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The chaperone function of DnaK requires the coupling of ATPase activity with substrate binding through residue E171

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Central to the chaperone function of Hsp70 stress proteins including *Escherichia coli* DnaK is the ability of Hsp70 to bind unfolded protein substrates in an ATP-dependent manner. Mg^{2+}/ATP dissociates bound substrates and, furthermore, substrate binding stimulates the ATPase of Hsp70. This coupling is proposed to require a glutamate residue, E175 of bovine Hsc70, that is entirely conserved within the Hsp70 family, as it contacts bound Mg^{2+}/ATP and is part of a hinge required for a postulated ATP-dependent opening/closing movement of the nucleotide binding cleft which then triggers substrate release. We analyzed the effects of *dnaK* mutations which alter the corresponding glutamate-171 of DnaK to alanine, leucine or lysine. *In vivo*, the mutated *dnaK* alleles failed to complement the Δ*dnaK52* mutation and were dominant negative in *dnaK*$^+$ cells. *In vitro*, all three mutant DnaK proteins were inactive in known DnaK-dependent reactions, including refolding of denatured luciferase and initiation of λ DNA replication. The mutant proteins retained ATPase activity, as well as the capacity to bind peptide substrates. The intrinsic ATPase activities of the mutant proteins, however, did exhibit increased K_m and V_{max} values. More importantly, these mutant proteins showed no stimulation of ATPase activity by substrates and no substrate dissociation by Mg^{2+}/ATP. Thus, glutamate-171 is required for coupling of ATPase activity with substrate binding, and this coupling is essential for the chaperone function of DnaK.

Key words: Escherichia coli/heat shock proteins/Hsp70/protein folding/stress response

Introduction

Hsp70 chaperones are essential constituents of the cellular machinery for folding, repair and degradation of proteins (reviewed in Morimoto *et al.*, 1990; Gething and Sambrook, 1992; Hartl *et al.*, 1992). Their activity has been implicated in central cellular processes, including folding of proteins during *de novo* synthesis, translocation of proteins, replication of DNA and proteolysis. They bind to fully or partially unfolded polypeptides and maintain them in loosely folded conformations, thereby preventing off-pathway folding reactions. It is unclear whether Hsp70-mediated folding of protein substrates occurs during or after their release from the chaperone. Interactions of Hsp70 proteins with substrates are controlled by Mg^{2+}/ATP which stimulates substrate dissociation. This control by Mg^{2+}/ATP is essential for virtually all chaperone functions of Hsp70 (reviewed in Gething and Sambrook, 1992). In addition, the ATPase activity of Hsp70 proteins is itself stimulated by binding of substrates (Flynn *et al.*, 1989; Sadis and Hightower, 1992; Blond-Elguindi *et al.*, 1993). Thus, a coupling of ATPase activity with substrate binding is central to the chaperone function of Hsp70.

The members of the Hsp70 family, including the DnaK heat shock protein of *Escherichia coli*, are composed of (i) an N-terminal ATPase domain of 44 kDa that exhibits >50% amino acid identity between different Hsp70 proteins, and (ii) a C-terminal domain of 25 kDa that is less well conserved (Nover and Scharf, 1991) and that, for DnaK, was shown to comprise the substrate binding site (J.McCarty, H.Schröder and B.Bukau, unpublished data). The 3-D structure of the ATPase domain of bovine Hsc70 in the ADP and P_i bound form has been solved to high resolution (Flaherty *et al.*, 1990). It consists of two subdomains that are separated by a deep central cleft. Two crossed α-helices connect the two subdomains and divide the cleft into (i) an upper cleft at the bottom of which nucleotide and Mg^{2+} are bound and (ii) a lower cleft (Figure 1). Key residues required for formation of this structure are conserved within the Hsp70 family (Holmes *et al.*, 1993). In particular, for Hsc70 and DnaK, the high sequence similarity between these proteins (51% identical residues) implies that their overall ATPase domains are very similar in structure. Surprisingly, there is high structural similarity between the Hsc70 ATPase, actin and hexokinase, despite low sequence similarity (Flaherty *et al.*, 1990, 1991; Kabsch *et al.*, 1990; Bork *et al.*, 1992; Holmes *et al.*, 1993). Comparative analysis of the three protein structures led to the proposal that binding of ATP rotates the two ATPase subdomains of these proteins relative to each other, thereby opening or closing the upper and the lower cleft (Holmes *et al.*, 1993). For actin, opening/closing motions are observed in the normal mode analysis (Tirion and ben-Avraham, 1993). For DnaK, a significant subdomain movement was indicated by the existence of ATP-dependent changes in the proteolytic cleavage pattern (Liberek *et al.*, 1991b) and in the fluorescence and infra-red spectra (Banecki *et al.*, 1992).

The hinge points of the proposed opening/closing movement are confined to a few residues of the two α-helices connecting the ATPase subdomains. In Hsc70, this hinge is connected to bound Mg^{2+}/ATP via a strictly conserved glutamate (E175 for Hsc70; E171 for DnaK) (Holmes *et al.*, 1993) (Figure 1). This residue is in one of the two connecting helices and, along with two aspartates (D10 and D199 for Hsc70; D8 and D194 for DnaK), is involved in the

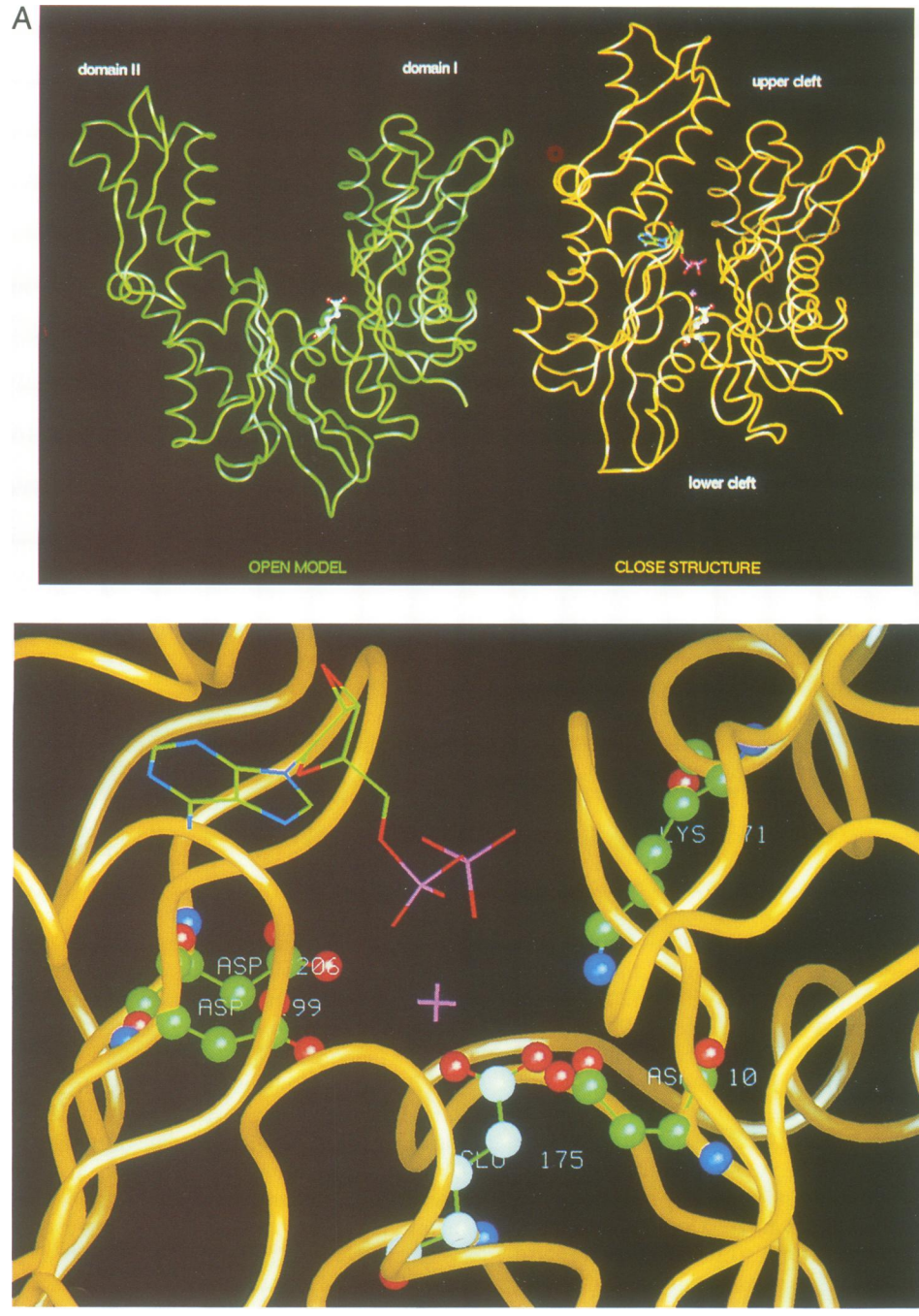

Fig. 1. (**A**) Structure of the closed form and model of the hypothetical open form of the ATPase domain of Hsc70. Lower and upper nucleotide binding cleft, domains I and II of the ATPase fragment (Flaherty *et al.*, 1990), residue E175 (E171 of DnaK) (white), and bound ADP are indicated. The model of the open form was generated by molecular modeling taking the known open structure of hexokinase (Anderson *et al.*, 1978) as a framework and moving domain II of Hsc70 as a rigid body to fit with the framework, as described in Holmes *et al.* (1993). The very high level of sequence identity (51%) between the DnaK and Hsc70 ATPases implies that they will have almost identical structures at the molecular level (r.m.s. difference between Cα atoms of 1 Å or less) (Chothia and Lesk, 1986). In particular, the similarity in the active site will be even larger as all key residues are conserved. Therefore, the interpretations made here, which are based on the structure of Hsc70, presumably can be directly extrapolated to DnaK. (**B**) Molecular detail of the Hsc70 nucleotide binding site and the residues involved in positioning of the Mg^{2+} ion. Bound ADP and the position of the Mg^{2+} ion (+) are indicated. The Mg^{2+} ion was not present in the original coordinates of Hsc70 (Flaherty *et al.*, 1990) and for this presentation the equivalent position of Ca^{2+} in the actin structure was used (Kabsch *et al.*, 1990). E175 (E171 of DnaK) is shown, together with other residues involved in positioning of the Mg^{2+} ion (D199 and D10) or contributing to the environment of E175 (D206 and K71). The color code is: oxygen, red; nitrogen, blue; carbon, white (E171) or green (other residues).

positioning of the Mg^{2+} (via water molecules). By interacting with two residues in each of the two subdomains, the Mg^{2+} ion acts as a bridge between the two subdomains, and the integrity of this binding is probably important for the stabilization of the closed form. We hypothesize that the connection of the Mg^{2+} ion to the hinge through E171/E175 is required for an ATP-dependent subdomain movement of the ATPases of Hsp70 proteins, and thereby enables the coupling of ATPase activity with substrate binding (Figure 1).

We investigated the mechanism and the importance for chaperone activity of this coupling. In particular, we

Table I. *In vivo* phenotypes of *dnaK-E171 A, L, K* mutations

Plasmid-encoded *dnaK* alleles	Growth at 30°C					Growth at 42°C					λ*vir* plaque formation	
	0	50	100	250	500	0	50	100	250	500	0	50
Δ*dnaK52* background												
dnaK$^+$	+	+	+	+	+	−	+	+	(+)	−	100	100
dnaK-E171A	+	+	+	+	+	−	−	−	−	−	0	0
dnaK-E171L	+	+	+	+	+	−	−	−	−	−	0	0
dnaK-E171K	+	+	+	+	+	−	−	−	−	−	0	0
dnaK$^+$ background												
dnaK$^+$	+	+	+	+	(+)	+	+	+	+	(+)	100	100
dnaK-E171A	+	+	+	(+)	±	+	(+)	±	−	−	1.4[a]	0.6[a]
dnaK-E171L	+	+	+	(+)	±	+	+	(+)	−	−	12[a]	0.8[a]
dnaK-E171K	+	+	+	(+)	±	+	+	(+)	−	−	6[a]	0

Δ*dnaK52* mutants (BB1553) and *dnaK*$^+$ cells (BB1996) that carry pDMI,1 as well as p*dnaK*, encoding the *dnaK*$^+$ and *dnaK-E171 A, L, K* alleles, were tested for growth at 30 and 42°C and for λ*vir* plaque formation. Growth was tested by determining the ability of the cells to form colonies on LB/Ap/Kn plates containing IPTG at concentrations as indicated in the head row (μM). +, normal number and size of colonies; (+), slightly reduced number and size of colonies; ±, very small translucent colonies; −, no colonies. λ*vir* plaque assays were performed as described (Miller, 1972) on LB/Ap/Kn agar plates containing 0 or 50 μM IPTG. The numbers of plaques are expressed as percent of plaques formed on cells carrying p*dnaK* encoding the *dnaK*$^+$ gene.

[a]Plaques were turbid and smaller than the plaques formed on cells encoding *dnaK*$^+$.

investigated the role of E171 of DnaK in this process by analyzing mutant DnaK proteins that have E171 replaced by A, L and K. We provide evidence that E171 is essential for the coupling of ATPase activity with substrate binding and release, and that this coupling is required for the chaperone function of DnaK both *in vivo* and *in vitro*.

Results

In vivo phenotypes of dnaK-E171A, L, K mutations

Our rationale for introducing the particular changes at E171 of DnaK was to replace this residue by amino acids that are expected to interfere to various degrees with Mg^{2+} coordination because of (i) small size and lack of charge (alanine), (ii) lack of charge (leucine) or (iii) replacement of the negative charge by a positive charge (lysine). We introduced specific mutations in *dnaK*$^+$ encoded by pUHE21-2fdΔ12 plasmids (p*dnaK*) to generate *dnaK-E171A*, *dnaK-E171L* and *dnaK-E171K* (collectively *dnaK-E171A, L, K*). These alleles encode DnaK mutant proteins which have E171 replaced by A, L and K, referred to as E171A, E171L and E171K (collectively E171A, L, K) mutant DnaK proteins. Expression of the p*dnaK*-encoded *dnaK* alleles is controlled by a T7 promoter/$lac_{O4/O3}$ promoter−operator system, allowing repression in the presence of *lacI*^{q} (encoded by pDMI,1) and induction upon addition of IPTG.

We investigated whether the *dnaK-E171A, L, K* mutations affect the *in vivo* functions of *dnaK*. We determined the ability of the mutant alleles to complement two major cellular defects of Δ*dnaK52* mutants lacking DnaK$^+$ (Paek and Walker, 1987; Bukau and Walker, 1989), the failure to grow at high (>40°C) temperature and to propagate bacteriophage λ. For this analysis we constructed Δ*dnaK52* strains carrying pDMI,1 as well as plasmids encoding the different mutant alleles. Expression of *dnaK*$^+$ from a plasmid allowed growth at 42°C when the *dnaK*$^+$ allele was expressed after induction by IPTG in the concentration range 50−250 μM. In contrast, expression of the *dnaK* mutant alleles did not allow growth of the cells at 42°C, as judged by their inability to form colonies on agar plates (Table I), even at conditions of full induction which caused accumulation of the mutant proteins to high levels (see below). These cells failed to grow even at the more moderate temperature of 40°C (data not shown). In addition, IPTG-induced expression of the *dnaK* mutant alleles in Δ*dnaK52* cells did not enable lytic growth of λ*vir* at 30°C. This was judged by the inability of λ*vir* to form plaques on a lawn of bacteria grown in the presence of 0 and 50 μM IPTG (Table I). In contrast, λ*vir* plaque formation was observed when the *dnaK*$^+$ allele was expressed from p*dnaK*. Thus, the *dnaK-E171A, L, K* alleles do not complement the Δ*dnaK52* mutation, indicating that they encode non-functional mutant DnaK proteins.

We further characterized the *dnaK-E171A, L, K* alleles by examining the phenotypes associated with their expression in *dnaK*$^+$ cells. IPTG-induced expression of each of the three plasmid-encoded mutant *dnaK* alleles in a wild-type background resulted in phenotypes similar to those associated with *dnaK*$^-$ cells (Table I). The ability to form colonies at 42°C was impaired after induction with as little as 50 (in the case of *dnaK-E171A*) or 100 μM (in the case of *dnaK-E171L, K*) IPTG. λ*vir* was unable to form plaques on a lawn of bacteria grown in the presence of 50 μM IPTG and was even reduced in plaque formation in the absence of IPTG. The *dnaK-E171A* mutation displayed an even greater dominant negative character than the *dnaK-E171L, K* mutations, because the onset of growth and λ*vir* propagation defects occurred at lower IPTG concentrations for this particular mutant. From these experiments we conclude that the *dnaK-E171A, L, K* alleles are dominant negative *in vivo* with respect to the tested phenotypes. Furthermore, all three mutations exhibited a slight dominant lethality. Strong overexpression of the *dnaK-E171A, L, K* alleles, but not of the *dnaK*$^+$ allele, in the presence of 500 μM IPTG prevented colony formation of *dnaK*$^+$ cells at 30°C (Table I). This toxicity by high level expression of the mutant alleles was not observed in a Δ*dnaK52* mutant background.

We determined the cellular levels of DnaK wild-type and

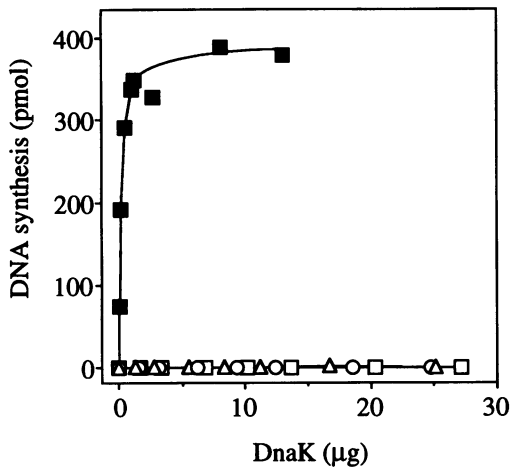

Fig. 2. E171 A, L, K mutant DnaK proteins are non-functional in λ DNA replication. DnaK$^+$ and E171 A, L, K mutant proteins were added to a λ DNA replication mixture lacking DnaK. The amount of newly synthesized *ori*λ plasmid DNA was determined and is expressed as picomoles of ^{3}H-labeled DNA. (■), DnaK$^+$; (△), E171A; (□), E171L; (○), E171K.

mutant proteins at various inducing conditions to investigate the efficiency by which the mutant DnaK proteins interfere with DnaK$^+$ functions to produce a dominant negative phenotype (data not shown). This evaluation was performed by densitometric evaluation of immunoblots of the appropriate cell extracts developed with DnaK-specific antisera. Prior to extraction, cells growing logarithmically at 30°C were induced by IPTG and further incubated at 30 or 42°C for 3 h. In chromosomal *dnaK*$^+$ cells grown at 30°C, plasmid-encoded DnaK proteins (DnaK$^+$ or E171A, K, L mutant DnaK proteins) induced with 50, 100 and 250 μM IPTG reached ~85, 175 and 275% of the levels of chromosomally produced DnaK$^+$, respectively. Similar results were found for Δ*dnaK52* mutant cells at 30°C. Consistent with previous reports, we found that in wild-type cells the level of DnaK$^+$ was 3- to 4-fold higher at 42 than at 30°C. Under these conditions, in both wild-type and Δ*dnaK52* backgrounds, plasmid-encoded DnaK proteins induced with 50 μM IPTG reached levels equivalent to that of chromosomally encoded DnaK found in wild-type cells at this temperature.

Chaperone activities of the E171A, L, K mutant DnaK proteins in vitro

The activities of the E171A, L, K mutant DnaK proteins were investigated in two well-characterized *in vitro* assays indicative for the chaperone function of DnaK: the disassembly of protein complexes and the refolding of denatured proteins. The activity of the DnaK variants in the disassembly of protein complexes was assayed by the ability to replicate λ DNA *in vitro* (Mensa-Wilmot *et al.*, 1989). The essential role of DnaK in this process is to promote the disassembly, in cooperation with the DnaJ and GrpE heat shock proteins, of the nucleoprotein preinitiation complex formed at the origin of replication in the λ chromosome, thereby activating DnaB helicase (Alfano and McMacken, 1989; Dodson *et al.*, 1989; Zylicz *et al.*, 1989). Initiation of λ DNA replication was strictly DnaK$^+$-dependent and reached an optimal level with ~2 μg added DnaK$^+$ (Figure 2). In contrast, the initiation of λ DNA replication *in vitro* in the presence of the E171A, L, K mutant DnaK proteins was completely defective, even when large amounts of DnaK, up to 25 μg, were used in the assay.

The activity in protein refolding was assayed by the ability of the mutant and wild-type DnaK proteins to mediate refolding of denatured firefly luciferase. Efficient refolding of luciferase denatured by either heat treatment or guanidinium−HCl was recently demonstrated to require the DnaK, DnaJ, GrpE chaperones (Schröder *et al.*, 1993; H.Schröder and B.Bukau, unpublished results). The presence of DnaK$^+$ in the refolding mixture led to efficient luciferase reactivation, with >90% of the activity present prior to denaturation recovered. In contrast, all three of the mutant DnaK proteins failed to increase luciferase reactivation above the background level obtained in the absence of added DnaK (Figure 3). These two tests clearly demonstrate that all three E171A, L, K mutant DnaK proteins are non-functional as chaperones *in vitro*.

ATPase and substrate binding activities of E171A, L, K mutant DnaK proteins

To identify defects in the biochemical activities of the E171A, L, K mutant DnaK proteins that compromise their function as chaperones, we assayed two key activities of DnaK: ATP hydrolysis and substrate binding. We determined the K_m and V_{max} values of the ATPase activity (Table II). The K_m values were significantly increased for all three E171A, L, K mutant DnaK proteins, by 6.5-, 12- and 18-fold, relative to the K_m for ATP of DnaK$^+$. The V_{max} values were 4- and 2.5-fold higher for the E171A and E171L mutant DnaK proteins, respectively, but considerably higher (13-fold) for the E171K mutant DnaK protein, relative to the V_{max} for DnaK$^+$. Thus, all three changes at residue E171 reduce the affinity of DnaK for ATP while, strongly in the case of the E171K mutant DnaK protein, increasing the rate of ATP hydrolysis at high ATP concentrations relative to DnaK$^+$. This ability to hydrolyze ATP demonstrates that the E171A, L, K mutant DnaK proteins are not significantly altered in their overall structures of the ATPase domain and that, at saturating ATP concentrations, it is not the failure to hydrolyze ATP that is responsible for the lack of chaperone function of these proteins.

We considered the possibility that, despite the use of a highly rigorous purification protocol for obtaining the E171A, L, K mutant DnaK proteins, other ATPases contaminate our protein preparations and contribute to the measured ATPase activities. To test this possibility, we examined the purity of our preparations by re-assaying for functions of DnaK after anti-DnaK immunodepletion. Aliquots of the DnaK$^+$ and E171A, L, K mutant DnaK protein preparations were applied to a Sepharose column to which affinity-purified polyclonal DnaK antibodies were covalently linked. Over 95% of the applied DnaK was retained on the column as determined by Bradford analysis. For all DnaK preparations tested, we found no significant ATPase activities in the flowthrough fractions. Therefore most, if not all, of the ATPase activities contained in the DnaK preparations used in the experiments described here are intrinsic to DnaK.

We also determined the ability of the E171A, L, K mutant DnaK proteins to bind substrates. As test substrate we used peptide C (13 amino acids in length) of vesicular stomatitis

virus glycoprotein (Flynn *et al.*, 1989). Peptide C binds with high affinity (K_D values in the low micromolar range) to the substrate binding sites of Hsc70, BiP (Flynn *et al.*, 1989) and DnaK (R.Jordan and R.McMacken, manuscript in preparation). The mutant proteins bound peptide C with high affinity, yielding K_D values from 10 (E171L, E171K) to 17 μM (E171A) (Table II). These K_D values are slightly increased (4- to 6-fold) as compared with the K_D of DnaK$^+$. These data indicate that the mutations introduced at E171 do not drastically alter the ability of DnaK to bind substrates.

The E171A, L, K mutant DnaK proteins fail to couple ATPase activity to substrate binding and release

A primary goal of altering E171 was to investigate the hypothesis that this residue is required for the coupling of ATPase activity with substrate binding and release. This hypothesis can be tested by examining (i) whether the ATPase activities of the E171A, L, K mutant DnaK proteins can be stimulated by substrate binding, and (ii) whether release of substrates occurs upon ATP addition. We determined the rates of ATP hydrolysis of these mutant proteins in the presence of saturating concentrations of both peptide C (500 μM) and ATP (at least 5-fold above the K_m of the particular mutant protein). Clearly, the presence of peptide C did not increase the rates of ATP hydrolysis of the E171A, L, K mutant DnaK proteins (Table II). In contrast, peptide C stimulated the rate of ATP hydrolysis of DnaK$^+$ 7-fold. This stimulation is similar to the reported stimulation of the ATPases of Hsc70 (Flynn *et al.*, 1989; Sadis and Hightower, 1992), BiP (Flynn *et al.*, 1989; Blond-Elguindi *et al.*, 1993) and DnaK (R.Jordan and R.McMacken, manuscript in preparation) by peptide and protein substrates.

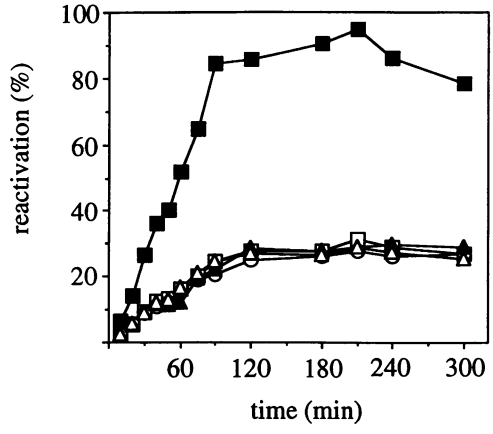

Fig. 3. E171 A, L, K mutant DnaK proteins are non-functional in the refolding of denatured luciferase. Guanidinium–HCl denatured luciferase was diluted into refolding buffer containing DnaJ and GrpE, as well as DnaK$^+$ and E171 A, L, K mutant proteins as indicated. The activity of refolded luciferase was determined in a bioluminescence assay and presented as percent of luciferase activity prior to denaturation. (■), DnaK$^+$; (○), E171A; (□), E171L; (△), E171K; (▲), no added DnaK.

We then tested the ability of Mg^{2+}/ATP to release peptides bound to E171A, L, K mutant, as well as to wild-type DnaK proteins. In these experiments, DnaK proteins were preincubated with saturating concentrations of ^{3}H-labeled peptide C ($>K_D$ for DnaK binding) to allow formation of peptide–DnaK complexes (30 min, 30°C). ATP (1 mM) was added for 1 min to allow peptide release, and DnaK–peptide complexes were separated from unbound peptide by rapid gel filtration and quantified by liquid scintillation counting. For DnaK$^+$, in agreement with other studies (R.Jordan, A.Mehl and R.McMacken, manuscript in preparation), >80% bound peptide C was released by ATP (Figure 4A). ATP acts by increasing the rate of release of bound peptide (J.McCarty, A.Buchberger and B.Bukau, submitted). In contrast, for the E171L and E171K mutant DnaK proteins the presence of ATP led to no significant decrease in the level of bound peptide. In the case of the E171A mutant DnaK protein, the complexes formed with peptide C were unstable even in absence of ATP, allowing no conclusion to be drawn. These experiments were repeated with a peptide (22 amino acids in length) derived from the bacteriophage λ cIII protein. cIII peptide binds to DnaK$^+$ with an affinity significantly higher than peptide C (J.McCarty, A.Buchberger and B.Bukau, submitted), and binds E171A, L, K mutant DnaK proteins with high affinity (data not shown). For DnaK$^+$, >70% of bound cIII peptide was released by ATP (Figure 4B). Again, the E171L and E171K mutant DnaK proteins showed no significant decrease, and the E171A mutant DnaK protein showed only a slight decrease, in the level of peptide bound in the presence of ATP. Together, the lack of ATPase stimulation and ATP-induced peptide release indicate that the mutational changes at E171 of DnaK disrupt the coupling of DnaK's ATPase activity with substrate binding indicating an essential role for E171 in this process.

Amino acid substitutions at E171 interfere with conformational changes in DnaK induced by ATP

The amino acid substitutions at E171 we demonstrated to interfere with the functional coupling of ATPase activity with

Table II. ATPase and substrate binding activities of E171 A, L, K mutant DnaK proteins

Protein	ATPase			Substrate binding
	K_m[a]	V_{max}[b]	Peptide C stimulation[c]	K_D for peptide C[a]
DnaK$^+$	3.9 ± 0.7	0.035	7.0×	2.6 ± 0.2
E171A	25.6 ± 7.8	0.140	0.9×	16.7 ± 2.5
E171L	47.8 ± 10.0	0.091	1.1×	10.4 ± 1.9
E171K	70.5 ± 6.6	1.040	0.9×	10.1 ± 1.5

[a]Expressed in μM.
[b]Expressed in pmol ATP hydrolyzed/pmol DnaK/min.
[c]Steady state ATPase activities in the presence of 500 μM peptide C were determined at saturating ATP concentrations (≥5-fold above the particular K_m) and are presented as factors of stimulation relative to the ATPase activities in the absence of peptide C.

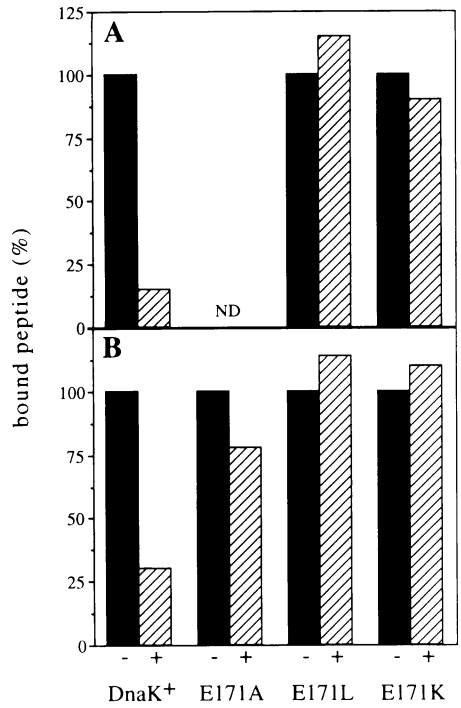

Fig. 4. E171 A, L, K mutant DnaK proteins are defective in ATP-dependent release of bound peptides. DnaK$^+$, E171A, E171L and E171K mutant DnaK proteins were incubated with ^{3}H-labeled peptide C (**A**) or cIII peptide (**B**) with (+) or without (−) subsequent addition of ATP, followed by separation of DnaK−peptide complexes from free peptide by rapid gel filtration. The amount of DnaK-bound labeled peptide found in the absence of ATP was taken as 100%.

substrate binding might interfere with a structural coupling of the ATPase domain with the substrate binding domain. For DnaK$^+$, structural coupling is manifested by conformational changes occurring both in the ATPase domain and the substrate binding domain following ATP addition (Liberek *et al.*, 1991b). These changes were characterized previously by analysis of the kinetics and pattern of protease digestion of DnaK (Liberek *et al.*, 1991b). We used this technique to examine structural coupling defects of the E171A, L, K mutant DnaK proteins. For DnaK$^+$, the kinetics and the pattern of trypsin digestion we observed (Figure 5) are similar to those described (Liberek *et al.*, 1991b); minor differences that exist are probably due to differences in the digestion and staining protocols. As reported previously, the addition of ATP to DnaK$^+$ causes (i) the rate of degradation to increase, (ii) prominent 31 and 33 kDa fragments to disappear, and (iii) 19 and 46 kDa fragments to become more prominent. It is important to emphasize that the stable 46 kDa proteolytic fragment corresponds to the ATPase domain of DnaK, as shown by immunoblot analysis using an ATPase domain-specific DnaK mAb (data not shown). This implies that major protease cleavage sites in DnaK affected by ATP reside within the C-terminal substrate binding domain. This allows us to monitor the ATP-dependent structural coupling of the ATPase domain with the substrate binding domain. In sharp contrast to DnaK$^+$, for all of the E171A, L, K mutant DnaK proteins, addition of ATP did not cause these alterations (Figure 5). For the E171A mutant DnaK protein, the rate and the pattern of degradation did not change upon ATP

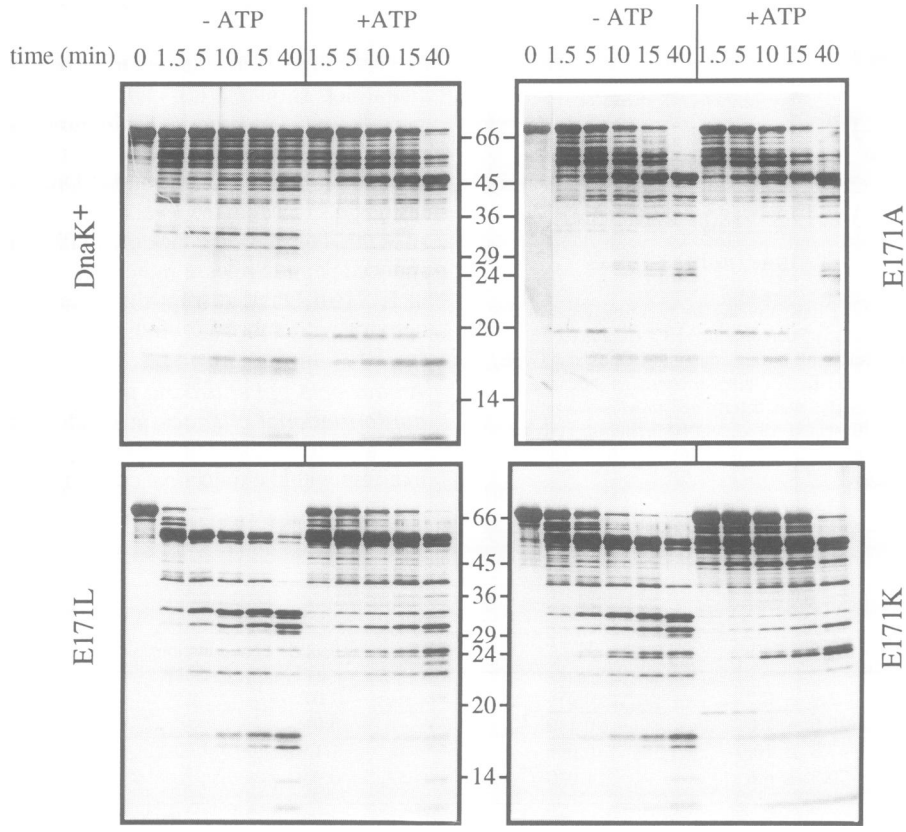

Fig. 5. E171A, L, K mutant DnaK proteins are altered in ATP-dependent conformational changes. DnaK$^+$, E171A, E171L and E171K mutant DnaK proteins were incubated with trypsin in the presence or absence of ATP. At the indicated time points, aliquots were taken and subjected to SDS−PAGE followed by silver staining of the gels. Molecular weight standards (in kDa) are indicated.

addition and were almost identical to those of DnaK$^+$ with ATP. In the case of the E171L and E171K mutant DnaK proteins, the rate and pattern of degradation were similar to each other, but different from those of DnaK$^+$ and of the E171A mutant DnaK protein, with and without ATP. Major differences are that (i) a new prominent 60 kDa degradation fragment accumulated with or without ATP, (ii) the 46 kDa fragment is not observable with or without ATP, (iii) the rate of degradation was very rapid without ATP but slower with ATP, and (iv) ATP-induced alterations in the pattern of degradation were far less pronounced as compared with DnaK$^+$. While strict interpretations of the changes in proteolytic degradation in the mutant proteins relative to wild-type are not possible at this point, it is clear that substitutions at residue E171 alter the ATP-dependent structural coupling of the ATPase domain with the substrate binding domain.

Discussion

The primary findings of this study are that (i) residue E171 of DnaK (E175 of bovine Hsc70) plays a non-essential role in ATP binding and hydrolysis, (ii) E171 is required for the coupling of ATPase activity with substrate release, and (iii) this coupling is essential for the chaperone function of DnaK.

A role for E171 in ATP binding and hydrolysis is indicated by the increased K_m and V_{max} values of the ATPase activity of DnaK associated with amino acid substitutions at residue E171. The biochemical and structural evidence so far only allows a tentative interpretation of the structural basis for these alterations in the ATPase activity. In the case of the E171A and E171L mutant DnaK proteins, the Mg^{2+} ion might bind with lower affinity compared with DnaK$^+$, thereby shifting the equilibrium between the open and closed forms of the ATPase domain (Figure 1) to the open form. As a consequence, the rate of nucleotide dissociation might increase, which is subsequently observed as an increased K_m for ATP. In the case of the E171K mutant DnaK protein, molecular modeling based on crystal structure data indicates that the charge reversal at position 171 alters the local structure of the nucleotide binding site, in part by elimination of a salt bridge normally formed between E171 and a nearby lysine (corresponding to the E175−K75 salt bridge in Hsc70). Such local structural alterations may result in a reduced affinity of the E171K mutant DnaK protein for ATP. In addition, the positively charged ϵ-amino group of lysine at position 171 might prevent the efficient binding of the Mg^{2+} ion. Studies in progress are aimed at an understanding of the increase in V_{max} also associated with the lysine replacement at E171. The preservation of ATPase activity in all three mutant proteins rules out the proposal (Tirion and ben-Avraham, 1993) that the residues corresponding to E171 of DnaK act as a catalytically essential general base.

The requirement of an intact coupling of ATPase activity with substrate binding for the chaperone function of DnaK is manifested by the inability of the E171A, L, K mutant DnaK proteins to sustain the biological activities of DnaK. *In vivo*, the *dnaK-E171A, L, K* mutations failed to complement temperature-sensitive growth and λ resistance of Δ*dnaK52* mutants. *In vitro*, the mutant proteins were unable to substitute for wild-type DnaK in λ DNA replication, presumably because they could not mediate the required protein disassembly reactions (Alfano and McMacken, 1989; Dodson *et al.*, 1989; Zylicz *et al.*, 1989). These mutant DnaK proteins also failed to promote refolding of denatured luciferase in a defined *in vitro* system (Schröder *et al.*, 1993). The functional defects of the mutant proteins observed *in vitro* are sufficient to explain the failure of the mutant proteins to function *in vivo*. Interestingly, in *dnaK*$^+$ cells expression of the *dnaK-E171A, L, K* alleles from plasmids caused a dominant negative phenotype for λ propagation and growth at high temperature. This phenotype was observed at cellular levels of the mutant DnaK proteins only slightly higher (for growth at high temperature) or even lower (for λ propagation) than normally found for DnaK$^+$, indicating that the mutant proteins are highly poisonous. This phenotype is in agreement with the recently described dominant negative phenotype in merodiploids of the *dnaK* mutation that leads to an E171→lysine change in DnaK (Wild *et al.*, 1992). This mutation was obtained by selection for mutants defective in heat shock gene regulation. The dominant negative phenotype of mutations altering E171 might result from sequestration by mutant DnaK protein of substrates that are essential for high temperature growth and λ propagation. This possibility is particularly attractive because we found the mutant proteins to be defective in ATP-mediated release of peptide substrates. Other possibilities to account for the dominant phenotype include titration of the cofactor GrpE which is essential for cell viability (Ang and Georgopoulos, 1989). Alternatively, as DnaK$^+$ is capable of oligomerization (Zylicz and Georgopoulos, 1984; Palleros *et al.*, 1992; J.McCarty and B.Bukau, unpublished results), it might be that the mutant DnaK proteins and DnaK$^+$ form hetero-oligomers with severe functional defects.

The key role of E171 in the coupling of ATPase activity and substrate release is indicated by both functional and direct structural evidence. Functionally, the E171A, L, K mutant DnaK proteins are defective in the linkage between ATP binding and substrate release. This was indicated by the lack of increase in the steady state rates of ATP hydrolysis of the mutant proteins upon addition of a peptide substrate (peptide C), in contrast to the 7-fold increase in the rate of ATP hydrolysis of DnaK$^+$ by addition of this peptide. Further, the mutant proteins did not efficiently release bound substrates upon addition of ATP. Structural evidence indicating disrupted linkage between the ATPase and the substrate binding domain is derived from studies examining conformational changes normally occurring in the ATPase and substrate binding domains of DnaK upon ATP addition. ATP addition alters the proteolytic susceptibility of DnaK$^+$, in particular of its C-terminus, thus emphasizing structural coupling between ATPase and substrate binding domains. This coupling was not observed for the E171A mutant DnaK protein. The lack of ATP-induced conformational changes in this mutant protein is strong support for a role of E171 in providing structural coupling of the two DnaK domains. Interestingly, the E171L, K mutant DnaK proteins differed in the ATP-dependent alterations of the proteolytic susceptibility not only to DnaK$^+$, but also to the E171A mutant DnaK protein. The reason for these differences is currently under investigation.

Identification of the central role of E171 in the coupling of the ATPase activity with substrate release allows us to hypothesize a coupling mechanism. We propose that the postulated ATP-dependent conformational change in the

ATPase domain (Holmes *et al.*, 1993) triggers conformational changes in the substrate binding site in the C-terminal domain that subsequently cause release of bound substrates. The conformational coupling might be established through a physical interaction of the substrate binding domain with the ATPase domain that is influenced by the opening/closing movement of the ATPase subdomains. One role of E171 in this coupling might be to control the equilibrium between the nucleotide-bound closed form and the nucleotide-free open form of the ATPase domain. This might rely on a function of E171 in tight binding and correct positioning of the Mg^{2+} ion and, consequently, of the ATP. A shift in this equilibrium to the open form or an alteration of the kinetics of this movement by substitution of E171 may account for the coupling defects of the mutant DnaK proteins investigated in this study.

A direct role of E171 in the interaction of the substrate binding domain with the ATPase domain is suggested by the effects of mutants in position 171 on the domain interaction even in the absence of added nucleotide as revealed by limited proteolysis experiments. It is possible that E171 is required for formation of an ATP-controlled binding structure involving the hinge region. Consistent with such a role, the residues of actin-like proteins, that correspond to E171 of DnaK, all occupy a critical position in the connection between ATPase subdomains I and II (Bork *et al.*, 1992; Holmes *et al.*, 1993; Tirion and ben-Avraham, 1993). They are located at the bottom of the nucleotide binding pocket, just above the lower cleft that separates the two subdomains (see Figure 1 for Hsc70 ATPase). In this lower cleft, just below the residues corresponding to E171 of DnaK, a number of conserved residues exist (Bork *et al.*, 1992) that are surface exposed in the 3-D structure of the nucleotide-bound forms of actin and Hsc70. From model building it appears that these residues change their surface accessibility in dependence of the nucleotide state of the protein (Holmes *et al.*, 1993; A.Valencia and C.Sander, unpublished data). These residues are therefore prime candidates for providing a binding site for the substrate binding domain of Hsp70 proteins. In support of this hypothesis, it has been shown that the lower cleft of the actin monomer does indeed provide a binding site for other monomers along the axis of the actin fiber (Holmes *et al.*, 1990). Finally, it is possible that E171 has another, even more direct, role in physically binding the substrate binding domain to the ATPase domain, e.g. by directly contacting residues of the substrate binding domain.

Material and methods

Bacterial strains, plasmids and growth conditions

Strains MC4100 (F$^-$ *araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 deoC1 ptsF25 rpsR flbB301*) (Casadaban, 1976) and BB1553 (MC4100 Δ*dnaK52*::Cm^r *sidB1*) (Bukau and Walker, 1990) have been described previously. BB1996 is a pDMI,1-carrying *recA*::Cm^r derivative of MC4100. Bacteria were grown at 30°C or at the indicated temperatures in Luria broth (Sambrook *et al.*, 1989) supplemented with kanamycin (40 μg/ml) and ampicillin (100 μg/ml) when required. Plasmids pDMI,1 (*lacI*^{q}, Kn^r) (Lanzer, 1988) and pDS12 (Ap^r) (Stüber and Bujard, 1982) have been described earlier. pUHE21−2fdΔ12 is a derivative of pDS12 carrying, in addition, the fd origin of replication and a 12 bp deletion upstream of the *Bam*HI site which allows translation of cloned genes to start at their authentic start codons.

Cloning techniques and site-specific mutagenesis

DNA manipulations were performed as described (Maniatis *et al.*, 1989). The *dnaK*$^+$ gene of MC4100 was amplified by PCR according to the instructions of the supplier of Taq polymerase (Boehringer Mannheim). The amplified *dnaK*$^+$ gene was cloned as a *Bam*HI−*Hin*dIII fragment into pUHE21-2fdΔ12, such that its expression is controlled by the $P_{A1}/lac_{O3/O4}$ promoter−operator system. Site-directed mutagenesis of the cloned *dnaK*$^+$ gene to generate *dnaK-E171A*, *dnaK-E171L* and *dnaK-E171K* was performed using the Mutagene kit from Bio-Rad. The entire DNA sequences of all cloned *dnaK* alleles were determined by the dideoxynucleotide method (Sanger *et al.*, 1977) and found to be as expected.

Purification of DnaK

Wild-type and mutant DnaK were overproduced in Δ*dnaK52* mutants (BB1553) which carry pDMI,1 and pUHE21-2fdΔ12 encoding the desired *dnaK* allele. Cultures of these cells were grown at 30°C to logarithmic phase and induced for 4 h with IPTG (1 mM final concentration). The DnaK proteins were purified as described (McCarty and Walker, 1991) with the following modifications. All liquid chromatography steps were performed in buffer A (25 mM HEPES−KOH, pH 7.6, 50 mM KCl, 2.5 mM $MgCl_2$, 1 mM EDTA, 10 mM 2-mercaptoethanol, 10% glycerol). The ammonium sulfate precipitate was resuspended and dialyzed against buffer A and, instead of a heparin agarose column, was applied to a DEAE Sepharose FF column (250 ml; Pharmacia). DnaK was eluted by a linear 50−550 mM KCl gradient (500 ml). For DnaK preparations of highest purity used in ATPase activity assays, the MonoQ fractions containing DnaK were pooled and subjected to gel filtration using a Superdex S200 column (Pharmacia). Fractions containing monomeric DnaK were pooled and kept at −80°C until further use. All DnaK preparations used were free of contaminations by DnaJ, GrpE and GroEL, as judged by immunoblot analysis. Except for the λ DNA replication tests, all *in vitro* assays in this study were performed using at least three independently purified DnaK preparations. The protein concentrations were based on amino acid analysis of DnaK.

λ DNA replication

In vitro replication of λ DNA was carried out essentially as described (Mensa-Wilmot *et al.*, 1989). The reaction was performed in 30 μl of the following mixture: 40 mM HEPES−KOH, pH 7.6, 100 mM potassium glutamate, 11 mM magnesium acetate, 4 mM ATP, 180 μM of each dATP, dCTP and dGTP, 80 μM [^{3}H]dTTP (2.4 μCi/nmol total deoxynucleotide), 50 μg/ml BSA, 215 ng oriλ-carrying pRLM4 DNA, 195 ng λ O, 100 ng λ P, 175 ng DnaB, 540 ng SSB, 50 ng DnaJ, 75 ng GrpE, 100 ng primase, 80 ng DNA polymerase III, 230 ng GyrA, 240 ng GyrB and mutant or wild-type DnaK proteins in amounts as indicated. This mixture was incubated for 40 min at 30°C followed by liquid scintillation counting of TCA-precipitable radiolabeled material.

Luciferase refolding assays

A stock solution of firefly luciferase (64 μM in 1 M glycylglycine, pH 7.4) (Sigma) was diluted 6.4-fold into unfolding buffer (25 mM HEPES−KOH, pH 7.6, 50 mM KCl, 5 mM $MgCl_2$, 5 mM β-mercaptoethanol, 6 M guanidinium−HCl) and denatured by incubation at room temperature for 1 h. Denatured luciferase was diluted (80 nM final concentration) into refolding buffer (25 mM HEPES−KOH, pH 7.6, 50 mM KCl, 5 mM $MgCl_2$, 5 mM dithiothreitol, 1 mM ATP) containing 160 nM DnaJ, 200 nM GrpE and 460 nM DnaK, as indicated. Aliquots of 2 μl were withdrawn at the indicated times, diluted into 250 μl assay buffer (25 mM glycylglycine, 15 mM $MgSO_4$, 5 mM ATP) and analyzed for bioluminescence activity in a Biolumat (Bertholdt), as previously described (Schröder *et al.*, 1993).

ATPase activity determinations

The rates of ATP hydrolysis were determined as described (Liberek *et al.*, 1991a). Reactions were performed at 30°C in mixtures containing buffer R [40 mM HEPES−KOH, pH 7.6, 50 mM KCl, 11 mM $Mg(OAc)_2$], 0.39 μM DnaK, [α-^{32}P]ATP (0.5 μCi/nmol, Amersham) and peptide C (Flynn *et al.*, 1989), as indicated. The range of ATP concentrations used for the K_m and V_{max} determinations was: DnaK$^+$, 2−30 μM; E171A, 10−120 μM; E171L, 25−400 μM; E171K, 50−400 μM. K_m and V_{max} values were calculated using the non-linear regression program Enzfitter (Elsevier).

For immunodepletion experiments, purified polyclonal DnaK antibodies (Bukau *et al.*, 1993) were covalently coupled to CNBr-activated Sepharose 4B beads (Pharmacia) at a concentration of 5 mg antibodies per 1 ml of beads, according to the manufacturer's instructions. 100 μl of a solution of purified DnaK were applied to a DnaK immunoaffinity column (250 μl) and eluted with 500 μl buffer R. The flowthrough contained <5% of the applied DnaK, whereas in control experiments 90% of BSA that was applied to the column was recovered in the flowthrough. Aliquots of the flowthrough were assayed for ATPase activity.

Substrate binding determinations

Peptide C binding to wild-type and mutant DnaK proteins was determined by equilibrium dialysis in a microdialyzer (Hoeffer) according to the manufacturer's instructions. The dialysis membrane (EMD103, Hoeffer) had a molecular weight cut-off of 12–14 kDa. Each half chamber of the microdialyzer was filled with 50 μl buffer R containing 0.1 mg/ml BSA. DnaK was added to one half chamber at a concentration of 9.5 μM; [^{3}H]peptide C (0.01–0.02 μCi/nmol) was added to both half chambers at concentrations ranging from 1 to 200 μM. Radioactive labeling of peptide C was carried out by reductive methylation (Tack *et al.*, 1980). Dialysis was performed at room temperature for 12 h. Then, aliquots of each half chamber were taken and the amount of radiolabeled peptide C was determined by liquid scintillation counting. The concentrations of free and DnaK-bound [^{3}H]peptide C in each half chamber were calculated and the K_D of DnaK for peptide C was determined using the Enzfitter program.

Release of DnaK-bound peptide by ATP was tested as follows. A reaction mixture (50 μl) was set up containing buffer R, DnaK and [^{3}H]peptide C (0.2 μCi/nmol) or [^{3}H]cIII peptide (0.05 μCi/nmol) (ESLLERITRKLRDGWKRLIDIL; Kornitzer *et al.*, 1991) at concentrations as follows: peptide C, 15 μM; cIII peptide, 5 μM; DnaK, 8.55 μM (for peptide C) and 5.7 μM (for cIII peptide). Peptide release of DnaK–E171A was tested with 11.4 μM E171A and 20 μM cIII peptide. After 30 min incubation at 30°C, an aliquot was removed to determine the amount of total radioactivity, and the remainder was further incubated for 1 min at 30°C in the presence or absence of ATP (4 mM final concentration) and then applied to a Sephadex G25 nick column (Pharmacia). Three-drop fractions were collected and the amount of radiolabeled peptide was determined by liquid scintillation counting. Under these conditions, unbound peptides eluted in fractions 11–20, whereas DnaK–peptide complexes eluted in fractions 6–8.

Proteolytic degradation of DnaK

11.4 μg DnaK was preincubated in buffer T (40 mM Tris–HCl, pH 7.6, 8 mM $Mg(OAc)_2$, 20 mM NaCl, 20 mM KCl, 0.3 mM EDTA, 2 mM DTT) for 30 min at 30°C with or without 5 mM ATP. After starting the proteolytic digestion of DnaK by addition of 0.15 μg trypsin (Merck), aliquots were taken at specified time points, added to an equal volume of 2 × SDS sample buffer, boiled immediately and analyzed by denaturing SDS–PAGE (Laemmli, 1970). Gels were silver stained according to Blum *et al.* (1987).

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Note added in proof

In a recent paper [(1993) *J. Biol. Chem.*, **268**, 24323–24329], O'Brien and McKay report the active site conformation of the Hsc70 ATPase domain in the Mg^{2+}-ADP and P_i bound state. The details of the Mg^{2+} coordination, including a hydrogen bond between residue E175 and a water molecule in the primary coordination sphere of magnesium, are in full agreement with the model proposed in our paper.