c*, The buried nature of the bound peptide substrate is shown in the space-filling representation of the standard view. The peptide is shown in blue, DnaK in green, side chains of residues which contribute to the interaction of helix B with $L_{1,2}$, $L_{3,4}$, $L_{4,5}$, $L_{5,6}$ are pink. All representations were done using INSIGHT II, Biosym.

the substrate binding cavity of DnaK, and two flanking basic segments which probably interact electrostatically with the negatively charged surroundings of the substrate binding pocket (Fig. 4*a*). The hydrophobic cores of individual binding sites consist of 2–4 hydrophobic residues in most cases, with Leu being by far the most abundant and enriched residue (Fig. 4*b*). Nearly 90% of good DnaK binding sites identified in protein sequences contain Leu. Acidic residues are excluded from hydrophobic cores and disfavoured in flanking regions of DnaK binding sites (Fig. 4). These characteristics fit well with the hydrophobic character of the binding cavity and the negative electrostatic potential surrounding the cavity. Based on the results of this study an algorithm was established that predicts DnaK binding sites in natural sequences with an accuracy of 84 % for all and 95 % for good sites investigated[43].

Chaperone activities

DnaK binding sites within protein sequences occur statistically every 36 residues[43]. There is no significant clustering of binding sites within polypeptide chains (except N-terminal binding sites constituted by hydrophobic signal sequences) and the sites do not show specificity with respect to origin and size of the protein. This is consistent with the promiscuity with which DnaK associates with protein substrates from *E. coli* and other organisms. The association of DnaK with these sites is very unlikely to involve a sliding of the chaperone along the polypeptide chain: such a sliding movement would be incompatible with the architecture of the substrate binding cavity of DnaK.

The hydrophobic nature of the core of DnaK binding sites identified in protein sequences suggests that these sites are typically located in the interior of folded proteins, in agreement with earlier proposals for eukaryotic Hsp70s[27,44]. Most binding sites are indeed completely buried within the folded structures of the proteins tested[43]. The association of DnaK with these proteins thus requires a substantial, local or complete, unfolding of the polypeptide chain. Not only the hydrophobic side chains of a binding site but also the corresponding peptide backbone needs to be separated from the remainder of the protein by at least 10 Å (Fig. 2*c*). Such sites may be exposed *in vivo* upon protein misfolding, for example, induced by heat treatment[23,24], or during the folding and targeting of newly synthesized proteins[2,8]. Some proteins, for example, the regulatory σ^{32} and RepA proteins and λ P, bind to DnaK in their folded, and possibly native states[22,40, 45–47]. The structural features of these substrates that allow association with DnaK are presently unknown.

To some extent, DnaK distinguishes secondary structure elements by recognizing primary amino-acid sequence. Screening of conformationally unrestricted 13-mer peptides for DnaK binding revealed that good binding sites preferentially localize to

Table 1 Conservation of DnaK residues contributing to van der Waals interactions with the substrate peptide NRLLLTG.

DnaK residue	van der Waals contacts to DnaK position[2]	Degree of conservation[1] Identical (%)	Conserved (%)	Conservation exchanges	Other exchanges[3]
Ile 401	0	63	100	Leu, Val	
Thr 403	0,–2	95	95		
Met 404[4]	–1, +1	6	41	Ile, Val, Leu	51% Ala
Val 407	–2	87	98	Ile, Leu, Met	
Thr 409	–2	91	93	Ser	
Ala 429[4]	–1, +1	36	36		57% Thr
Gln 433	+2	100	100		
Ala 435	+2	13	13		51% Gly
Val 436	0	99	100	Ile, Met	
Ile 438	0	91	97	Val	
Ile 472	-	93	100	Leu, Val	

[1]Based on 209 entries founds in the Swiss prot database.
[2]Data taken from ref. 36
[3]Only the frequent exchanges are shown.
[4]The arch forming residues are shaded.

the β-strands of the corresponding folded proteins[43]. The strict disfavouring of the β-strand breaking residues Glu and Asp in DnaK binding sites may contribute to this preference. Furthermore, the hydrophobic core motif of the binding sites has a preference for stretches of consecutive hydrophobic residues which are atypical for amphiphilic helices. DnaK also binds to peptide segments which can adopt helical conformations[43] (J.S. McCarty and B.B., unpublished), but they are anticipated to be extended when associated with DnaK.

It is unclear whether the binding of sequences found preferentially in β-strands is a prerequisite for DnaK's activity as chaperone. β-sheets are stabilized through interactions between β-strands and a disruption of these interactions during protein misfolding frequently generates structurally unstable strands prone to aggregation. DnaK may thus be tailored to assist β-strands with their specific folding problems. It is tempting to speculate that the association of DnaK with specific β-strands of protein subunits provides a mechanism to control oligomerization reactions. In this context it is interesting to note that DnaK binding sites rarely occur in helical oligomerization regions of proteins.

Evolutionary conservation of substrate recognition

Key features of the hydrophobic substrate binding cavity of DnaK are conserved among Hsp70 proteins (Table 1). In particular, position 0 is highly conserved and only in few cases subject to conservative changes[36]. The arch-forming Met 404 and Ala 429 of DnaK are also conserved among prokaryotic, and some eukaryotic Hsp70 homologues (including most BiP proteins). In most eukaryotic Hsp70's, however, the architecture of the arch is inverted by a replacement of Met 404 with Ala and of Ala 429 with Tyr. It cannot be excluded that this switch alters the substrate recognition at positions –1 and +1 since the arch-forming residues of DnaK contribute most of the van der Waals interactions with the substrate at these positions. In addition, substrate recognition differences between Hsp70 members may also be caused by variations in the surface charge distribution surrounding the substrate binding cavity[36]. While the surface potential is negative on both sides outside the DnaK substrate binding cavity, for BiP the potential is slightly positive on one side. This difference may account, to some extent, for differences in substrate binding by BiP compared to DnaK.

To determine the binding specificity of BiP, Rothman and coworkers analyzed randomized soluble peptide mixtures that bound to this chaperone[27] using bovine BiP. Sequencing of a pool of heptameric peptides isolated in complex with BiP revealed that bulky aliphatic hydrophobic amino acids were enriched in the centre of the peptides, and Gln and Arg were enriched at the N- and C-terminal ends respectively[27]. This pattern of amino acids is in agreement with the nature of the substrate binding site of DnaK[36] and its recognition motif[43]. However, the determined minimal peptide length of seven residues required for high affinity binding to BiP[27] exceeds the conserved size of the hydrophobic substrate binding cavity of DnaK by two residues. This discrepancy is probably due to a solubility requirement for the peptides used in this experiment which counterselects for highly hydrophobic short peptides.

To determine individual BiP binding sequences and the binding motif, Gething and coworkers[44] employed a phage panning approach using recombinant murine BiP. In BiP-binding octameric sequences Trp, Leu and Phe were enriched while Ala, Glu and Lys were disfavoured. This amino-acid pattern differs from that of DnaK[41,43] and of BiP as identified in the experiments described above[27]. The existence of differences in the substrate binding specificity of DnaK and BiP was further suggested by *in vitro* studies[48,49]. One interpretation of the results obtained using the phage panning approach, however, is difficult to reconcile with the conserved structure of the substrate binding cavity of DnaK. It was proposed that the substrate binding site of BiP contains of four alternating pockets for aromatic and large hydrophobic residues (in particular Trp), each separated by one residue[44]. In retrospect, it appears that the number of BiP-binding peptides used in this study was too small to establish a binding motif model. This problem now becomes apparent with the finding that the conserved hydrophobic substrate binding cavity of DnaK binds a hydrophobic core of only five, and not seven, residues. The lack of knowledge of the precise positioning of the BiP binding sites within the octameric peptides identified by panning also restricts the ability to identify a binding motif with the available data.

The degree of divergence in substrate specificity within the Hsp70 family remains an open question of considerable relevance for understanding the biological activities of these chaperones.

Control of substrate binding by ATP

DnaK binds substrates with K_d values typically of 50 nM–5 μM [22,37,50–52]. In the absence of ATP, DnaK–substrate complexes are stable on the time scale of physiological processes ($t_{1/2}$ for σ^{32}, 40 min; $t_{1/2}$ for peptide substrates, 1–15 min[22,51]; J. S. McCarty and B. B., unpublished). The ability to stably associate with substrates provides the basis for a regulatory mechanism that utilizes the nucleotide status of the chaperone to tightly control substrate interactions. Such a control mechanism is an intrinsic

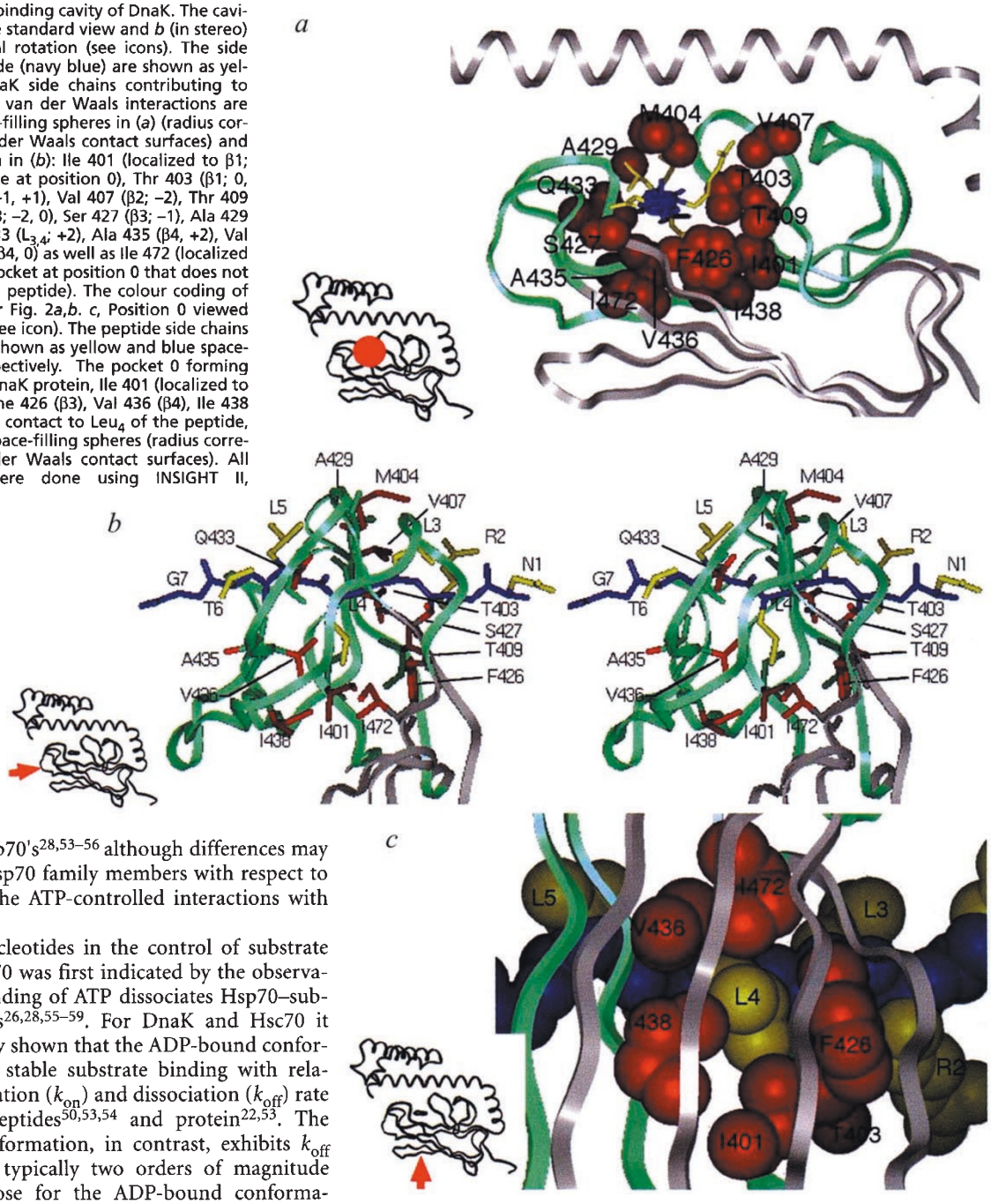

Fig. 3 The peptide binding cavity of DnaK. The cavity is shown in *a*, the standard view and *b* (in stereo) and *c*, after vertical rotation (see icons). The side chains of the peptide (navy blue) are shown as yellow sticks. The DnaK side chains contributing to peptide binding by van der Waals interactions are shown as red space-filling spheres in (*a*) (radius corresponding to van der Waals contact surfaces) and stick representation in (*b*): Ile 401 (localized to β1; contacts the peptide at position 0), Thr 403 (β1; 0, –2), Met 404 ($L_{1,2}$; –1, +1), Val 407 (β2; –2), Thr 409 (β2; –2), Phe 426 (β3; –2, 0), Ser 427 (β3; –1), Ala 429 ($L_{3,4}$; –1, +1), Gln 433 ($L_{3,4}$; +2), Ala 435 (β4, +2), Val 436 (β4, 0), Ile 438 (β4, 0) as well as Ile 472 (localized to β6; part of the pocket at position 0 that does not contact Leu_4 of the peptide). The colour coding of the ribbon is as for Fig. 2*a*,*b*. *c*, Position 0 viewed from underneath (see icon). The peptide side chains and backbone are shown as yellow and blue space-filling spheres, respectively. The pocket 0 forming side chains of the DnaK protein, Ile 401 (localized to β1), Thr 403 (β1), Phe 426 (β3), Val 436 (β4), Ile 438 (β4), Ile 472 (β6; no contact to Leu_4 of the peptide, are shown as red space-filling spheres (radius corresponding to van der Waals contact surfaces). All representations were done using INSIGHT II, Biosym.

feature of all Hsp70's[28,53–56] although differences may exist between Hsp70 family members with respect to the kinetics of the ATP-controlled interactions with substrates.

A role for nucleotides in the control of substrate binding by Hsp70 was first indicated by the observation that the binding of ATP dissociates Hsp70–substrate complexes[26,28,55–59]. For DnaK and Hsc70 it was subsequently shown that the ADP-bound conformation exhibits stable substrate binding with relatively low association (k_{on}) and dissociation (k_{off}) rate constants for peptides[50,53,54] and protein[22,53]. The ATP-bound conformation, in contrast, exhibits k_{off} values that are typically two orders of magnitude higher than those for the ADP-bound conformation[50,53,54]. The k_{on} values are also higher compared to the ADP-bound conformation, though to a more variable degree (2–40-fold) depending on the particular Hsp70 and substrate species. This coupling of nucleotide state and substrate binding is also manifested by the ability of substrates to stimulate the ATPase activity of Hsp70's by up to 20-fold[27,28,30,52].

The association of the ADP-bound conformation of Hsp70 with substrates that are only transiently available, for example, during folding reactions, is probably far too slow to be of physiological relevance. For DnaK it was shown that DnaJ itself binds chaperone substrates[4,20,22,23,60] and stimulates the hydrolysis of DnaK-bound ATP[28–30,61]. DnaK in the ATP-bound conformation is able to associate with DnaJ–substrate complexes, followed by a stabilization of DnaK–substrate complexes through DnaJ-mediated rapid conversion of DnaK to the ADP-bound conformation. This 'locking-in' mechanism is probably central to the role of DnaJ in DnaK-dependent folding reactions[4,22,23,62] and may be conserved within the Hsp70 family[32,33]. Another co-chaperone, GrpE, seems to be restricted to bacterial and mitochondrial Hsp70 systems where it acts as a nucleotide exchange factor. By facilitating the release of chaperone-bound nucleotide[28–30] by up to 5000-fold[75] GrpE allows the rapid association of ATP and subsequent substrate release[22,63]. GrpE thereby acts to speed up the functional cycle. The recent structural determination of the GrpE dimer in complex with the ATPase domain of DnaK[74] reveals that one

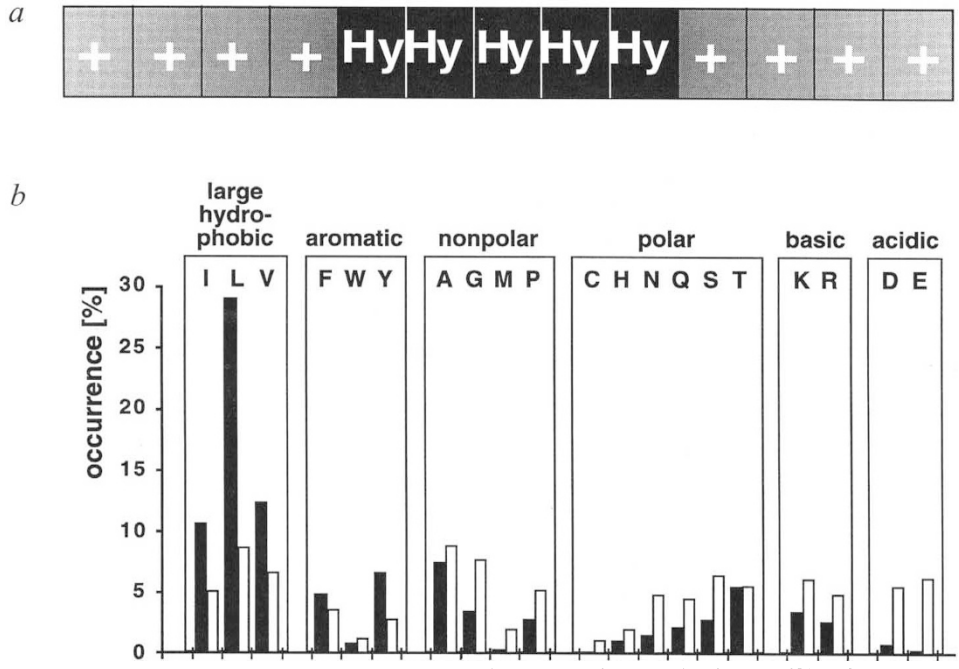

Fig. 4 DnaK binding sites in the sequences of protein substrates. *a*, The DnaK binding motif in substrates consists of a core of up to five large hydrophobic or aromatic residues (Hy) and flanking regions enriched in basic residues (+) which are of decreasing importance with increasing distance from the core. *b*, Amino acid distribution (%) within the hydrophobic cores of 90 good DnaK binding sites identified in protein sequences (black bars) compared to the averaged distribution within the whole library used for this study (white bars)[43].

subunit of GrpE contracts DnaK thereby inducing a subdomain rotation of 14° leading to an opening of the nucleotide binding cleft. In addition, sturtural and biochemical findings indicate a role of GrpE in substrate dissociation, which is likely to be mediated through an interaction between the N-terminal segment of GrpE and the substrate binding domain of DnaK[74]. DnaJ and GrpE together provide a tight control system for the DnaK ATPase cycle and, consequently, of DnaK–substrate interactions.

Coupling mechanism

Nucleotide-induced conformational changes have been identified in Hsp70 proteins using a variety of experimental approaches[55,56,58,59,64–69]. These changes occur upon binding, not hydrolysis, of ATP[55,56,59,64,66,68] and thus are correlated to events leading to substrate release. They must depend on a highly specific interaction of the chaperone with ATP, since they are not observed in the absence of Mg^{2+} or K^+, which are both required for the correct coordination of ATP[70], and with ATP-analogues and ADP[55,56,68,69]. For bovine brain Hsc70[68] and DnaK[70] it was demonstrated by small angle X-ray scattering analysis that they are elongated prolate molecules which, upon binding of ATP, reduce their radii of gyration by 10–15 Å. Furthermore, the ATP-induced changes in fluorescence of a Trp located in the ATPase domains of DnaK and Hsc70 depend on the presence of the substrate binding domains[64,66]. Together, these results suggest that ATP triggers an association of the substrate binding domain with the ATPase domain (Fig. 5). These alterations cause conformational changes within the substrate binding domain itself, as evidenced by partial proteolysis analysis of DnaK[66].

The C termini of Hsc70 and DnaK are dispensable for the coupling mechanism. Fragments of these chaperones lacking 96 (Hsc70) and 101 (DnaK) C-terminal amino acids still show ATP-induced conformational changes and ATP-induced substrate release[64,66,68] (H. Schröder and B. B., unpublished). The coupling mechanism thus involves an interaction of the ATPase domain with a short stretch of ~150 adjacent amino acids comprising the substrate binding site.

Models for a coupling mechanism

Several laboratories have identified mutations in the ATPase domain of DnaK and BiP that affect coupling. These mutations are all located close to the active site and result in reduced ATPase activities, presumably caused by interference with the binding of

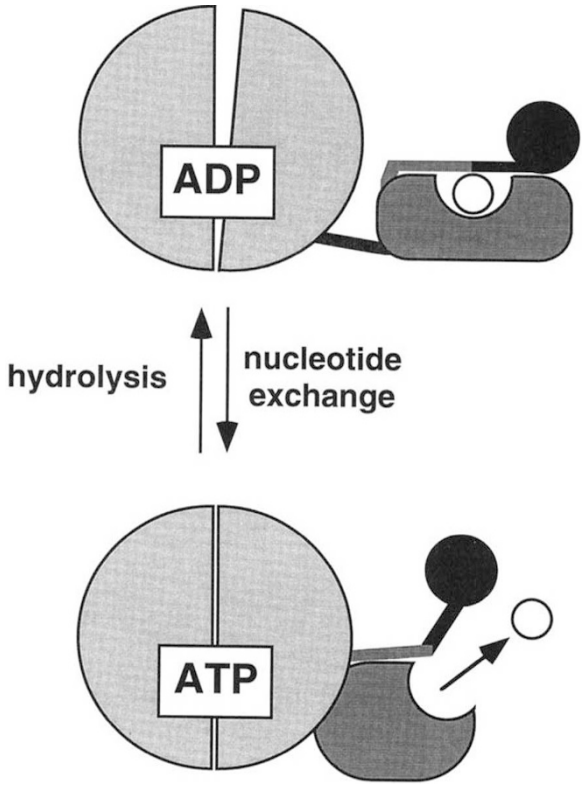

Fig. 5 Model for the mechanism of coupling of ATP binding with substrate release. DnaK is drawn schematically, with the gray scale for the different structural elements adopted from Fig. 1. The nucleotide binding site is depicted as box in the centre of the ATPase domain (two half circles), the substrate as small circle. In the ADP-bound state (top), substrate is tightly bound by DnaK under a lid formed by α-helix B of the substrate binding domain. In the ATP-bound state (bottom), the two ATPase subdomains adopt a slightly different conformation leading to an altered interaction of the ATPase domain with the substrate binding domain. This interaction induces structural alterations in the substrate binding domain and, consequently, the opening of the lid helix and substrate release.

Mg/ATP[52,59,71]. None of these mutants provide an insight into the molecular mechanism of interdomain coupling, although they do emphasize the essential role of correctly positioning the Mg/ATP complex for this process. So far, no mutation in the substrate binding domain is known that affects coupling.

There are two proposals to explain coupling, which deal with different mechanistic aspects and are not mutually exclusive. The first proposal focuses on the conformational changes within the ATPase domain that mediate nucleotide-controlled communication with the substrate binding domain[52,72]. It is based on the fact that the ATPase domains of Hsp70's are structurally highly similar to actin and hexokinase. For both these related proteins there is evidence that the nucleotide binding cleft closes and opens during the nucleotide binding/release cycle[72,73]. An opening/closing of the nucleotide binding domain also exists for Hsp70 proteins as revealed by the determination of the ATPase domain of DnaK in complex with GrpE[74]. Such a mechanism has been proposed to directly affect the association with the substrate binding domain[52,72]. This cavity would be suitably positioned to allow direct, nucleotide-controlled association with the substrate binding domain.

The second proposal focuses on the events in the substrate binding domain leading to ATP-controlled substrate release[36]. It is based on the finding that the substrate binding domain of DnaK was crystallized in two forms. One structure has a kink at residues 536–538 of the lid-forming helix B (Fig. 2*a*, not shown) that leads to a rigid body rotation of the C-terminal helical subdomain by 11° and consequently in a loss of the contacts between helix B and the outer loops $L_{3,4}$ and $L_{5,6}$. The increased flexibility of the loops destabilize the interactions with the bound substrate, including those with the arch. The crystal form containing the kink has been interpreted as a snapshot of the early peptide dissociation process, with full release requiring further displacement of the lid and the outer loops[36]. This 'latch' mechanism is proposed to be important for the ATP-induced dissociation of bound substrate. An interaction of the ATPase domain with helix A is proposed to lead to conformational changes of helices A and B[36].

The latch hypothesis is supported by a proteolysis study that identified Arg 467 as a major tryptic cleavage site which becomes accessible in the ATP-bound conformation of DnaK[66]. Arg 467 is located at the tip of outer loop $L_{5,6}$ and contributes a key contact to Asp 540 of helix B. Further supporting evidence comes from analysis of a DnaK mutant protein (Met 1–Asn 637) lacking 101 C-terminal residues (H. Schröder and B.B., unpublished). This protein is truncated exactly at the hinge point within helix B and, consistent with the latch hypothesis, has a k_{off} for peptides that is elevated about sixfold compared to DnaK$^+$. However, this mutant protein still exhibits coupling as judged by the ATP-dependend peptide release and peptide-stimulated ATPase activities. This suggests that ATP imposes additional destabilizing effects on substrate binding.

Open questions and perspectives

Despite the significant progress being made towards dissecting the molecular basis of Hsp70–substrate interactions many aspects of this process remain unknown. A more complete understanding of the DnaK substrate binding cavity, for example, determining the precise positioning of various peptides within the cavity, is vital. As is solving the puzzle of how entire proteins associate with Hsp70. Furthermore, it is important to explore the degree by which the substrate specificity varies within the Hsp70 family as such diversity may contribute to the functional diversity of family members. It should also be kept in mind that the known crystal structure of the DnaK substrate binding domain provides only a snapshot of the highly dynamic Hsp70–substrate interaction cycle. In particular, elucidation of the mechanism of control of substrate binding by nucleotides and co-chaperones is a major task that will require a number of sophisticated experimental approaches.

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1. Morimoto, R. I., Tissieres, A. & Georgopoulos, C. The biology of heat shock proteins and molecular chaperones. *Cold Spring Harbor Laboratory Press* (1994)
2. Hartl, F. U. Molecular chaperones in cellular protein folding. *Nature* **381**, 571–580 (1996)
3. Gething, M.-J. H. & Sambrook, J. F. Protein folding in the cell. *Nature* **355**, 33–45 (1992)
4. Langer, T., Lu, C., Echols, H., Flanagan, J., Hayer, M. K. & Hartl, F. U. Successive action of DnaK, DnaJ and GroEL along the pathway of chaperone-mediated protein folding. *Nature* **356**, 683–689 (1992)
5. Frydman, J., Nimmesgern, E., Ohtsuka, K. & Hartl, F. U. Folding of nascent polypeptide chains in a high molecular mass assembly with molecular chaperones. *Nature* **370**, 111–117 (1994)
6. Buchberger, A., Schröder, H., Hesterkamp, T., Schönfeld, H.-J. & Bukau, B. Substrate shuttling between the DnaK and GroEL systems indicates a chaperone network promoting protein folding. *J. Mol. Biol.* **261**, 328–333 (1996)
7. Freeman, B. C. & Morimoto, R. I. The human cytosolic molecular chaperones hsp90, hsp70 (hsc70) and hdj-1 have distinct roles in recognition of a non-native protein and protein refolding. *EMBO J.* **15**, 2969–2979 (1996)
8. Bukau, B., Hesterkamp, H. & Luirink, J. Growing up in a dangerous enviroment: a network of multiple targeting and folding pathways for nascent polypetides in the cytosol. *Trends Cell Biol.* **6**, 480–486 (1996)
9. Craig, E. A., Gambill, B. D. & Nelson, R. J. Heat shock proteins: molecular chaperones of protein synthesis. *Microbiological Reviews* **57**, 402–414 (1993)
10. Bult, C. J., White, O., Olsen, G. J., Zhou, L., Fleischmann, R. D., Sutton, G. G., Blake, J. A., M., F. L., A., C. R., al., e. & Venter, J. C. Complete genome sequence of the methanogenic archaeon, Methanococcus jannaschii. *Science* **273**, 1058–1073 (1996)
11. Georgopoulos, C. The emergence of the chaperone machines. *TIBS* **17**, 295–299 (1992)
12. James, P., Pfund, C. & Craig, E. C. Functional specificity among Hsp70 molecular chaperones. *Science* **275**, 387–389 (1997)
13. Schatz, G. & Dobberstein, B. Common principles of protein translocation across membranes. *Science* **271**, 1519–1526 (1996)
14. Georgopoulos, C., Liberek, K., Zylicz, M. & Ang, D. Properties of the heat shock proteins of *Escherichia coli* and the autoregulation of the heat shock response. In *The biology of heat shock proteins and molecular chaperones* (R. I. Morimoto, A. Tissieres & C. Georgopoulos, eds.) 209–250 (Cold Spring Harbor Laboratory Press; 1994)
15. Chappell, T. G., Welch, W., Schlossman, D. M., Palter, K. B., Schlesinger, M. J. & Rothman, J. E. Uncoating ATPase is a member of the 70 kilodalton family of stress proteins. *Cell* **45**, 3–13 (1986)
16. Sherman, M. & Goldberg, A. L. Involvement of the chaperon DnaK in the rapid degradation of a mutant protein in *Escherichia coli*. *EMBO J.* **11**, 71–77 (1992)
17. Straus, D. B., Walter, W. A. & Gross, C. A. *Escherichia coli* heat shock gene mutants are defective in proteolysis. *Genes & Dev.* **2**, 1851–1858 (1988)
18. Straus, D., Walter, W. & Gross, C. DnaK, DnaJ, and GrpE heat shock proteins negatively regulate heat shock gene expression by controlling the synthesis and stability of σ^{32}. *Genes & Dev.* **4**, 2202–2209 (1990)
19. Craig, E. A. & Gross, C. A. Is hsp70 the cellular thermometer? *TIBS* **16**, 135–140 (1991)
20. Wickner, S., Hoskins, J. & McKenney, K. Monomerization of RepA dimers by heat shock proteins activates binding to DNA replication origins. *Proc. Natl. Acad. Sci. USA* **88**, 7903–7907 (1991)
21. Blaszczak, A., Zylicz, M., Georgopoulos, C. & Liberek, K. Both ambient temperature and the DnaK chaperone machine modulate the heat shock response in *Escherichia coli* by regulating the switch between σ^{70} and σ^{32} factors assembled with RNA polymerase. *EMBO J.* **14**, 5085–5093 (1995)
22. Gamer, J., Multhaup, G., Tomoyasu, T., McCarty, J. S., Rüdiger, S., Schönfeld, H.–J., Schirra, C., Bujard, H. & Bukau, B. 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 (1996)
23. Schröder, H., Langer, T., Hartl, F.-U. & Bukau, B. DnaK, DnaJ, GrpE form a cellular

chaperone machinery capable of repairing heat-induced protein damage. *EMBO J.* **12**, 4137–4144 (1993)
24. Skowyra, D., Georgopoulos, C. & Zylicz, M. 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 (1990)
25. Bukau, B. Regulation of the *E. coli* heat shock response. *Molec. Microbiol.* **9**, 671–680 (1993)
26. Flynn, G. C., Chappell, T. G. & Rothman, J. E. Peptide binding and release by proteins implicated as catalysts of protein assembly. *Science* **245**, 385–390 (1989)
27. Flynn, G. C., Pohl, J., Flocco, M. T. & Rothman, J. E. Peptide-binding specificity of the molecular chaperone BiP. *Nature* **353**, 726–730 (1991)
28. McCarty, J. S., Buchberger, A., Reinstein, J. & Bukau, B. The role of ATP in the functional cycle of the DnaK chaperone system. *J. Mol. Biol.* **249**, 126–137 (1995)
29. Liberek, K., Marszalek, J., Ang, D., Georgopoulos, C. & Zylicz, M. *Escherichia coli* DnaJ and GrpE heat shock proteins jointly stimulate ATPase activity of DnaK. *Proc. Natl. Acad. Sci. USA* **88**, 2874–2878 (1991)
30. Jordan, R. & McMacken, R. 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 (1995)
31. Höhfeld, J., Minami, Y. & Hartl, F. U. Hip, a novel cochaperone involved in the eukaryotic Hsc70/Hsp40 reaction cycle. *Cell* **83**, 589–598 (1995)
32. Cyr, D. M., Lu, X. & Douglas, M. G. Regulation of Hsp70 function by a eukaryotic DnaJ homolog. *J. Biol. Chem.* **29**, 20927–20931 (1992)
33 Levy, E. J., McCarty, J., Bukau, B. & Chirico, W. J. Conserved ATPase and luciferase refolding activities between bacteria and yeast Hsp70 chaperones and modulator. *FEBS Lett.* **368**, 435–440 (1995)
34. Wang, T.-F., Chang, J. & Wang, C. Identification of the peptide binding domain of Hsc70. *J. Biol. Chem.* **268**, 26049–26051 (1993)
35. Flaherty, K. M., Deluca-Flaherty, C. & McKay, D. B. Three-dimensional structure of the ATPase fragment of a 70K heat-shock cognate protein. *Nature (London)* **346**, 623–628 (1990)
36. Zhu, X., Zhao, X., Burkholder, W. F., Gragerov, A., Ogata, C. M., Gottesman, M. & Hendrickson, W. A. Structural analysis of substrate binding by the molecular chaperone DnaK. *Science* **272**, 1606–1614 (1996)
37. Burkholder, W. F., Zhao, X., Zhu, X. & Hendrickson, W. A. Mutations in the C-terminal fragment of DnaK affecting peptide binding. *Proc. Nat. Acad. Sci. USA* **93**, 10632–10637 (1996)
38. Landry, S. J., Jordan, R., McMacken, R. & Gierasch, L. M. Different conformations for the same polypeptide bound to chaperones DnaK and GroEL. *Nature* **355**, 455–457 (1992)
39. Richarme, G. & Kohiyama, M. Specificity of the *Escherichia coli* chaperone DnaK (70-kDa heat shock protein) for hydrophobic amino acids. *J. Biol. Chem.* **268**, 24074–24077 (1993)
40. Wickner, S., Hoskins, J. & McKenny, K. Function of DnaJ and DnaK as chaperones of origin specific DNA binding by RepA. *Nature (London)* **350**, 165–167 (1991)
41. Gragerov, A., Zeng, L., Zhao, X., Burkholder, W. & Gottesman, M. E. Specificity of DnaK-peptide binding. *J. Mol. Biol.* **235**, 848–854 (1994)
42. Kramer, A., Schuster, A., Reineke, U., Malin, R., Volkmer-Engert, R., Landgraf, C. & Schneider-Mergener, J. Combinatorial cellulose-bound peptide libraries: screening tools for the identification of peptides that bind ligands with predefined specificity. *Methods (Comp. Meth. Enzymol.)* **6**, 388–395 (1994)
43. Rüdiger, S., Germeroth, L., Schneider-Mergener, J. & Bukau, B. Substrate specificity of the DnaK chaperone determined by screening of cellulose-bound peptide libraries. *EMBO J.* **16**, 1501–1507 (1997)
44. Blond-Elguindi, S., Cwirla, S. E., Dower, W. J., Lipshutz, R. J., Sprang, S. R., Sambrook, J. F. & Gething, M.-J. H. Affinity panning of a library of peptides displayed on bacteriophages reveals the binding specificity of BiP. *Cell* **75**, 717–728 (1993)
45. Alfano, C. & McMacken, R. Ordered assembly of nucleoprotein structures at the bacteriophage λ replication origin during the initiation of DNA replication. *J. Biol. Chem.* **264**, 10699–10708 (1989)
46. Gamer, J., Bujard, H. & Bukau, B. Physical interaction between heat shock proteins DnaK, DnaJ, GrpE and the bacterial heat shock transcriptional factor σ^{32}. *Cell* **69**, 833–842 (1992)
47. Liberek, K., Galitski, T. P., Zylicz, M. & Georgopoulos, C. The DnaK chaperone modulates the heat shock response of Escherichia coli by binding to the σ^{32} transcription factor. *Proc. Natl. Acad. Sci. USA* **89**, 3516–3520 (1992)
48. Fourie, A. M., Sambrook, J. F. & Gething, M.-J. H. Common and divergent peptide binding specificities of Hsp70 molecular chaperones. *J. Biol. Chem.* **269**, 30470–30478 (1994)
49. Gragerov, A. & Gottesman, M. E. Different peptide binding specificities of Hsp70 family members. *J. Mol. Biol.* **241**, 133–135 (1994)
50. Schmid, D., Baici, A., Gehring, H. & Christen, P. Kinetics of molecular chaperone action. *Science* **263**, 971–973 (1994)
51. McCarty, J. S., Rüdiger, S., Schönfeld, H.-J., Schneider-Mergener, J., Nakahigashi, K., Yura, T. & Bukau, B. 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 (1996)
52. Buchberger, A., Valencia, A., McMacken, R., Sander, C. & Bukau, B. The chaperone function of DnaK requires the coupling of ATPase activity with substrate binding through residue E171. *EMBO J.* **13**, 1687–1695 (1994)
53. Greene, L. E., Zinner, R., Naficy, S. & Eisenberg, E. Effect of nucleotide on the binding of peptides to 70-kDa heat shock protein. *J. Biol. Chem.* **270**, 2967–2973 (1995)
54. Takeda, S. & McKay, D. B. Kinetics of Peptide Binding to the Bovine 70 kDa Heat Shock Cognate Protein, a Molecular Chaperone. *Biochemistry* **35**, 4636–4644 (1996)
55. Palleros, D. R., Reid, K. L., Shi, L., Welch, W. J. & Fink, A. L. ATP-induced protein-Hsp70 complex dissociation requires K^+ but not ATP hydrolysis. *Nature* **365**, 664–666 (1993)
56. Theyssen, H., Schuster, H.-P., Bukau, B. & Reinstein, J. The Second Step of ATP Binding to DnaK Induces Peptide Release. *J. Mol. Biol.* **263**, 657–670 (1996)
57. Munro, S. & Pelham, H. R. B. An Hsp70-like protein in the ER: identity with the 78 kDa glucose-regulated protein and immunoglobulin heavy chain binding protein. *Cell* **46**, 291–300 (1986)
58. Liberek, K., Skowyra, D., Zylicz, M., Johnson, C. & Georgopoulos, C. The *Escherichia coli* DnaK chaperone, the 70-kDa heat shock protein eukaryotic equivilant, changes conformation upon ATP hydrolysis, thus triggering its dissociation from a bound target protein. *J. Biol. Chem.* **266**, 14491–14496 (1991)
59. Wei, J., Gaut, J. R. & Hendershot, L. M. *In Vitro* Dissociation of BiP-Peptide Complexes Requires a Conformational Change in BiP after ATP Binding but Does Not Require ATP Hydrolysis. *J. Biol. Chem.* **270**, 26677–26682 (1995)
60. Hoffmann, H. J., Lyman, S. K., Lu, C., Petit, M. A. & Echols, H. Activity of the Hsp70 chaperone complex-DnaK, DnaJ, and GrpE-in initiating phage λ DNA replication by sequestering and releasing λ P protein. *Proc. Natl. Acad. Sci. USA* **89**, 12108–12111 (1992)
61. Wall, D., Zylicz, M. & Georgopoulos, C. 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 (1994)
62. Szabo, A., Langer, T., Schröder, H., Flanagan, J., Bukau, B. & Hartl, F. U. The ATP hydrolysis-dependent reaction cycle of the *Escherichia coli* Hsp70 system-DnaK, DnaJ and GrpE. *Proc. Natl. Acad. Sci. USA* **91**, 10345–10349 (1994)
63. Banecki, B. & Zylicz, M. Real Time Kinetics of the DnaK/DnaJ/GrpE Molecular Chaperone Machine Action. *J. Biol. Chem.* **271**, 6137–6143 (1996)
64. Ha, J.-H. & McKay, D. B. Kinetics of Nucleotide-Induced Changes in the Tryptophane Fluorescence of the Molecular Chaperone Hsc70 and Its Subfragments Suggest the ATP-Induced Conformational Change Follows Initial ATP Binding. *Biochemistry* **34**, 11635–11644 (1995)
65. Banecki, B., Zylicz, M., Bertoli, E. & Tanfani, F. Structural and functional relationships in DnaK and DnaK756 heat-shock proteins from *Escherichia coli*. *J. Biol. Chem.* **267**, 25051–25058 (1992)
66. Buchberger, A., Theyssen, H., Schröder, H., McCarty, J. S., Virgallita, G., Milkereit, P., Reinstein, J. & Bukau, B. Nucleotide-induced conformational changes in the ATPase and substrate binding domains of the DnaK chaperone provide evidence for interdomain communication. *J. Biol. Chem.* **270**, 16903–16910 (1995)
67. Shi, L., Kataoka, M. & Fink, A. L. Conformational Characterization of DnaK and Its Complexes by Small-Angle X-ray Scattering. *Biochemistry* **35**, 3297–3308 (1996)
68. Wilbanks, S. M., Chen, L., Tsuruta, H., Hodgson, K. O. & McKay, D. B. Solution small-angle X-ray scattering study of the molecular chaperone hsc70 and its subfragments. *Biochem.* **34**, 12095–12106 (1995)
69. Fung, K. L., Hilgenberg, L., Wang, N. M. & Chirico, W. J. Conformations of the Nucleotide and Polypeptide Binding Domains of a Cytosolic Hsp70 Molecular Chaperone Are Coupled. *J. Biol. Chem.* **271**, 21559–21565 (1996)
70. Wilbanks, S. M. & McKay, D. B. How Potassium Affects the Activity of the Molecular Chaperone Hsc70. II. Potassium Binds Specifically in the ATPase Active Site. *J. Biol. Chem.* **270**, 2251–2257 (1995)
71. Kamath-Loeb, A. S., Lu, S. Z., Suh, W.-C., Lonetto, M. A. & Gross, C. A. Analysis of Three DnaK Mutant Proteins Suggests That Progression through the ATPase Cycle Requires Conformational Changes. *J. Biol. Chem.* **270**, 30051–30059 (1995)
72. Holmes, K. C., Sander, C. & Valencia, A. A new ATP-binding fold in actin, hexokinase and Hsc70. *Trends Cell Biol.* **3**, 53–59 (1993)
73. Tirion, M. M. & ben-Avraham, D. Normal mode analysis of G-actin. *J. Mol. Biol.* **230**, 186–195 (1993).349
74. Harrison, C.J. , Hayer-Hartl, M., Diliberto, M., Hartl, F.U. & Kuriyan, J. Crystal structure of the nucleotide exchange factor GrpE bound to the ATPase domain of the molecular chaperone DnaK. *Science* **in the press**.
75. Packschier, L. *et al.* The molecular chaperone GrpE accelerates nucleotide exchange of DnaK by an associative displacement mechanism. *Biochemistry* **in the press.**