Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 7139–7143, August 1992
Genetics

Partial loss of function mutations in DnaK, the *Escherichia coli* homologue of the 70-kDa heat shock proteins, affect highly conserved amino acids implicated in ATP binding and hydrolysis

(dominant negative mutants/heat shock response/ATPase)

JADWIGA WILD*, ASHWINI KAMATH-LOEB*, EVA ZIEGELHOFFER*, MICHAEL LONETTO*, YASUO KAWASAKI†, AND CAROL A. GROSS*‡

*Department of Bacteriology, University of Wisconsin-Madison, Madison, WI 53706; and †Institute for Virus Research, Kyoto University, Kyoto 606, Japan

Communicated by Julius Adler, April 6, 1992

ABSTRACT **A set of 37 mutations in DnaK, the *Escherichia coli* homologue of the 70-kDa heat shock proteins, was isolated using a selection for high constitutive expression of heat shock proteins. Of these, 11 mutants were able to carry out some but not all functions of DnaK. These partial function mutants were divided into two classes. Class I mutants are recessive and permit replication of bacteriophage λ and growth of cells up to 40°C. Class II mutants are dominant, do not permit growth of λ, and are temperature-sensitive for growth above 34°C. Mutations in both classes alter amino acids that are highly conserved in the 70-kDa heat shock protein family. The dominant negative mutations provide strong genetic evidence that at least one form of DnaK is multimeric. Moreover, every dominant negative mutation occurs at an amino acid that has been hypothesized to be intimately involved in the process of ATP binding and hydrolysis. Our findings provide strong support for the hypothesis that such mutations are excellent tools for identifying amino acids that play critical roles in protein function.**

The 70-kDa heat shock proteins (hsp70) form a major ubiquitous family of proteins whose rate of synthesis is highly responsive to changes in temperature. Prokaryotic cells have a single heat-inducible member of this family, called DnaK, whereas eukaryotic cells have multiple family members. Some eukaryotic hsp70s are temperature-inducible but others are synthesized constitutively or even negatively regulated by temperature upshift. The hsp70s are essential in both prokaryotic and eukaryotic cells, share extensive sequence homology, and exhibit a number of similar biochemical properties including a high binding affinity for ATP and a weak ATPase activity. Interaction with other proteins, either to maintain or alter their conformation in an ATP-dependent fashion, appears to be the basic biological activity of hsp70 (1–3). As specific examples of this type of function, various hsp70s have been implicated in maintaining substrates in a translocation-competent conformation (4–6), assembling and disassembling multimeric proteins (7), facilitating protein degradation (8–10), and regulating the heat shock (hs) response (11–13).

Despite this general consensus for the function of hsp70s, there has been no detailed analysis of how these proteins carry out this role. One way to approach this problem is to select mutations in hsp70 genes and to analyze those mutants that are partially defective in hsp70 function. We have carried out such studies in *Escherichia coli* where the analysis of hsp70 function is simplified by the fact that it contains only a single hsp70 member. We report on the selection, molecular analysis, and physiological characterization of 11 mutations in *dnaK* that lead to loss of some but not all functions of the DnaK protein.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Genetic Methods. The *dnaK* mutations were analyzed in MC1061 (14). Cells were grown in LB or in M9/glucose medium supplemented with all amino acids (aa) except L-methionine and appropriate antibiotics at the standard concentrations (15). Transformations and phage P1 and λ manipulations were as described (15, 16). The *rpoD–lacZ* operon fusion was transferred from pJW3 into λRS45 (17) to give λJW10. To construct *dnaK*$^+$/*dnaK*$^-$ merodiploids, *dnaK*$^-$ mutations were transduced into strain MC1061λ*imm21dnaK*. pJW1 contains the *htpG* promoter fused to the chloramphenicol acetyltransferase gene and pJW3 carries an operon fusion of the *rpoD* hs promoter to *lacZ*. pDC400, pJW4, pJW7, and pEZ2, -3, -6, -9, and -10 contain fragments of *dnaK* that span the entire coding region of the gene (Fig. 1).

Assays. Temperatures at which mutant cells plated with 100% efficiency were considered permissive for growth. λ plating was assayed in NZYM top agar by λ*c*I60 or λ*c*I60*dnaK* growth on a bacterial lawn (15). Lysogen formation was assessed by quantifying kanamycin resistance (Kan^R) or *dnaK*$^+$ after infection with λ*imm21*Kan^R or λ*imm21dnaK*. Mini-F replication proficiency was assessed by maintenance of the mini-F plasmid pKV5110 (19). The GroEL and DnaK synthesis rates of cells growing exponentially at 30°C in M9/glucose were quantified by pulse-labeling for 1 min, chasing for 1 min with nonradioactive L-methionine, and immunoprecipitating ^{35}S-labeled DnaK or GroEL with the corresponding antibodies. Where used, isopropyl β-D-thiogalactoside (IPTG) was added two doublings prior to labeling. L-[^{3}H]Leucine-labeled cells were added to correct for losses during sample preparation (20). Puromycyl fragment degradation was performed as described (9). Percent soluble cpm in 10% (wt/vol) trichloroacetic acid was plotted as a function of time after removal of puromycin. Binding to ATP was assayed qualitatively by ATP-Sepharose (Pharmacia LKB) column chromatography (21). DNA from colony lysates was amplified by the polymerase chain reaction (PCR, Perkin–Elmer/Cetus protocol). Purified single-stranded genomic DNA amplified from at least two independent asymmetric PCRs was sequenced using Sequenase Version 2.0 (United States Biochemical).

Selection of Mutants with Elevated HSP Synthesis. MC1061 with the chloramphenicol acetyltransferase gene expressed

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Abbreviations: hsp70, family of 70-kDa heat shock proteins; hsc70, 70-kDa heat shock cognate protein; hs, heat shock; aa, amino acid(s); IPTG, isopropyl β-D-thiogalactoside.

‡To whom reprint requests should be addressed.

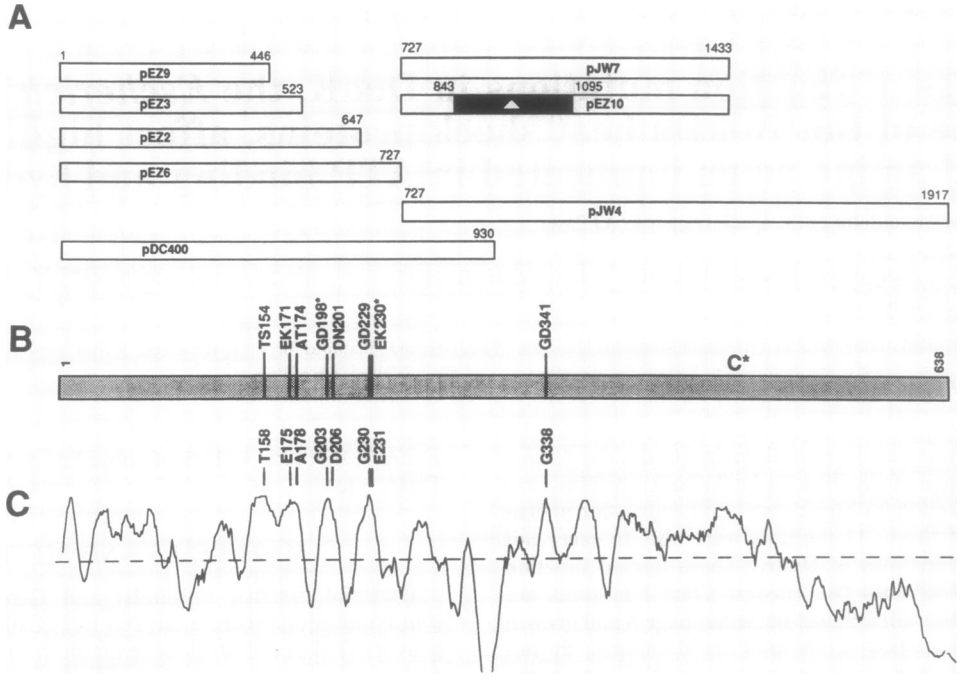

FIG. 1. Location and sequence of *dnaK* mutations. (*A*) Plasmids used for mapping. The open boxes indicate the fragment of *dnaK* carried on each plasmid. Flanking numbers refer to base pairs of *dnaK*. The solid box in pEZ10 represents a deletion of base pairs 843–1097 in pJW7. (*B*) Location of mutations. The shaded box represents *dnaK*. Mutational substitutions (see Table 1) are marked by vertical lines. The asterisks indicate the three mutations identified in one of the *dnaK* mutants. The position of the C-terminal change, designated C*, was deduced from the observation that the mutant protein is 75% the length of wild-type DnaK by SDS/PAGE. This mutation has not been sequenced. (*C*) Aligned sequences of 29 hsp70-related proteins. The aa positions in the 70-kDa hs cognate protein (hsc70) that correspond to the location of *dnaK* mutations are shown above the aligned sequences. *dnaK* mutations TS154(T158), EK171(E175), GD198(G203), DN201(D206), and GD229(G230) are located at completely conserved aa; AT174(A178) and EK230(E231) are located at positions conserved in 28 of 29 hsp70s. Sequences were aligned with PILEUP (GCG) (18) using the default scoring matrix and gap parameters. The graph of sequence identity was made using PLOTSIMILARITY (GCG) with a window of 10. The highest scoring portions of the graph indicate completely conserved sequences of at least 10 aa. The hsp70 alignment was from Elizabeth Craig (personal communication).

from the *htpG* hs promoter (pJW1) and a *lacZ* gene expressed from the *rpoD* hs promoter (λJW10) forms white colonies on McConkey/lactose plates and does not grow on chloramphenicol at 150 μg/ml. Cells with high levels of chloramphenicol acetyltransferase and β-galactosidase were selected by plating on McConkey/lactose/chloramphenicol (150 μg/ml) plates and picking red colonies.

RESULTS

Selection of *dnaK* Mutants. We utilized the fact that mutations in *dnaK* lead to increased expression of the hs genes (13, 22) to select additional *dnaK* mutations. Transductional analysis of the 62 mutants obtained indicated that all carried a mutation 50% linked to a Tn*10* transposon located near the *dnaK* gene. Of these, 37 were shown by complementation or marker rescue analysis to carry a mutation in *dnaK*. The rest had a mutation at the adjoining *dnaJ* locus or were *dnaK dnaJ* double mutants. The fact that we did not obtain mutations in *grpE*, an expected target gene, may relate to the general difficulty of isolating mutations in this gene (23).

Most *dnaK* mutant strains are temperature-sensitive for growth at 42°C and do not permit growth of bacteriophage λ (24). Both phenotypes are recessive (25). The 26 *dnaK* mutants with these phenotypes were not characterized further. The remaining 11 *dnaK* mutants fell into two classes and were analyzed further. The observations that class I *dnaK* mutants support bacteriophage λ growth whereas class II mutants do not support λ growth even in the presence of $dnaK^+$ suggest that class II mutations are dominant.

Mapping and DNA Sequence Analysis. Mutations were located within the *dnaK* gene by marker rescue resulting from recombination between cloned fragments of the wild-type *dnaK* gene and the resident mutant gene. Fragments covering the mutation yield wild-type recombinants that grow at 44°C (Fig. 1*A*). With the exception of one mutant strain, a single substitution was identified in the interval located by marker rescue. Ten of the mutations were located between aa 154 and 230, a region that contains only 12% of the protein. GD341, the remaining mutation, was located 100 aa downstream of this region. The two class I mutants, TS154 and AT174, exhibited conservative aa changes whereas substitutions in the dominant-negative class II mutants often led to an alteration in the aa charge (Fig. 1*B*). Five mutants with the same aa substitution GD229 were the sole representatives of a unique phenotypic subclass, characterized by lethality upon coexpression of GD229 and wild-type DnaK.

One dominant negative mutant had two substitutions, GD198 and EK230, in the N-terminal region and an additional C-terminal change (not yet sequenced) that resulted in a truncated DnaK ≈75% the length of wild-type protein. The dominant negative phenotype was conferred by the N-terminal mutations as a truncated DnaK with the wild-type sequence permitted λ growth.

Phenotypes of *dnaK* Mutants. We examined the ability of the mutant strains to carry out various activities ascribed to DnaK. All of the mutant strains were defective in regulating the hs response. They exhibited a 3- to 6-fold increase in the rate of hs protein synthesis at 30°C (Table 1), which was comparable to that exhibited by the wild-type strain at the peak of the hs response. The *dnaK* mutant strains were also defective in degrading puromycyl fragments and in maintaining a plasmid with a mini-F origin of replication (Table 1).

The basis for the phenotypic distinction between class I and class II mutants was the ability of the former to support the growth of bacteriophage λ (Table 1). Class I mutants were

Table 1. Analysis of the *dnaK* mutant phenotypes

dnaK mutation	Permissive temperature	Plating properties		Lysogen formation	Replication of mini-F	Synthesis of GroEL		Proteolysis	Binding to ATP-Sepharose
		λ	λ*dnaK*			30°C	42°/30°C		
dnaK$^+$	16–44°C	+	+	+	1.00	1.0	5.0	1.00	+
*dnaK756**	16–40°C	−	+	+	<0.01	2.3	8.5	0.55	+†
Class I mutants									
TS154	16–40°C	+	+	+	<0.01	4.7	16.9	0.59	ND
AT174	16–40°C	+	+	+	<0.01	5.1	14.3	0.47	+
Class II mutants									
EK171	30–34°C	−	−	+	<0.01	4.3	8.2	0.41	+
GD198, EK230,C‡	16–34°C	−	−	+	<0.01	2.9	4.9	0.34	+
DN201	16–34°C	−	−	+	<0.01	4.9	11.3	0.33	+
GD229	16–34°C	−	−	+§	<0.01	6.0	9.6	0.41	−
GD341	16–34°C	−	−	+	<0.01	4.8	8.6	0.34	−

dnaK mutations are denoted by the single-letter code of a wild-type aa followed by the substituted aa and position. Transformation efficiency of mini-F plasmid pKV5110 at 30°C is presented relative to that of pBR322. The efficiency of transformation of *dnaK*$^+$ by pBR322 was 9.4 × 10^5 transformants per μg of DNA. Data for GroEL indicate the rate of GroEL synthesis at 30°C relative to that of the wild-type strain at 30°C and the GroEL synthesis rate at 5 min after shift to 42°C relative to that of the wild-type strain at 30°C. Data are from one experiment. Experiments were repeated two to four times; values were within 10% of each other. Proteolysis is the rate of puromycyl fragment degradation 20 min after removal of puromycin normalized to the degradation rate of the wild-type strain. +, Positive result; −, negative result. ND, not determined.

*The *dnaK756* mutation (26) has been recently sequenced (39).

†Binding to ATP-Sepharose is described in ref. 40.

‡This mutant carries two indicated aa substitutions and an additional C-terminal mutation.

§Dominant lethal mutant forms lysogens with λ but not with λ*dnaK*$^+$.

distinguished in several additional respects from those in class II. Class I mutants permitted growth to 40°C whereas class II mutants permitted growth to only 34°C. In addition, only class I mutants exhibited a significantly higher rate of hs gene expression than the wild-type strain at the peak of the hs response (Table 1).

We have also qualitatively examined the ability of the mutant proteins to bind ATP, by monitoring their retention on an ATP-Sepharose column. All but two of the class II mutant strains produced DnaK proteins that bound well to ATP-Sepharose (Table 1). The two that bound poorly (GD229 and GD341) exhibited the most severe phenotypes of the mutants analyzed, as indicated by the merodiploid analysis described below.

Merodiploid Analysis of Dominant *dnaK* Mutants. To determine whether the class II *dnaK* mutations were dominant for all phenotypes, we constructed merodiploid strains carrying mutant and wild-type *dnaK* genes and reexamined the phenotypes displayed by the original mutant strain (Table 2). Four of the class II mutants varied in the extent to which the phenotypes were dominant. The GD341 and EK171 merodiploids, at one extreme, were mutant for almost all phenotypes tested, indicating complete dominance of the mutation. In contrast, the merodiploid carrying mutations GD198, EK230, and C (see above) was dominant only for its λ phenotype. Finally, the merodiploid carrying DN201 was recessive for all phenotypes tested including λ growth. The fact that DN201 did not support the growth of λ*dnaK* may result from a quantitative difference in the amount of wild-type DnaK. In the merodiploid, 50% of the DnaK is wild-type whereas, immediately after infection at low multiplicity by λ*dnaK*, very little of the DnaK is wild-type.

Table 2. Analysis of the phenotypes of *dnaK*$^-$/*dnaK*$^+$ merodiploid strains

dnaK mutation	Growth at 42°C	λEOP	Replication of mini-F	GroEL synthesis	Proteolysis
dnaK$^+$	+	+	1.00	WT	1.0
GD341	−D	−D	0.01 D	High D	0.4 D
EK171	+R	−D	0.06 D	High D	0.6 D
GD198, EK230, C*	+R	−D	0.30 R	WT R	1.0 R
DN201	+R	+R	0.90 R	WT R	1.1 R

Merodiploid strains were λ*imm21dnaK* lysogens containing the *dnaK* mutation on the chromosome. The assays are as described in Table 1 except for GroEL synthesis (30°C), which was determined by electrophoresing equal cpm of ^{35}S-labeled total cell extracts by SDS/PAGE on a 10% gel and estimating the intensity of the GroEL band. WT, wild-type level; D, dominant mutant phenotype; R, recessive mutant phenotype; λEOP, efficiency of plating λ phage.

*For description, see Table 1 footnote ‡.

A Dominant Lethal Mutation in *dnaK*. We were unable to construct a merodiploid strain of GD229 by using either λ*dnaK* or F′*dnaK*. To determine whether GD229 is inviable in the presence of *dnaK*$^+$, we introduced into GD229 a plasmid carrying the *dnaK*$^+$ gene under control of the inducible *lacUV5* promoter. By changing the concentration of IPTG, we were able to correlate the viability of GD229/*dnaK*$^+$ with the amount of wild-type DnaK. At 0.2 mM IPTG, where mutant and wild-type DnaK were present in approximately equimolar amounts, cell death ensued (see Fig. 2), confirming that our inability to construct merodiploid strains resulted from lethality. A merodiploid analysis performed at 0.1 mM IPTG (at this concentration of IPTG, sufficient DnaK is made to complement the *dnaK756* mutation but not to kill mutant GD229) indicated that all GD229 phenotypes were dominant.

DISCUSSION

As a first step in a structure–function analysis of the ubiquitous hsp70s, we have undertaken to select and analyze mutations in *dnaK* that result in partially functional proteins. Such mutations are useful for analyzing functional domains as they are unlikely to disrupt protein structure and result in misfolding, aggregation, and/or degradation of the mutant protein. Two distinct classes of *dnaK* mutants retained partial protein function. Class I consists of two recessive mutants able to carry out some but not all DnaK functions. Class II consists of five dominant mutants whose ability to interfere with wild-type function indicates that they still carry out some functions of DnaK.

The defects of three of the mutant strains in λ and mini-F replication are particularly interesting. The two class I mutants, TS154 and AT174, promote λ but not mini-F replication. Conversely, the class II mutant with multiple substitu-

tions promotes mini-F but not λ replication in the presence of wild-type DnaK. This separation of activities could result from a quantitative difference in the requirements for some biochemical activity. However, since we have mutants specifically defective in each type of replication, it seems more likely that these mutational changes eliminate a discrete activity of DnaK required by each replicon, for example, release of λP from the *oriλ* preprimosomal complex (27–29) or proper folding/oligomerization of the mini-F initiator protein RepE (19).

The Dominant Negative Phenotype. We show that dominant negative mutations occur frequently in *dnaK*. Dominant negative mutations usually occur in genes encoding multimeric proteins and result from mixed oligomers in which mutant subunits "poison" some of the activities of the wild-type subunit. Occasionally, dominant negative mutations are obtained in genes encoding a monomeric protein. Such a mutant protein titrates out a substrate present in limiting concentrations, rendering it inaccessible to the wild-type protein and resulting in the dominance of the mutant phenotype (30). DnaK and other members of the hsp70 family purify as monomers (31), suggesting a monomeric unit as the functional state. However, there are indications of a higher-order state; hsc70 binds clathrin triskelia as a trimer (32) and binding protein BiP/GRP78 exists in functionally distinct interconvertible monomeric and oligomeric forms (33, 34). The merodiploid phenotype of GD229 provides strong genetic support for the proposal that DnaK exists in a multimeric state or that monomers functionally interact. In this mutant, overproduction of wild-type DnaK to a concentration equivalent to that of GD229 results in cell death (Fig. 2). Lethality cannot result from titration of a limiting substrate

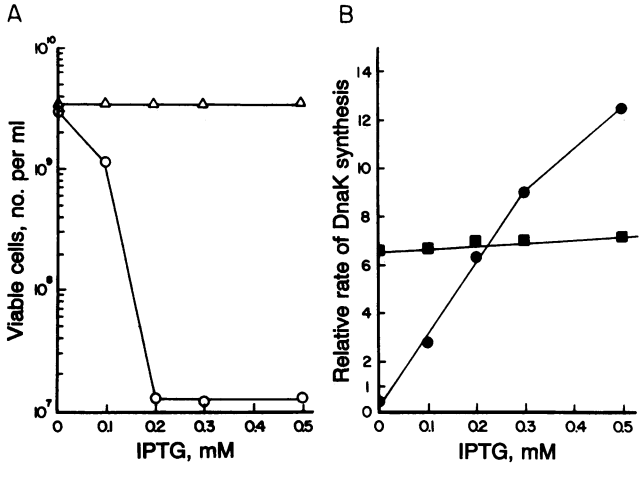

FIG. 2. Lethality of GD229 in the presence of *dnaK*$^+$ correlates with increased amounts of wild-type DnaK. (*A*) Viability of GD229 with *dnaK*$^+$. The number of viable cells per ml of overnight cultures of the *dnaK* mutant GD229 carrying pNRK416 (○) or isogenic wild-type MC1061 carrying pNRK416 (△) grown on M9/glucose/ampicillin plates supplemented with the indicated IPTG concentrations. pNRK416 encodes *dnaK*$^+$ under control of the IPTG-inducible *lacUV5* promoter. (*B*) The rate of DnaK synthesis from pNRK416. DnaK synthesis from pNRK416 (●) in cells growing exponentially at 30°C in M9/glucose/ampicillin supplemented with IPTG. A strain in which chromosomal *dnaK* is truncated (described in Table 1, footnote ‡) was used for this measurement so that endogenous DnaK would not interfere with detection of DnaK from pNRK416. Data are expressed as the rate of plasmid DnaK synthesis relative to that exhibited by wild-type strain at 30°C. The synthesis rate reflects the steady-state level of these proteins since both mutant and wild-type proteins are stable (J.W., data not shown). The rate of DnaK synthesis in GD229 (■) is 6- to 7-fold higher than that of a wild-type strain at 30°C (see also Table 1). At 0.2 mM IPTG, the rate of synthesis of DnaK from pNRK416 is approximately equal to that of GD229.

by an aberrant mutant protein because it results from overproduction of wild-type DnaK. Instead, lethality is easily understood if there is a functional interaction between wild-type and GD229 monomers. For example, mixed complexes of wild-type DnaK competent for ATP binding and hydrolysis and GD229 defective for these activities could generate proteins with gross defects in behavior.

Structural Implications of the *dnaK* Mutations. To our knowledge, this is the first instance in which a collection of mutations resulting in partially functional proteins could be compared to an extensive data base of protein sequence. Sequence analysis of 29 members of the hsp70 family shows that each of the mutations we isolated affects an aa previously identified to be located in a highly conserved region of the protein (Fig. 1*C*). All of the class II (dominant negative) mutants and one of the class I mutants contain a mutational change affecting a completely conserved aa and the remainder affect an aa conserved in 28 of 29 hsp70s (see Fig. 1). Based on these findings, we believe that, in general, mutations resulting in partially functional proteins should be useful in identifying conserved domains of a protein.

The three-dimensional structure of the N-terminal 44-kDa ATPase domain of bovine hsc70 has recently been determined by x-ray crystallographic analysis (35) and is very similar to that of rabbit skeletal muscle actin although the two proteins share only 15% aa conservation (36). Based upon this combined structural information, Flaherty *et al.* (36) have inferred the aa residues crucial for ATP binding and hydrolysis. To our surprise, every one of our dominant negative mutations affects an aa expected to be involved in these processes. Moreover, although only 39 aa are identical between hsc70 and actin, three dominant negative mutations occur at such residues. In contrast, although the two class I mutations are located in the regions generally involved in the ATPase mechanism, their functions are not easily inferred from the crystal structure.

Two mutations, GD198 and DN201, are at positions expected to interfere with the hydrolysis of ATP that is postulated to occur by attack on the γ-phosphate by a water molecule H-bonded to Thr-199 (Thr-204 in hsc70). Two other mutations, GD229 and GD341 (Gly-230 and Gly-338 in hsc70), affect glycine residues proposed to be part of the adenine-binding pocket, and a third, EK230, is also likely to perturb formation of this structure. The glycine altered in GD229 (Gly-230 in hsc70) may be directly involved in forming the binding site for adenine by H bonding to a distant lysine. In addition the glycine altered in GD341 (Gly-338 in hsc70) is immediately adjacent to another glycine (Gly-339 in hsc70) that is so close to the α-phosphate of ATP that no side chain can be accommodated at this position. Gly → Asp changes at these two positions might alter the protein conformation sufficiently to prevent ATP binding since the side chains of aspartic acid would point directly into the adenine-binding pocket. Consistent with this expectation, the GD229 and GD341 mutant proteins bind very poorly to ATP-Sepharose. Although we cannot eliminate the possibility that poor binding to ATP results from an altered conformation in the mutant proteins, our results also suggest a direct participation of these aa in the adenine-binding pocket. Finally, the glutamic acid altered in EK171 (Glu-175 in hsc70) affects a residue inferred by analogy to actin to be involved in binding a Ca^{2+} ion. Although bound Ca^{2+} is essential for the ATPase activity of actin (37), Ca^{2+} severely inhibits the ATPase reaction catalyzed by wild-type DnaK (38). If Glu-171 (in DnaK) was involved in binding Ca^{2+}, a Glu → Lys substitution should decrease Ca^{2+} binding and inhibition. However, this is not the case. The EK171 ATPase is as severely inhibited by Ca^{2+} as wild type (A.K.-L., unpublished data). Thus, it is unclear where Ca^{2+} binds to DnaK.

The potential use of dominant negative mutants in a functional analysis of a protein has been elegantly presented (30). Our results provide strong validation for the hypothesis that dominant negative mutants are a powerful tool for identifying and characterizing the activities of multimeric proteins. Although there are 638 aa in DnaK, the 6 aa substituted in the dominant negative mutants are those predicted from the x-ray crystallographic structure (35, 36) to be intimately involved in the central enzymatic activity of DnaK—the binding and catalysis of ATP. Surprisingly, the observation that four of these mutant proteins are still able to bind ATP suggests that some of the conserved aa can be altered one at a time without significantly changing the ATP binding site.

We thank N. Kusukawa and T. Yura for supplying plasmid pNRK416, R. Hendrix for the GroEL antibodies, and E. Craig for the hsp70 alignment. We thank E. Craig, H. Echols, and K. Holmes for critical reading of the manuscript. We also thank the reviewers for their insightful comments on this manuscript. This work was supported by a Public Health Research Grant GM36278 from the National Institutes of Health to C.A.G. A.K.-L. was the recipient of a Public Health Service Postdoctoral Fellowship.

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