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DnaK as a thermometer: Threonine-199 is site of autophosphorylation and is critical for ATPase activity

(*Escherichia coli*/heat shock/molecular chaperone/regulation/σ^{32})

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ABSTRACT **DnaK, the sole *Escherichia coli* member of the highly conserved 70-kDa heat shock protein (HSP70) family of proteins, autophosphorylates when incubated with ATP *in vitro*. We show that threonine-199 is the amino acid that becomes phosphorylated and we demonstrate that threonine-199 is critical for the ATPase activity of DnaK. We also report that both the ATPase and autophosphorylating activities of DnaK increase very strongly over the range of temperatures that is physiologically relevant for *E. coli* growth. The temperature dependence of either or both of these activities could be of significance with respect to the postulated role of DnaK as a molecular chaperone in helping cells ameliorate the deleterious consequences of elevated temperature. Furthermore, we postulate that DnaK plays a key role in regulation of the heat shock response by serving as a cellular thermometer that directly senses the environmental temperature.**

DnaK is the sole *Escherichia coli* member of the highly conserved family of 70-kDa heat shock proteins (HSP70s) that act as molecular chaperones (1, 2). The expression of *dnaK* is induced by heat shock and other forms of stress in a σ^{32}-dependent manner. *dnaK* missense mutants display a variety of phenotypes, including the inability to replicate bacteriophage λ DNA, temperature sensitivity for growth (3), and a defect in the initiation of chromosome replication (4). DnaK plays critical roles in regulation of the heat shock response and negatively affects σ^{32} translation, stability (5), and activity (6). Analyses of a null mutant (Δ*dnaK52*) have shown that cells lacking DnaK can grow at 30°C but not at higher temperatures (7, 8). However, a Δ*dnaK52* mutant growing at 30°C displays a variety of severe phenotypes including extensive filamentation, slow growth, defects in chromosomal partitioning, and high levels of synthesis of the HSPs (8–10). At 30°C, all of these phenotypes can be suppressed by *rpoH* mutations that reduce σ^{32} activity (10). These results indicate that DnaK is not essential for growth at 30°C and these first three phenotypes are due to the inappropriately high expression of the remaining heat shock genes at 30°C. Thus, the major physiological role of DnaK at 30°C appears to be to negatively regulate the heat shock response (10). However, it appears that DnaK is critical for cellular survival at higher temperatures. Many *dnaK* mutants including Δ*dnaK52* are unable to grow at higher temperatures such as 42°C and Kusukawa and Yura (11) have observed that high levels of DnaK are required primarily at higher temperatures.

Like other members of the HSP70 family, DnaK has a weak ATPase activity and functions as a molecular chaperone (1, 2). For example, DnaK has been shown to be necessary for initiation of λ and P1 replication (12, 13) and to be sufficient to reactivate heat-denatured RNA polymerase in a manner dependent on ATP hydrolysis (14). DnaK associates with the GrpE protein both *in vivo* and *in vitro* (15) and recently it has been demonstrated that DnaK ATPase is stimulated when assayed in the presence of DnaJ and GrpE (16).

In vitro DnaK becomes autophosphorylated on a threonine if incubated in the presence of ATP (17), as do various other members of the HSP70 family (18). The autophosphorylation of DnaK has been found to be strongly stimulated by Ca^{2+} (19, 20). DnaK has been reported to be phosphorylated *in vivo* on serine and threonine under certain conditions (21) and evidence has been reported indicating that various eukaryotic members of the HSP70 family also become phosphorylated *in vivo* (22–25). In this study, we undertook identification of the threonine of DnaK that becomes autophosphorylated *in vitro*. We found that this threonine was crucial for ATPase activity as well and we also discovered a strong temperature dependence of the DnaK ATPase and autophosphorylation activities that could have physiological significance.

MATERIALS AND METHODS

ATPase Assays. ATPase rates were determined in a 50-μl reaction mixture containing 100 mM buffer [Mes (pH 6.2) or Hepes (pH 8.1)], 10% (vol/vol) glycerol, 35 mM KCl, 5 mM $MgCl_2$, 2.5 μg of wild-type or mutant DnaK, 70 μM ATP, 10 μCi of [α-^{32}P]ATP (Amersham; 3000 Ci/mmol; 1 Ci = 37 GBq), and 5 mM 2-mercaptoethanol after preincubations for 5 min at the indicated temperature. The reaction was terminated and rates were determined as described (26) and corrected for spontaneous hydrolysis with reactions under identical conditions and length of incubation that contained no added protein. Protein concentration for DnaK and mutant protein was determined with Bio-Rad protein assay reagent and correlated to concentration with immunoglobulin standard and absorbance of DnaK in guanadine hydrochloride at 280 nm (27).

Site-Directed Mutagenesis. All DNA manipulations were as described (28) and mutagenesis was performed using the Muta-Gene kit (Bio-Rad). pJM1 was constructed by subcloning the *Bam*HI fragment from pBB1 (B. Bukau and G.C.W., unpublished results) containing the *dnaKJ* operon into the SK+ plasmid (Stratagene) and was maintained at 30°C to substantially lower the copy number (29). Oligonucleotide-directed deletion of this plasmid placed *dnaKJ* under P_{lac} control (pJM2). This plasmid was maintained in MC4100 cells containing $LacI^q$ and grown in rich medium with 0.1% glucose at 30°C and was mutagenized with an oligonucleotide degenerate at a single base to yield derivatives encoding mutant DnaK.

Protein Purification. DnaK was purified as described (20), except for use in determining ATPase rates, where the purification was modified to eliminate contaminating AT-

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Abbreviations: HSP, heat shock protein; HSC, heat shock cognate protein.

Pases. Two liters of Δ*dnaK52* (10) cells carrying a plasmid encoding wild-type or mutant DnaK were grown to late logarithmic phase at 30°C and induced by shifting to 37°C and adding 1 mM isopropyl β-D-thiogalactopyranoside. Lysis was performed as described (20) and protein was precipitated by adding 280 g of ammonium sulfate per liter followed by centrifugation for 30 min at 30,000 rpm (70 Ti rotor; Beckman). The resulting pellet was resuspended in 5–10 ml of buffer A (25 mM imidazole, pH 7.0/10% sucrose/25 mM NaCl/5 mM $MgCl_2$/5 mM 2-mercaptoethanol), dialyzed extensively against buffer A, and applied to a 2-ml heparin/agarose column (Pharmacia). The flow-through was then repeatedly applied to a 2.5-ml ATP/agarose column (Sigma; A2767), washed with 10 column vol of buffer A containing 500 mM NaCl followed by 2 column vol of buffer A, and eluted with buffer Q [25 mM Hepes, pH 7.6 (with KOH)/50 mM KCl/1 mM EDTA/5 mM 2-mercaptoethanol/10% glycerol] containing 5 mM ATP. The fraction containing the highest concentration of protein was applied to a Sephadex 75 column (10/30) on an FPLC (Pharmacia) and eluted with buffer Q. Wild-type DnaK and mutant forms eluted as two overlapping peaks; the fractions for each peak were pooled separately and reapplied to a 1-ml Mono Q column equilibrated with buffer Q. A 10-ml gradient was run from 50 to 500 mM KCl in buffer Q, collecting 0.5-ml fractions.

RESULTS

Identification of the Phosphorylated Residue of DnaK. C-18 reverse-phase column separation of tryptic fragments from DnaK phosphorylated *in vitro* revealed two labeled peaks (Fig. 1*A*), with the major peak containing 97% of counts retained on the column. We rechromatographed the two fractions with the most counts from the major peak on a C-4 column. For each fraction, all the radioactive material bound

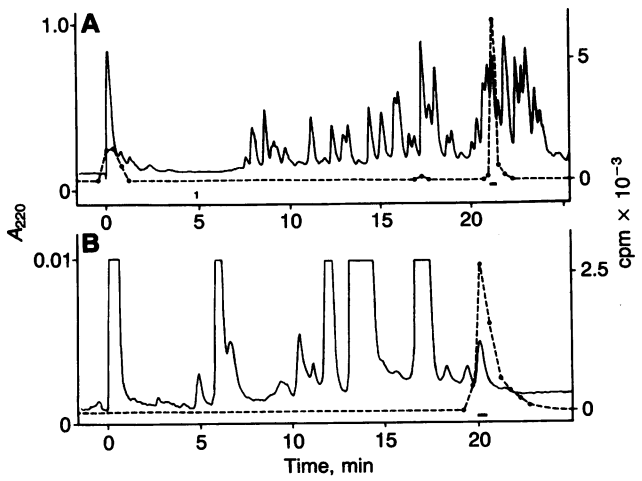

FIG. 1. HPLC purification of the phosphorylated tryptic fragment of DnaK. DnaK was phosphorylated at 37°C by dialyzing 10 mg of DnaK against 5 mM ATP (50 mM Mes, pH 6.2/10% glycerol/5 mM 2-mercaptoethanol/5 mM $MgCl_2$/10 mM $CaCl_2$) and was mixed with 10 μg of DnaK phosphorylated (20) with [γ-^{32}P]ATP of high specific activity. CPTK trypsin (Worthington)-digested protein was prepared as described (30) and separated on HPLC (Waters) utilizing C-18 followed by C-4 columns. (*A*) C-18 column (Vydac) separation with 0–100% B (31) buffer over 60 min at 2 ml/min. (*B*) A fraction from the peak containing 97% of the counts retained on the C-18 column was reapplied to a C-4 column (Bakerbond; wide-bore, 5 μm) and was separated with 20–40% B buffer over 30 min at 2 ml/min. A fraction of this material was then subjected to microsequencing analysis. Shown here are the portions of the gradients that contained radioactive material as determined by Cerenkov radiation from the whole sample in a Beckman scintillation counter. Fractions used in further purification steps are indicated by a bar.

Peptide	TIAVYDLGGG FDISIIEISEVDGE
DnaK	189TIAVYDLGGG**T**FDISIIEISEVDGEK
Bovine HSC70	195VLIFDLGGG**T**FDVSILTIEDG
Human HSP70	195VLIFDLGGG**T**FDVSILTIDDG

FIG. 2. Comparison of peptide sequence generated from microsequencing of purified phosphorylated peptide with DNA-predicted sequences of DnaK (32) and HSP70 homologs (33, 34). Microsequencing was performed on an Applied Biosystems 477A protein sequenator with an on-line phenylthiohydantoin derivatized amino acid analyzer. Blank space indicates a residue giving much lower yield than other cycles and not assignable as the predicted threonine.

to the C-4 column and eluted as a single peak (Fig. 1*B*). In each case, the optical density of the resulting fractions correlated with the amount of radioactivity present in each fraction. Twenty-five cycles of microsequencing (Fig. 2) of the material contained in this peak revealed that it corresponded, with the exception of one amino acid, to the first 25 residues of a DnaK tryptic fragment predicted by the DNA sequence of *dnaK* (32). The cycle for the 11th residue had a much lower yield and could not be assigned as the predicted threonine. This would be the expected result if Thr-199 were phosphorylated.

Mutations Altering the Site of DnaK Autophosphorylation. To verify our identification of Thr-199 as the phosphorylated residue and to assess the significance of the autophosphorylation to DnaK activity, we used site-directed mutagenesis to alter Thr-199 of DnaK to Ala, Val, and Asp. Wild-type (DnaK T199) and mutant proteins (DnaK TA199, TV199, and TD199, respectively) were then purified after isopropyl β-D-thiogalactopyranoside induction from an *E. coli* strain carrying the Δ*dnaK52* deletion mutation with a *sidA* suppressor, which suppresses a septation defect and allows faster growth (10). Under the conditions used in their purification (4°C; pH 8.1), all three mutant DnaK proteins bound to ATP/agarose in a manner indistinguishable from that of wild-type protein. However, we found that all three purified mutant proteins were completely deficient in autophosphorylation at 37°C or 52°C (Fig. 3), a result that strongly supports our assignment of Thr-199 as the site of autophosphorylation. The lack of even low levels of autophosphorylation suggests that the minor (3%) phosphorylated peak observed on C-18 separation of wild-type DnaK tryptic fragments (see Fig. 1) is a different peptide, possibly resulting from aberrant trypsin digestion (30, 35), that also contains Thr-199.

Autophosphorylation Site Mutants of DnaK Are Defective in ATPase Activity. We next examined the ATPase activities of highly purified proteins. Because wild-type DnaK has a very weak ATPase activity (0.15 ATP hydrolyzed per DnaK per min at 37°C), great care was taken to purify the wild-type and three mutant proteins under identical conditions. All three mutant proteins behaved during all steps of the purification in a manner indistinguishable from wild type. In contrast to the similarity in behavior of the proteins during purification, analysis of two separate fractions from each protein revealed that all three mutant proteins hydrolyzed ATP at a rate that

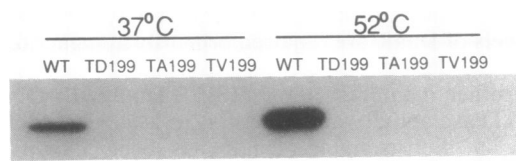

FIG. 3. Comparison of autophosphorylation activity of wild-type and mutant DnaK proteins. Wild-type (DnaK T199) and mutant proteins with aspartic acid, alanine, and valine at position 199 (DnaK TD199, TA199, and TV199 encoded on plasmids pJM3, pJM4, and pJM5, respectively) were phosphorylated in the presence of 5 mM Mg^{2+} and 10 mM Ca^{2+} as described (20) at 37°C and 52°C.

Table 1. ATPase rates of wild-type and mutant DnaK proteins at 37°C (ATP cleaved per DnaK per min)

Fraction	DnaK+ T199	Mutant DnaK TV199	Mutant DnaK TA199	Mutant DnaK TD199
A	0.15	0.001	0.003	0.009
B	0.15	0.001	0.007	0.011

Rates were determined at pH 8.1 from time points (other than zero) where <5% of ATP was converted to ADP and were corrected for spontaneous hydrolysis. Fractions A and B were from protein separated during a gel-filtration step during purification. All the fractions assayed, including fractions A and B from the same protein, were from the same position on a KCl anion-exchange gradient on a FPLC Mono Q column (Pharmacia).

was less than 1/15th that of wild-type protein (Table 1). The presence of either alanine or valine at position 199 resulted in ATPase rates essentially indistinguishable from spontaneous hydrolysis of ATP, while the slight remaining ATPase activity observed for DnaK TD199 could be associated with either the mutant DnaK or with a minor contaminating protein. At 30°C, this same wild-type DnaK demonstrated an initial ATPase rate of 0.06 ATP cleavage per DnaK protein per min, a rate that is 20 times lower than previous reports under similar conditions (17, 36). Although we have noticed that the presence of plasmids encoding the mutant proteins alter cell growth and heat shock protein expression, careful physiological characterization of the mutant phenotypes will require subcloning from the high copy number SK+ vector.

DnaK ATPase Activity and Autophosphorylation Are Highly Temperature Dependent. We discovered that autophosphorylation and ATPase activities of DnaK are both highly temperature dependent. At pH 6.2, the optimal pH for autophosphorylation, autophosphorylation activity was found to increase 400-fold between 20°C and 50°C, while ATPase activity increased 70-fold in the range of 20°C to 53°C (Fig. 4*A*). HPLC analysis of tryptic fragments of DnaK phosphorylated at various temperatures showed that the same peptide is phosphorylated at all temperatures (data not shown). Above 53°C, both activities declined precipitously as a function of temperature. We also observed a similar increase of 80-fold in the ATPase activity at the more physiological pH of 7.6 in the temperature range of 20°C to 53°C (data not shown). At pH 8.1, near the optimum pH for ATPase activity at 37°C (pH 8.8), ATPase activity increased less sharply above 37°C and exhibited the same decrease in activity at temperatures above 53°C. Autophosphorylation at this pH increased 20-fold between 20°C and 45°C, before decreasing dramatically (Fig. 4*B*). Autophosphorylation and ATPase activities were not increased at a given temperature by preincubation at a higher temperature, indicating that the increases in activities are not a permanent alteration in DnaK. In addition, we assayed two other agents, ethanol and Cd^{2+}, which are known to mimic heat shock as a stress agent *in vivo*. In each case, no increase in autophosphorylation could be detected with purified DnaK under a variety of conditions and ATPase activity was not increased by the addition of 10 mM Cd^{2+}. Although there are slight differences in temperature of maximum activity, these results are consistent with there being a relationship between the ATPase and autophosphorylating activities of DnaK. Furthermore, they raise the possibility that DnaK itself could function as a direct sensor of the environmental temperature.

At pH 6.2, the phosphate on DnaK was stable to incubation with excess ATP and ADP (up to 1 hr postincorporation) at several incubation temperatures. Also, no loss of incorporated phosphate was detected by incubation at temperatures that were either higher (63°C) or lower (37°C) than the original temperature of incorporation (50°C). These results indicate that dephosphorylation or cycling of incorporated phosphate does not occur at any temperature tested at pH 6.2, and therefore such an activity could not explain the low incorporation observed above 50°C.

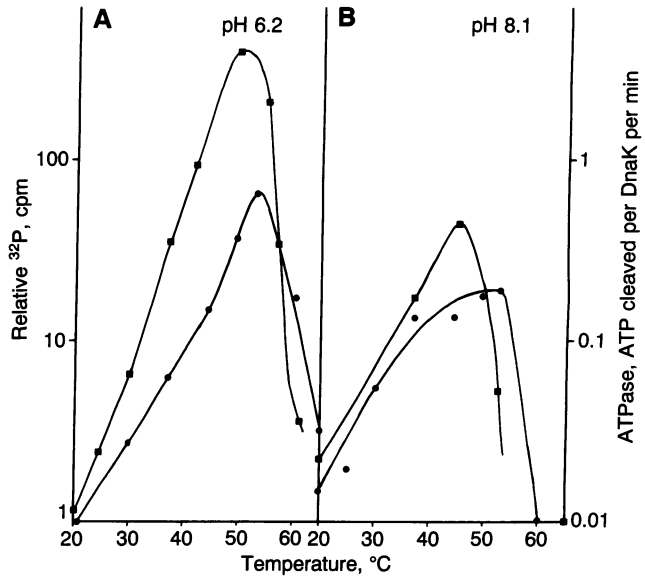

FIG. 4. ATPase (●) and autophosphorylation (■) activities of DnaK as a function of temperature. ATPase rates were determined from time points (other than zero) where <5% of ATP was converted to ADP. Phosphorylation was determined in reactions as described (20) at the indicated temperature with the following buffers and divalent cations: at pH 6.2, 100 mM Mes/5 mM $MgCl_2$/10 mM $CaCl_2$ (*A*); at pH 8.1, 100 mM Hepes/10 mM $CaCl_2$ (*B*). Reaction mixtures were allowed to preincubate for 5–30 min and reactions were terminated at 15 min by addition of equal volumes 2× PAGE loading buffer and immediately boiled. Gel fragments containing DnaK from 10% PAGE gels were counted on a Beckman scintillation counter in a digestant fluor (Fluorosol; National Diagnostics, Manville, NJ).

DISCUSSION

In this paper, we have shown that the ATPase and autophosphorylating activities of DnaK increase very strongly over the range of temperatures that is physiologically relevant to the growth of *E. coli*. The temperature dependence of either or both of these activities could be of significance with respect to the postulated role of DnaK as a molecular chaperone in helping to ameliorate the deleterious consequences of elevated temperature and also to its known roles in regulation of the heat shock response. In addition, we have shown that Thr-199 is the amino acid of DnaK that becomes phosphorylated during the autophosphorylation reaction and, furthermore, that Thr-199 is critically important for the ATPase activity of DnaK. Thr-199 corresponds to Thr-204 of the bovine 70-kDa heat shock cognate protein (HSC70) derivative whose crystal structure has recently been determined (37) and, by analogy, would be expected to be located extremely close to the γ-phosphate of the bound ATP.

The temperature stimulation of DnaK ATPase and autophosphorylation activities could permit DnaK to function more efficiently as a molecular chaperone at high temperatures. Thus, the strategy for *E. coli* survival at higher temperatures might include not only increasing the expression of DnaK (38) but also increasing the activity of DnaK. Other organisms, including eukaryotes, could utilize a similar strategy. Since the release of bound peptides by HSP70s requires ATP hydrolysis (1, 39), a faster rate of ATP hydrolysis by DnaK at higher temperatures might allow it to carry out more efficiently such postulated functions as mediating the folding of proteins and the disaggregation of complexes of denatured proteins (1, 40, 41). The stimulation of DnaK

ATPase by temperature is large compared to the stimulation of HSP70 ATPase by various peptides reported by Flynn *et al.* (39) and indeed appears to be an independent effect since we have recently found that DnaK ATPase activity is stimulated 4- to 5-fold by peptide C (39) at all temperatures tested in the range 30°C–50°C at pH 7.6 (unpublished results). Furthermore, the subset of DnaK molecules that become autophosphorylated could play some special role in DnaK function such as binding to unfolded proteins (as postulated by C. Panagiotidis, G. Giatanaris, W. Burkholder, M. E. Gottesman, and S. Silverstein, personal communication). Our suggestion that DnaK might be more active at higher temperatures *in vivo* could help to explain Kusukawa and Yura's (11) observation that GroE plays a key protective role in supporting growth at normal physiological temperatures (20°C–40°C), whereas DnaK is important primarily at higher temperatures. In addition, temperature-dependent activities of DnaK (and perhaps other heat shock proteins) could account for the discrepancy in providing thermotolerance between a heat treatment at 42°C and a nonthermal induction of HSPs. VanBogelen *et al.* (42) reported that induction of HSPs by isopropyl β-D-thiogalactopyranoside in cells containing P_{tac}-*rpoH* failed to provide thermotolerance. Furthermore, in light of the facts that *E. coli* can only sustain balanced growth up to 49°C and can survive only short incubation periods above this temperature (43), it is interesting that the ATPase and autophosphorylation activities of DnaK decline sharply at temperatures above 53°C. This correlation suggests that DnaK may be one of the key molecules whose properties determine the physiological growth range of *E. coli*.

The stimulation of the ATPase and autophosphorylation activities of purified DnaK with increasing temperature is sufficiently strong that either could be used to measure temperatures in the range of 20°C–50°C with a considerable degree of precision. Thus, at least *in vitro*, DnaK directly senses temperature within a physiologically relevant range and can be used as a thermometer. This leads us to hypothesize that, *in vivo*, DnaK may function as a cellular thermometer that directly senses temperature. For example, if the rapid degradation of σ^{32} (5) requires association of σ^{32} with DnaK (5, 44), then the stabilization of σ^{32} observed after a heat shock (5) could be due to an increase in the amount of unbound σ^{32} present in the cell. If hydrolysis of ATP results in dissociation of σ^{32} from DnaK, as is observed for peptide release from HSP70s (39), then an increased rate of ATP hydrolysis at higher temperature could result in an increase in the pool of free σ^{32} and an increase in heat shock gene expression. Since HSP70s have been implicated in regulation of the heat shock gene in eukaryotes (for reviews see refs. 44 and 45), it is possible that direct sensing of temperature by a HSP70 could play a role in regulation of the heat shock response in eukaryotes as well, possibly by affecting interactions between a HSP70 and HSF (the heat-shock transcription factor).

Our use of the word "thermometer" differs fundamentally from recent use of the same term by Gross and coworkers (5, 44), who have proposed that a homeostatic mechanism involving the level of free DnaK in a cell provides a thermometer for reacting to temperature changes. They suggest that DnaK interacts with "substrates," such as partially denatured proteins and misfolded nascent polypeptides that are postulated to arise after an increase in temperature, thereby temporarily depleting the pool of free DnaK. We are raising the possibility that DnaK functions as a thermometer by directly sensing the environmental temperature, whereas Craig and Gross (44) have suggested that DnaK is indirectly sensing the environmental temperature by monitoring the cellular consequences of an increase in temperature. The two models for how DnaK senses temperature are not mutually exclusive and both could be operative under certain conditions with DnaK serving to integrate the direct and indirect effects of temperature. It seems reasonable that proteins would unfold as an organism shifts to temperatures that are near or above the upper limit of its growth range and thus that, under such circumstances, the sensing of such partially denatured proteins by DnaK as proposed by Craig and Gross (44) would contribute to the induction of the heat shock response as well as any direct sensing of the temperature by DnaK. The suggestions that DnaK can be titrated by unfolded or misfolded proteins is supported by a considerable body of evidence summarized by Craig and Gross (44).

Our hypothesis that direct sensing of the environmental temperature by DnaK plays a role in regulation of the heat shock response is particularly attractive for explaining the DnaK-regulated increase in heat shock gene expression that is observed when cells are shifted from lower temperatures to temperatures that are below 39°C, the optimal growth temperature for *E. coli* (46, 47). For example, Lemaux *et al.* (48) showed that major HSPs of *E. coli*, such as GroEL, are induced when cells are shifted from 28°C to 33°C or 36°C and Yamamori and Yura (49) showed that the set of HSPs is induced upon a shift from 30°C to 34°C. Our hypothesis does not demand that proteins unfold or misfold during shifts to temperatures that are below an organism's optimal growth temperature in order to induce a heat shock response. Furthermore, even when cells are shifted from lower temperatures to 42°C, a temperature used in many studies of the *E. coli* heat shock response, the cells are being shifted to a temperature at which they have a slightly higher growth rate than at 37°C (47). This raises the questions as to whether, during shifts to 42°C, enough unfolding or misfolding of cellular proteins occurs to constitute the primary signal for induction of the heat shock response and leads us to suggest that direct sensing of the environmental temperature by DnaK might be of primary importance under these conditions as well.

The model proposed by Craig and Gross is attractive for explaining the induction of HSPs by various agents and treatments in the absence of any change in temperature. However, the induction of heat shock gene expression by expression of unfolded repressor (50), misfolded foreign proteins (51), or the amino acid analog canavanine (51) results in a slow steady induction of heat shock gene expression. This differs markedly from that observed after a heat shock: a rapid transient increase in heat shock gene expression followed by a rapid decrease that leads to a higher steady-state level of HSPs (5). Direct sensing of the environmental temperature by DnaK could account for the rapid induction of heat shock gene expression after a heat shock, where the maximal induction is ≈5 min after the shift (5). Furthermore, the observed rapid decrease in σ^{32} activity associated with a downshift from a steady-state temperature of 42°C to 30°C (6) could be economically accounted for by the model we propose. In this case, the decrease in DnaK activity associated with a lower temperature would lead directly to an increased interaction of DnaK with σ^{32}.

The amino acid corresponding to Thr-199 of DnaK is conserved in every member of the HSP70 family of proteins including human HSC70 (33) and the recently crystallized bovine HSC70 44-kDa ATPase fragment (37). Based on comparison to the crystal structure of the bovine HSC70 fragment (37) and the Chou–Fasman (52) prediction of DnaK secondary structure, Thr-199 is near the midpoint of a highly conserved loop structure that lies between two highly conserved β-sheet structures. In all members of the HSP70 family, the threonine corresponding to Thr-199 is preceded by an aspartic acid and a run of three glycines and is followed by phenylalanine and aspartic acid (Fig. 2). This high degree of conservation makes it extremely likely that autophospho-

rylation of the many other HSP70 and HSC70 proteins discussed in the introduction occurs on the corresponding threonine of these proteins. It is interesting to note that a HSP90 has been shown to autophosphorylate at a serine under very similar conditions to DnaK (53, 54) and contains the sequence SAGGSFT in both the murine 84- and 86-kDa forms of the protein (53). We suggest that this may form a loop structure analogous to the structure in DnaK and that autophosphorylation may occur at Ser-169.

Thr-204 of the bovine HSC70 ATPase fragment, which corresponds to Thr-199 of DnaK, is localized in the interior of the protein within several angstroms of the γ-phosphate of the bound Mg^{2+} ATP (D. McKay, personal communication) and hydrogen bonds with a water molecule that is a strong candidate for an in-line attack on the γ-phosphate of ATP (55). Its predicted interior location makes it highly unlikely that phosphorylation of Thr-199 results from intermolecular kinase activity or that it could be related to the putative kinase activity of DnaK for several proteins that was postulated by Itikawa *et al.* (56). Furthermore, Flaherty *et al.* (55) have recently predicted phosphorylation of Thr-204 based on comparison of the highly similar actin and bovine HSC70 three-dimensional structures and we have observed autophosphorylation of HSC70 at 37°C to an extent similar to DnaK autophosphorylation (data not shown). It is interesting that actin, unlike DnaK, hydrolyzes Ca^{2+} ATP and contains valine in the position corresponding to Thr-204 of HSC70 (55). Finally, we note that the temperature stimulation of both the DnaK ATPase and autophosphorylation activities could be explained economically by postulating that increasing temperature results in a gradual change in the conformation of DnaK that changes the spatial relationship of Thr-199 to the bound ATP.

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