Isolation and Characterization of an *Escherichia coli* DnaK Mutant with Impaired ATPase Activity

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A temperature-sensitive mutant of DnaK, the principal *Escherichia coli* member of the 70 kDa heat shock protein family, has been isolated. The mutation, dnaK25, lies in the putative ATP binding pocket of DnaK. It consists of a C to T transition that changes the highly conserved proline 143 to serine. Mutant strains do not support the propagation of bacteriophage λ or of plasmids that require DnaA for replication. They are also defective in the utilization of mannose and sorbitol. ATPase activity of the mutant protein is reduced 20-fold relative to wild-type DnaK; nucleotide exchange by both proteins is markedly increased by GrpE. The DnaK25 ATPase is still stimulated by DnaJ and GrpE and by peptide substrates. However, the affinity of most peptides tested for stimulating the DnaK25 ATPase is reduced significantly. These results indicate that a mutation in the N-terminal nucleotide binding domain can alter substrate interactions with the C-terminal substrate binding site. Nucleotide exchange by both wild-type DnaK and DnaK25 proceeds at a much faster rate than ATP hydrolysis, and therefore cannot be the rate limiting step of ATP hydrolysis under the conditions used in these experiments. Consistent with this, peptides, which stimulate ATP hydrolysis, have no effect on nucleotide exchange. Peptides thus appear to stimulate the ATPase by acting at another step, such as increasing the rate of phosphate bond cleavage.

Keywords: DnaK; heat shock proteins; Hsp70; ATP hydrolysis; peptide binding

1. Introduction

DnaK is the principal *Escherichia coli* member of the highly conserved, ubiquitous family of 70 kDa heat shock proteins (Hsp70s). The Hsp70s function as molecular chaperones. They assist in the proper folding of polypeptides, inhibit protein aggregation, target misfolded protein for degradation, facilitate polypeptide translocation, and participate in disassembling oligomeric structures (Gething & Sambrook, 1992; Hartl et al., 1992; Ang et al., 1991; Craig et al., 1993).

DnaK acts as a molecular chaperone in *E. coli*. *In vitro*, DnaK blocks the aggregation and permits the recovery of heat-inactivated RNA polymerase (Skowyra et al., 1990), rhodanese (Langer et al., 1992), and firefly luciferase (Schroder et al., 1993). DnaK dissociates dimeric RepA and aggregated DnaA replication proteins (Wickner et al., 1992; Hwang et al., 1990), and promotes the disassembly of the preprimosomal complex at ori-λ (Alfano & McMacken, 1989; Zylicz et al., 1989). *In vivo*, DnaK

Abbreviations used: Hsp70, 70 kDa heat shock protein; Hsp, heat shock protein; PTS, phosphotransferase.
stimulates the reactivation of thermally inactivated λ repressor and luciferase (Gaitanaris et al., 1990; Schröder et al., 1993) and prevents the aggregation of newly synthesized polypeptides (Gragerov et al., 1992).

DnaK is induced by heat shock and is required for growth at both high and low temperatures (Georgopoulos et al., 1982; Bukau & Walker, 1989). dnaK mutants are defective in the down-regulation of heat shock gene expression (Tilly et al., 1983; Strauss et al., 1990), initiation of chromosome replication (Sakakibara, 1988), chromosome segregation (Bukau & Walker, 1989a), cell division (Paek & Walker, 1987), flagellum synthesis (Shi et al., 1992), proteolysis (Keller & Simon, 1988; Strauss et al., 1988), ribosome assembly (Alix & Gérin, 1993), export of SecB-independent proteins (Wild et al., 1992), cosmoadaptation (Meury & Kobiyama, 1991), survival during starvation (Spence et al., 1990), and propagation of λ (Georgopoulos, 1977), P1 (Tilly & Yarmolinsky, 1989) and F-based replicons (Kawasaki et al., 1990).

The ability of the Hsp70s to hydrolyze ATP is thought to be crucial for their biological function (Getling & Sambrook, 1992; Hartl et al., 1992; Ang et al., 1991; Craige et al., 1993). The Hsp70s avidly bind ATP (Welch & Feramisco, 1985) and possess a weak ATPase activity that is stimulated by peptide and polypeptide substrates (Zyliszczak et al., 1983; Brauell et al., 1984; Flynn et al., 1989; DeLuca-Flaherty et al., 1990; Palleros et al., 1991; Sadas & Hightower, 1992; Gragerov et al., 1994). The ATPase of DnaK is also stimulated by the Hsps DnaJ and GrpE (Lieber et al., 1991a). dnaJ and grpE are required for dnaK-dependent functions in vivo (Ang et al., 1991). Eukaryotic homologs of Hsps have also been found to stimulate Hsp70 ATPase activity (Cyr et al., 1992), and this activity may be important for their in vivo function (Caplan et al., 1992). The ATPase of Hsc70 lies within the N-terminal domain of the protein (Chappell et al., 1987). The structure of the Hsc70 N terminus has been determined (Flaherty et al., 1990) and closely resembles the nucleotide binding domains of actin, hexokinase, and glyceral kinase (Flaherty et al., 1990, 1991; Hurley et al., 1993). The Hsp70 polypeptide binding site lies in the C-terminal domain (Chappell et al., 1987; Wang et al., 1993; Gragerov et al., 1994). Intact N and C-terminal domains are both required for peptide and polypeptide stimulation of Hsp70 ATP hydrolysis (R. McMacken, personal communication). Hsp70s form tight, salt-resistant complexes with their target polypeptides, and release them in an ATP-dependent manner while undergoing a conformational change (Pellam, 1986; Lieber et al., 1991b; Banecki et al., 1992; Palleros et al., 1993). ATP increases both the association and dissociation rates of peptide substrates with DnaK but shifts the equilibrium towards complex dissociation (Schmid et al., 1994). NMR studies with peptide model substrates suggest that peptides bind DnaK in an extended conformation, consistent with the notion that DnaK recognizes and binds unfolded proteins (Landry et al., 1992).

In addition to its weak ATPase activity, DnaK undergoes autophosphorylation in vitro (Zyliszczak et al., 1983). The functional significance of this reaction is unclear. Peptide substrates and GrpE inhibit autophosphorylation, whereas DnaJ has no effect (Panagiotidou et al., 1994). Mutations in threonine 199, the site of autophosphorylation, greatly reduce or abolish the DnaK ATPase (McCarty & Walker, 1991), but the corresponding threonine 204 of Hsc70 is not essential for ATP hydrolysis (O'Brien & McKay, 1993).

In our study of the biological role of DnaK, we isolated and characterized a pleiotropic, temperature-sensitive DnaK mutant with impaired ATPase activity. The residual ATPase of DnaK25 was still regulated by peptide substrates and the Hsps DnaJ and GrpE. However, the mutation, which lies in the nucleotide binding domain, reduced the affinity of most peptide substrates for stimulating ATP hydrolysis.

2. Materials and Methods

(a) Growth media and bacteriological techniques

Strains were grown on M9 minimal medium and plates supplemented with 0.2% (w/v) fructose, 20 μg/ml L-threonine, 10 μg/ml thiamine, and 0.08% (w/v) Casamino acids (Difco) (Miller, 1972). MacConkey indicator plates were prepared using 1.5% (w/v) MacConkey agar (Difco) and 1% (w/v) carbon source. Standard bacteriological techniques were used as described (Miller, 1972; Silhavy et al., 1984).

(b) Bacteria, plasmid and plasmid strains

The bacterial strains used were derivatives of X99 (F’·rpsL5 and K2). Strains P, (X99 dnaK thr:Tn10) and P (X99 dnaK25 thr:Tn10) were obtained by P1 transduction into X99. P1 lysates for transduction of dnaK25 were grown on a dnaK25 strain (5μ4, X99 ara- m− dnaK25 thr:Tn10(pSM22 dnaK) marked by a Tn10 insertion in the thr locus with a 50% linkage to dnaK25 and carrying dnaK on a plasmid to permit growth of P1. The recipient X99-derived strain in the back crosses from the merodiploid dnaK strains (see Results) was LammB− to block plating of induced λ prophage. The ΔλdnaK and Δλimm·dnaK transducing phage, and the pMOB4 dnaK′ plasmid have been previously described (Georgopoulos, 1977; Yachim et al., 1978; Zylisz & Georgopoulos, 1984). pSM22 carries a 3 kb DnaI fragment, including the entire dnaK gene and its regulatory elements, inserted into the Smal site of pBR322 (Churchward et al., 1984). pKV1022 contains the S' BglII fragment of dnaK, spanning the upstream region through nt 930 of the coding region. pJK10 contains an internal dnaK fragment (EcoRI-ClaI, nt 727 to 1437) and pJK4 carries a fragment including the C-terminal region of dnaK (BglII-HindIII, nt 930 to 2165).

(c) Selection of bacterial mutants

X99 bacteria were mutagenized with nitrosoguanidine as described (Miller, 1972). Approximately 106 surviving mutagenized cells were spread on plates seeded with a
mixture of 10^7 cfu and 10^5 cfu broth and incubated at 32°C for 24 to 48 h. Survivors were then screened for growth at 42°C. About 10% of the individual isolates were temperature sensitive. These rare colonies were purified and studied further. The combination of the two bacteriophage, each utilizing a different entry pathway, was used in order to exclude recipient mutants that occur at high frequency when selecting for resistance to a single bacteriophage (unpublished observations).

(d) Sequencing of dnaK25

The dnaK gene from the strain Ω73 was amplified by the polymerase chain reaction (PCR), digested with EcoRI (cutting within dnaK) and HindIII (cutting at a site incorporated into the 5' PCR primer), and the fragment including nucleotides 4 to 732 of the dnaK coding region was subcloned into pBluescript SK(+) and SK(-) (Stratagene). The coding and non-coding strands were sequenced using internal and flanking primers. The remaining 3' end of the gene was sequenced by cycle sequencing of the amplified dnaK25 PCR product (Innis et al., 1988). The PCR primers used were complementary to sites upstream and downstream of the dnaK coding sequence; their sequences were: 5' CCAGACTCTTTAAAGTGACCGAGTTAGTG-3' (inserting a HindIII site on the 3' end of the PCR fragment), and 5' GCCCGATCGCCGAGAACATTCCGTTCTCGG-3' (inserting a EcoRI site on the 3' end of the PCR fragment). The additional primers for sequencing were: 5' CCATCAGCCGCTTCAAAG-3', 5' CCTGTATCCGCGCTTGA-3', 5' ACCGGCTGAGAGACAT-3', 5' TACCCCGCTTCTCGGGC-3', 5' CTTTCCCGGAGAAGATA-3', 5' GCCAGAAGACTGGACAG-3' [all complementary to the non-coding strand]; 5' GTTTCGCCTGCTTCGT-3', 5' ACTGACCGAGAGATTG-3', 5' CCAAGAAGACTGGACAG-3', 5' GCCTTCTGCGTGGC-3', 5' GCCTTGAAACACTGAGT-3' [all complementary to the coding strand]. The SK primer (Stratagene), complementary to pBluescript, was also used. PCR reactions and cycle sequencing (Perkin Elmer Cetus) DNA fragment purification (QIAEX agarose gel extraction system, Qiagen), and single-stranded DNA sequencing (Sequenase 2.0, United States Biochemical) were all performed according to the manufacturer's specifications. The GenBank and SwissProt databases were searched using the Genesics Computer Group (GCC; Madison, WI) sequence analysis software package. The Hae70 ATPase domain crystal structure (Flicker et al., 1990) was examined using the Insight II molecular modeling package (Biosyn).

(e) Plasmid stability assay

The ability of a host to maintain an episome was determined by measuring plasmid loss during non-selective growth at 32°C. Wild-type or mutant strains were transformed with the plasmid to be assayed as described by Hanahan (1983). The transformants were purified and grown in the presence of the appropriate antibiotic to ensure plasmid retention. The cells were subsequently diluted into non-selective medium and grown for several generations. A sample from this culture was spread onto plates without antibiotics and the resulting colonies were replica tested on antibiotic containing plates. The fraction of antibiotic-resistant colonies over the number of colonies present on non-selective plates represents the measure of plasmid stability. A value of 1.0 indicates that all the cells were able to retain the episome. A minimum of 100 colonies was tested for each plasmid-host combination. All experiments were repeated at least twice with independent transformants assayed each time.

(f) Mannose uptake assays

Fresh overnight cultures of Ω72 (dnaK) and Ω73 (dnaK25) were diluted 1:100 and grown at 32°C to A660=0.5, 25 ml samples from each culture were washed twice with ice-cold M9 salts (Miller, 1972) and resuspended in 1.5 ml of M9 salts. Chloramphenicol was added to a final concentration of 100 µg/ml and the cells were kept on ice. Transport was assayed using 2-14C]mannose (Amersham) mixed with unlabeled sugar (see legend to Figure 2). Each culture was assayed twice per experiment. In the assay, 200 µl of cells were mixed with 1:6 µl of M9 salts and incubated for five minutes at room temperature. 200 µl of labeled sugar was added with mixing to begin the assay and at the indicated times 200 µl samples were spotted onto GF/C filters, precut with M9 salts, on a Millipore manifold under vacuum. Filters were immediately washed five times with 5 ml of ice-cold M9 salts. 200 µl of labeled mannose diluted tenfold in M9 salts were filtered and washed for background counts. 50 µl of assay mix was spotted onto a dry filter without filtration or washing to obtain total counts, divided by four. Filters were dried, mixed with 5 ml Ecosol (Du Pont/NEN) and radioactivity was measured by scintillation counting. Cells not in the assay were sonicated (for a tenfold or greater decrease of A660) and protein concentrations of the 10,000 g supernatants were determined to normalize the uptake rates. Protein concentrations were estimated using the Bradford protein assay (Bradford, 1976) (Bio-Rad) with BSA as a standard.

(g) Phage adsorption assay

dnaK, dnaK25, and malT' strains were grown overnight in M9 minimal media supplemented with fructose and maltose, harvested and resuspended in TMG (10 mM Tris·HCl (pH 7.4), 10 mM MgSO4, 0.01% gelatin, 5 x 10^9 cells/ml) with 2µl of phage (2.5 x 10^9 plaque forming units/ml) in TMG, incubated for ten minutes at 37°C and then diluted into TMG containing chloroform. Cells were pelleted by centrifugation and the titers of the free phage in the supernatants was determined.

(h) Purification of DnaK and DnaK25

DnaK was purified from the overproducing strain, Ω282 (N99/PMOB45dakK') grown overnight at 37°C in LB containing 20 µg/ml chloramphenicol. DnaK25 was purified from Ω473 grown overnight at 32°C in LB minimal medium supplemented as described above. Cells were collected by centrifugation, washed once with PBS and stored frozen as a pellet at ~20°C. Cell disruption with lysozyme and initial purification of DnaK' and DnaK25 on DE52 cellulose columns (Whatman) followed by ATP agarose affinity columns (C-8 linkage, Sigma A-2767) was performed as reported (Zylicz et al., 1987). DnaK was further purified on a hydroxyapatite column (Bio-Rad) followed by a DEAE-Sepharose column with NaCl gradient elution, as described (Zylicz & Georgopoulos, 1984). The hydroxyapatite step was omitted in the purification of DnaK25. An additional DEAP-Sepharose column with NaCl gradient elution was run in some DnaK25 purifications. DnaK and DnaK25 preparations were re-exchanged into 20 mM Tris·HCl (pH 7.5), 0.1 mM
EDTA, 0.5 mM DTT, 10% glycerol for storage by repeated dilution and concentration using Centricron-10 microconcentrators (Amicon). Preparation purity was judged by silver staining (Bio Rad) of SDS polyacrylamide gels (Laemmli, 1970).

(i) Peptide synthesis and purification

Peptide F6 was initially provided as a kind gift by Michael Sherman and Fred Goldberg (Sherman & Goldberg, 1991). Additional peptide F6 was synthesized by Nick Pleegh of the Columbia University Protein Chemistry Core Facility on an Applied Biosystems Peptide Synthesizer, Model 430A, using t-Boc protected L-amino acid derivatives. The peptide was deprotected and cleaved from the resin support by treatment with hydrogen fluoride, precipitated and washed with ethyl acetate, extracted with 10% acetic acid, and lyophilized. The peptide was then further purified by reversed-phase HPLC on a 21.4 mm x 250 mm Dynamax 300 A Å column in a 0-1% trifluoroacetic acid/acetonitrile linear gradient mobile phase. Sample homogeneity, estimated to be 98%, was determined by analytical HPLC using the same matrix and mobile phase, and peptide was quantified by amino acid analysis. Peptides XR and kW were synthesized by Chiron Miniotopes and purified by reversed-phase HPLC. They were estimated to be 90% homogenous based on analytical HPLC and were quantified by amino acid analysis. Peptides A and C were a kind gift from Greg Flynn and James Rothman, and were prepared as described (Flynn et al., 1989). All the peptides were provided lyophilized and were resuspended in water for use.

(j) ATPase assays

Reactions were carried out in 50 mM Hapes-KOH (pH 7.9), 60 mM KCl, 5 mM MgCl₂, 1 mM DTT, 10% glycerol, 0.2 mg/ml ovalbumin. The concentration of ovalbumin was reduced to 50 µg/ml for the peptide stimulation assays and to 83 µg/ml for the reactions including DnaJ and GrpE. All the assay components except ATP were mixed on ice and the reactions begun by adding ATP and shifting to the incubation temperature. ATP (Sigma) was added to the specified final concentrations with [γ-32P]ATP (3000 Ci/mmole, Amersham) included per reaction. At each time point 2 µl samples were spotted onto PEI-cellulose sheets (J.T. Baker) and developed in 8:2 (v/v) mix of 1 M acetic acid and 4 M LiCl to separate ADP from ATP. Labeled ADP and ATP were quantified on a Betascope 603 Blot Analyzer (Betagen). Background hydrolysis for each condition in the absence of DnaK was subtracted. No detectable increase in background hydrolysis was found in the presence of DnaJ, GrpE or peptides. Rate calculations were based on three or more time points per condition having levels of hydrolysis at least twice above background and less than 15% of total ATP. The assays with DnaJ and GrpE were performed at 32°C in the presence of 5 µM ATP using 100 nM wild-type DnaK with 50 nM GrpE and 200 nM DnaJ added, or 900 nM DnaK25 with 150 nM GrpE and 1.8 gM DnaJ added.

(k) Nucleotide exchange assays

Manipulations and centrifugations were performed in a cold room at 4°C. Each timepoint was determined using a separate incubation reaction. 200 nM wild-type DnaK or DnaK25 was incubated with 1 µM [γ-32P]ATP (29.2 mCi/ mmole, Du Pont/NEN) in 50 mM Tris - HCl (pH 7.8), 50 mM NaCl, 50 mM KCl, 10 mM MgCl₂, 10% glycerol (v/v), 2 mM DTT, 0.2 mg/ml ovalbumin (30 µl final volume) for five minutes at 30°C. Neither increasing the length of incubation from five minutes up to one hour nor increasing the concentration of [γ-32P]ATP from 1 µM to 10 µM resulted in a further increase in the levels of [γ-32P]ATP binding to wild-type DnaK or DnaK25. Bound [γ-32P]ATP was chased by adding an equal volume of buffer prewarmed to 30°C containing 4 mM unlabeled ADP and 4 mM sodium phosphate (pH 8.0) in the absence of presence of 1 mM peptide A or C or 800 nM GrpE (2 × final concentrations), and continuing the incubation at 30°C. At the indicated times following initiation of the chase, 50 µl samples were applied to Bio Spin 30 desalting columns (Bio-Rad), precipitated with buffer lacking ovalbumin, and centrifuged three times at 1000 g. The flow-throughs were each mixed with 10 ml Aquasol (Du Pont/NEN) and radioactivity was quantified by scintillation counting. 100% binding was determined by chasing with an equal volume of prewarmed buffer without ADP. Control experiments showed that no loss of binding occurred over the time of the assay following chase with buffer alone, so only an initial timepoint was taken in these experiments. Non-specific binding was subtracted; it was determined by incubating 200 nM DnaK or DnaK25 with premixed 1 µM [γ-32P]ATP and 4 mM unlabeled ADP for five minutes at 30°C and chasing with an equal volume of prewarmed buffer plus 4 mM inorganic phosphate, in the presence or absence of 800 nM GrpE.

(l) Autophosphorylation assays

Autophosphorylation reactions were carried out in a reaction volume of 50 µl containing 50 mM Hapes-KOH (pH 6.5), 20 mM CaCl₂, 5 mM 2-mercaptoethanol and 0.12 mg/ml ovalbumin. All reaction components except ATP were mixed on ice and the assays initiated by adding ATP and shifting to the incubation temperature. ATP was added to a final concentration of 1 µM with 0.8 µCi of [γ-32P]ATP (3000 Ci/mmole, Amersham) included per reaction. At each time point, 16 µl samples were taken and mixed with 4 µl of 5 X SDS-PAGE loading buffer (Laemmli, 1970). Following the assay, the samples were boiled for two minutes and loaded directly onto a 10% SDS polyacrylamide gel. The gels were silver stained to ensure equal loadings, dried and quantified on a Betascope 603 Blot Analyzer (Betagen).

3. Results

(a) Isolation, mapping and DNA sequencing of dnaK25

The dnaK25 mutant was isolated in a selection for E. coli mutants that blocked λ development at 32°C. The λK colonies that failed to grow at 42°C were further analyzed. One mutant, designated dnaK25, was also defective in the utilization of mannose and sorbitol. A plasmid bearing the wild-type dnaK allele suppressed all the phenotypes of this mutant, suggesting that the mutation lay in dnaK (Figure 1). Furthermore, although λ did not form plaques on the mutant strain, dnaK25 transducing phage plated, albeit at low efficiency. Subsequent T4 phage transductions of dnaK25 linkage marker indicated that the λ, T', Man- and Sorb- phenotypes cotransduced at the frequency expected for a dnaK allele (data not
shown). These observations indicated that all four phenotypes arose from one or more mutations closely linked to the dnaK locus.

Marker rescue experiments were performed to map more precisely the site of the dnaK25 mutation. ColE1 plasmids carrying overlapping fragments of dnaK were introduced into the mutant strain (Figure 1). None of the plasmids complemented dnaK25 for any of its phenotypes. However, strains carrying plasmid pKY1022 reverted to T^R at a high frequency (10^-4 to 10^-5). Co-reversion of the A^R, Man^- and Srb^- phenotypes was observed in all cases. The remaining plasmids did not increase the frequency of reversion above the background level (10^-8). These results suggested that the dnaK25 mutation(s) lay within a segment spanning nucleotides 1 to 732 of the coding region carried by pKY1022.

Sequencing of the entire dnaK25 coding region identified a single base-pair change, a C to T transition at nucleotide 427 in the dnaK coding strand. This transition is predicted to eliminate an RsaI recognition site. Restriction analysis of two independent PCR-amplified dnaK and dnaK25 sequences confirmed the loss of the RsaI site in the mutant (data not shown).

The dnaK25 mutation changes proline 143 to serine in the predicted amino acid sequence of the protein. Proline 143 is conserved in all of the Hsp70s for which full sequence data are available (SwissProt database, release 24.0, 12/92) and is also conserved in the ATP binding domain of actin (Flaherty et al., 1991). The residue corresponding to proline 143 in the ATP binding domain of Hsc70, proline 147, lies near the base of the nucleotide binding cleft in close proximity to glutamate 175 (C=O distance, 5.74 Å). The strong sequence conservation of the N-terminal domains of bovine Hsc70 and DnaK suggests that proline 143 may be similarly positioned in DnaK.

(b) Phenotypes of dnaK25

The dnaK25 mutant has characteristics in common with other dnaK mutants (Gething & Sambrook, 1992; Ang et al., 1991). At permissive temperature (32°C), the mutant overexpressed the heat shock proteins DnaK and GroEL (data not shown). When shifted to 44°C, DNA and RNA synthesis rapidly ceased whereas protein synthesis decreased more slowly (data not shown). The dnaK25 mutation also interfered with λ replication and the propagation of F-based plasmids (Table 1).

Propagation of plasmids pSC101 and RK2 was similarly inhibited by dnaK25 (Table 1). The defect is

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<td>pRK301</td>
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<td>pK1022</td>
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<td>pGB2</td>
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Wild-type and mutant strains were transformed with the specified plasmids and colonies were selected on antibiotic-containing plates at 32°C. The ability or inability of a plasmid to transform is indicated with + or −. Two or more experiments for every plasmid-strain combination were performed and at least 100 colonies were counted for each combination to determine transformation efficiencies. No difference between dnaK and dnaK25 strains was observed whenever a replica was capable of transforming the mutant. Plasmid stability was estimated as described in Materials and Methods. DnaA-dependence of the various replications is indicated with +. The plasmids used have been previously described (Churchward et al., 1984; Bolivar et al., 1977; Smirsky et al., 1981; Stalker et al., 1983; Kahn et al., 1979; Gaitanaris et al., 1986; Figurski et al., 1978). The pSC101 derivative carrying dnaK is pSIM2. ND, not determined; NA, not applicable.
Figure 2. Mannose transport by dnaK and dnaK25 strains. Initial uptake rates of $[^{14}C]$mannose by wild-type and mutant strains were determined at room temperature. Fresh overnight cultures of $\Omega 72$ (dnaK) and $\Omega 73$ (dnaK25) were diluted 1:100 and grown at 32°C to A$_{600}$ 0.5. Cells were washed twice and resuspended in M9 salts plus 100 µg/ml chloramphenicol. U Mannose was added to a final concentration of 100 µM (including 0.8 µCi $[^{14}C]$mannose), 3 to 5 times above the reported $K_{m}$ (Raphaeli & Saier, 1985). At the indicated times, samples were spotted onto GF/C filters under vacuum and washed with M9 salts. Labeled sugar in the absence of cells was spotted and washed to determine background radioactivity. Filters were dried, quantified, and the data were normalized by protein concentration as described in Materials and Methods. Less than 10% of labeled sugar was taken up by the latest time point, and all experimental values were at least twice above background. 95% confidence intervals are shown for duplicate assays of the same cultures.

recessive, since a pSC101 plasmid carrying the dnaK allele could stably transform a dnaK25 host. The DnaA replication initiation protein is essential for the function of the F, pSC101, and RK2 replicons (Frey et al., 1979; Scott, 1984; Hansen & Yarmolinsky, 1986; Gaylot et al., 1987). Plasmids pBR322, R100 and R6K, which do not utilize DnaA (Frey et al., 1979; Scott, 1984), could be maintained in the mutant strain. These results suggest that the dnaK25 mutant is partially impaired in DnaA function.

The dnaK25 mutant was defective in the utilization of mannose and sorbitol and formed white colonies on MacConkey indicator plates containing these sugars. Although mannose and sorbitol are phosphotransferase (PTS) pathway sugars (Postma & Lengeler, 1985), other PTS sugars (fructose, glucose and mannitol) were utilized by the mutant, as were maltose and lactose, which are PTS-independent carbohydrates. The Man$^+$ and Srb$^+$ phenotypes could be suppressed independently, since Srb$^-$ or Man$^-$ revertants were isolated which remained deficient in the utilization of the other sugar (data not shown). The defect in mannose utilization is due, at least in part, to reduced transport. Initial uptake rates of $[^{14}C]$mannose were fourfold lower in dnaK25 mutant cells than in wild-type cells (Figure 2).

We noted that idnaK$^+$ transducing phage plated with low efficiency on dnaK25 strains. The mutants dnaK7 and dnaK756, in contrast, fully support the growth of idnaK$^+$. We eliminated the possibility that the chromosomal dnaK allele was dominant to the transduced dnaK allele by constructing merodiploid strains. dnaK25 was introduced by P1 cotransduction with a linked Tn10 marker into a strain carrying a $\Delta$imm$^-$dnaK$^+$ prophage. All Tet$^-$ transductants screened were $\Delta$m$^-$, $\Delta$i$^-$, and were able to utilize mannose and sorbitol. To demonstrate the presence of the dnaK25 allele, four transductants were back-crossed into a non-lysogenic dnaK$^+$ strain. Two of the four back-crosses yielded transductants that displayed the full dnaK25 phenotype. We conclude that dnaK25 is recessive. Defects in mannose permease prevent the penetration of $\lambda$ (Williams et al., 1986), which may explain the reduced efficiency of plating of idnaK$^+$ transducing phage on dnaK25 strains. However, adsorption of $\lambda$ to a dnaK25 strain was reduced twofold relative to wild-type (data not shown), and this may also contribute to the idnaK plating phenotype.

(c) Biochemical characterization of DnaK25

The distinctive dnaK25 phenotypes prompted us to study the enzymatic activities of the mutant protein. The DnaK proteins from wild-type and dnaK25 strains were purified and their ATPase and autophosphorylation activities compared.

Characterization of the kinetics of ATP hydrolysis was complicated by the apparent high affinity of both wild-type DnaK and DnaK25 for nucleotide and their slow rates of ATP hydrolysis. Given these properties, we were not always able to assay ATPase activity with a large molar excess of ATP in the reaction. Under these conditions, one of the assumptions required to derive the Michaelis-Menten equation for irreversible single substrate reactions, that free substrate is in large molar excess over the enzyme-substrate complex, no longer holds. Kinetic parameters may be estimated by using an alternative equation derived without this assumption (see the legend to Table 2 for details).

We found that the ATPase activity of DnaK25 was significantly reduced relative to wild-type (Table 2). The wild-type enzyme has a $V_{max}$ corresponding to 0.058 (± 0.003) ATP/min per monomer of DnaK at 32°C, pH 7.9. In contrast, DnaK25 has a $V_{max}$ corresponding to 0.0021 (± 0.0002) ATP/min per monomer of DnaK25, approximately 20-fold lower than DnaK. The $K_m$ of wild-type DnaK and DnaK25 for ATP are roughly equal (20 (± 3) mM versus 25 (± 24) mM). The large error in our estimate of the DnaK25 $K_m$ results from the necessity of using concentrations of enzyme well above its apparent $K_m$ for ATP in order to accurately measure hydrolysis rates. Similar kinetic constants were determined for three independently purified DnaK25 preparations. We next asked if the DnaK25 ATPase was still subject to regulation by the Hps DnaJ and GrpE and by peptide substrates. ATP hydrolysis by both wild-type DnaK and DnaK25 was stimulated upon addition of either DnaJ or GrpE, and further stimulation was seen when DnaJ and GrpE were
added together (data not shown). Under our experimental conditions (see Materials and Methods), addition of DnaJ and GrpE together stimulated ATP hydrolysis twofold for wild-type DnaK and fourfold for DnaK25 (data not shown). Replacement of DnaJ and GrpE with equal concentrations of ovalbumin did not stimulate the ATPase of either DnaK or DnaK25. It was necessary to use tenfold higher protein concentrations in the DnaK25 reactions to detect basal ATPase activity, though the same stoichiometries of GrpE and DnaJ to DnaK were used in the reactions with wild-type DnaK and DnaK25. The higher protein concentrations may favor complex formation and perhaps explain the greater stimulation of the DnaK25 ATPase by the two Hsp70s.

GrpE has been shown to stimulate nucleotide exchange by DnaK (Liberek et al., 1991a). As a further test of the ability of GrpE to regulate DnaK25, we determined the rates of nucleotide exchange of wild-type DnaK and DnaK25 in the absence and presence of GrpE. Wild-type or mutant protein was preincubated at 30°C for five minutes

**Table 2**

| Kinetics of ATP hydrolysis by wild-type DnaK and by DnaK25 in the presence and absence of saturating F6 peptide |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Kₘ (nM)         | kₘ (min⁻¹)     |
| DnaK            | 20 ± 3          | 0.038 ± 0.003   |
| DnaK25          | 25 ± 24         | 0.0021 ± 0.0002 |
| DnaK25 + F6 peptide | 50 ± 14     | 0.015 ± 0.0006  |

Wild-type DnaK was assayed using 0.5 nM DnaK and 18, 24, 30, 60, 120, 360, and 1000 nM ATP. DnaK25 was assayed in the absence of F6 peptide using 450 nM DnaK25 and 150, 200, 300, 420, 660, 1000, and 2000 nM ATP, and in the presence of 1-12 nM F6 peptide using 45 nM DnaK25 and the same ATP concentrations. The ATPase assays were performed at 32°C. Because of the low ATPase activity of DnaK25, it was necessary to use high concentrations of enzyme in the assays to determine accurately the rates of ATP hydrolysis. Under these conditions, an assumption required to derive the Michaelis-Menten equation, that substrate be in large excess over enzyme, does not hold. Alternatives to the Michaelis-Menten equation have been developed for determining kinetic parameters when substrate is not in large excess (reviewed by Griffiths, 1979). An equation accurately describing the dependence of reaction velocity on total substrate concentration for an irreversible, single-substrate reaction can be derived without assuming that substrate is in excess by solving the quadratic equation for the concentration of the enzyme-substrate complex directly, assuming that \( d[E][S]/d[t] = 0 \) during the course of the reaction. Thus:

\[
\frac{V}{V_{max}} = \frac{[E]_0 + [S]_0}{[E]_0 + [S]_0 + [E]_0 [S]_0} + \frac{[S]_0 + K_m}{[S]_0 + [E]_0 [S]_0} \quad (1)
\]

(Reiner, 1969), where \([E]_0\) and \([S]_0\) are the initial concentrations of enzyme and substrate. Here we assumed that \( d[E][S]/d[t] = 0 \) during the linear portions of the progress curves at each concentration of ATP estimated \( V_{max}\) by inspection of the plateau region of the saturation curve, and solved for \( K_m \) and \( V_{max}\) in equation (1) by least squares non-linear regression fitting of the data. Indicated errors are the standard errors from the non-linear regression analyses only and do not include the errors from the estimates of the initial rates. The kinetic parameters for both wild-type DnaK and DnaK25 were determined using this approach; the kinetic parameters determined for wild-type DnaK by fitting the data to the Michaelis-Menten equation by non-linear regression were not significantly different.

\[ k_m = \frac{V_{max}}{[S]_0} (\text{nM min}^{-1})(\text{M}) \]

![Figure 3](image)

**Figure 3.** Nucleotide exchange by wild-type DnaK and DnaK25 in the presence and absence of GrpE. Each timepoint was determined using a separate incubation reaction. 200 nM wild-type DnaK or DnaK25 was preincubated with 1 μM [³H]ADP at 30°C for 5 min and then chased with 2 mM unlabeled ADP and 2 mM inorganic phosphate in the presence or absence of 400 nM GrpE. At the indicated times, samples were withdrawn and bound [³H]ADP was separated by rapid gel filtration. Non-specific binding, determined by mixing [³H]ADP with excess unlabeled ADP before preincubating with DnaK, was subtracted. The 0 min timepoint indicates the amount of [³H]ADP bound when buffer-alone was added as chase; no loss of binding occurred with time following chase with buffer alone (data not shown). See Materials and Methods for details.

with 1 μM [³H]ADP, and then chased with 2 mM unlabeled ADP in the presence or absence of GrpE. Bound [³H]ADP was separated from free [³H]ADP by rapid gel filtration. Under buffer conditions similar to those used in the ATPase assay, wild-type DnaK had a very rapid rate of nucleotide exchange at 30°C (t½ = 15 seconds, data not shown), which was further stimulated by addition of GrpE. Addition of inorganic phosphate greatly reduced the rate of nucleotide exchange, as previously described by Gao et al. (1993), and this allowed for more accurate comparison of the wild-type DnaK and DnaK25 exchange rates. Under these conditions, wild-type DnaK had a t½ for ADP exchange of 2-23 minutes (Figure 3). The exchange rate of DnaK25 was fourfold faster (t½ = 0-59). Inclusion of GrpE in the chase with excess unlabeled ADP resulted in nearly full exchange of bound [³H]ADP by the first timepoint with both wild-type DnaK and DnaK25. Thus the DnaK25 mutant protein still responds to GrpE stimulation of nucleotide exchange and ATP hydrolysis.

The binding of peptide and polypeptide substrates to DnaK and the other Hsp70s also stimulates ATP hydrolysis (reviewed by Gething & Sambrook, 1992; Hartl & Neupert, 1992; Ang et al., 1991; Craig et al., 1993). To study this reaction, we used five model peptides known to interact with DnaK or the Hsp70, BIP (Flyt et al., 1989; Sherman & Goldberg, 1991; Gragerov et al., 1994). ATPase assays were performed using ATP concentrations 10 to 40 times higher than the apparent Kₘ values of wild-type DnaK and DnaK25. Progress curves for ATP hydrolysis...
by wild-type DnaK and DnaK25 at varying concentrations of peptide NR are shown in Figure 4B, and the relative initial rates calculated from these curves are plotted against peptide NR concentration in the saturation curves shown in Figure 4A. Similar data for all five peptides are summarized in Table 3. The peptides exhibited a range of apparent affinities for stimulating the wild-type DnaK ATPase, from 4 μM to 1 mM. At saturating concentrations, peptides stimulated the wild-type ATPase three- to ninefold above basal levels (Table 3). For three of the peptides used in these experiments (NR, F6, KW) the relative affinities for stimulating ATP hydrolysis corresponded to the relative affinities for binding wild-type DnaK as determined by competitive binding experiments (Gragerov et al., 1994). Peptides NR, F6, and KW also inhibited autophosphorylation by wild-type DnaK with the same relative affinities (Panagiotidis et al., 1994). At saturating concentrations, all five peptides stimulated the DnaK25

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Figure 4. Stimulation of wild-type DnaK and DnaK25 ATP hydrolysis by peptide NR. A. Saturation of the peptide-stimulated ATPase activities of DnaK and DnaK25 by increasing concentrations of peptide NR. The initial rates of ATP hydrolysis at each concentration of peptide NR have been normalized separately for wild-type DnaK and DnaK25, so that 0% is the basal ATPase rate of wild-type DnaK or DnaK25 in the absence of peptide, and 100% is the rate of ATP hydrolysis at saturating [NR], determined by least squares non-linear regression fitting of the saturation curve data. See Table 3 for the estimates of $K_a$ and $V_{max}$ (expressed as maximum-fold stimulation) determined from the curves before normalization. B. Progress curves of ATP hydrolysis by wild-type DnaK (left) and DnaK25 (right) with increasing concentrations of peptide NR. The initial rates determined from these curves by least squares linear regression were used to plot the saturation curves shown in A.
Table 3
Stimulation of DnaK and DnaK25 ATP hydrolysis by peptide substrates

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>(K_s (\mu M))</th>
<th>(K_{max}) (DnaK)</th>
<th>(K_{max}) (DnaK25)</th>
<th>Maximum-fold stimulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>NR</td>
<td>NRELITG</td>
<td>3 ± 1</td>
<td>110 ± 30</td>
<td>3 4 ± 0 3</td>
<td>4 5 ± 0 4</td>
</tr>
<tr>
<td>C</td>
<td>KLGRLSSLFPRPK</td>
<td>21 ± 6</td>
<td>310 ± 90</td>
<td>3 4 ± 0 1</td>
<td>4 9 ± 0 3</td>
</tr>
<tr>
<td>F6</td>
<td>MQERTPLKDVAM</td>
<td>31 ± 4</td>
<td>470 ± 30</td>
<td>4 3 ± 0 2</td>
<td>8 4 ± 0 3</td>
</tr>
<tr>
<td>A</td>
<td>KRQIYTRLEMRNLGK</td>
<td>120 ± 20</td>
<td>2600 ± 100</td>
<td>3 0 ± 0 3</td>
<td>4 9 ± 1 3</td>
</tr>
<tr>
<td>KW</td>
<td>KWVALHFEG</td>
<td>1130 ± 80</td>
<td>980 ± 100</td>
<td>8 6 ± 0 3</td>
<td>4 3 ± 0 3</td>
</tr>
</tbody>
</table>

Wild-type DnaK was assayed at a concentration of 7.5 nM in the presence of 250 nM ATP and DnaK25 was assayed at a concentration of 200 nM in the presence of 1 μM ATP. Peptide concentrations ranged from 1 μM to 3 μM for peptide NR, 5 μM to 2 μM for peptide C, 5 μM to 1 μM for peptide F6, 12 μM to 1 μM for peptide A, and 100 μM to 3 μM for peptide K.W. The ATPase assays were performed at 37°C. Maximum-fold stimulation was calculated as the ratio of \(V_{max}\) (from the peptide saturation curve) to the basal rate of ATP hydrolysis in the absence of peptide. Values are indicated ± standard error. The standard errors are minimum estimates only determined from the least-squares non-linear regression fits of the peptide saturation curves and the least-squares linear regression fits of the basal ATPase progress curves. The amino acid composition of the peptides is designated by single-letter code.

ATPase to about the same extent as they did the wild-type ATPase. The apparent \(K_s\) values of peptides NR, A, C, and F6 for stimulating the DnaK25 ATPase, however, were at least an order of magnitude higher than for wild-type DnaK. Peptide KW, which had the lowest affinity for wild-type DnaK, with a \(K_s\) for ATPase stimulation of 1 mM, had an equally low affinity for DnaK25. Thus the dnaK25 mutation reduces the affinity of several peptide substrates for stimulating the DnaK25 ATPase but does not affect the overall fold-stimulation of the ATPase at saturating peptide concentrations.

We next examined how peptides stimulate the DnaK ATPase. Peptide F6 increased the \(V_{max}\) of ATP hydrolysis for DnaK25 but had little effect on \(K_s\) (Table 2). Similar results were found with wild-type DnaK at single ATP concentrations above and below the \(K_s\) for ATP (data not shown). We asked if peptides stimulated ATP hydrolysis by promoting nucleotide exchange, as suggested by Sadie & Hightower (1992). We found, however, that addition of either peptide A or peptide C to the unlabeled ADP chase at concentrations that are saturating for ATPase stimulation had no effect on the rate of nucleotide exchange (Figure 5).

In contrast to its impaired ATPase, the autophosphorylation activity of DnaK25 was equal to or greater than that of wild-type DnaK in the temperature range from 32 to 44°C. DnaK25 lost activity between 44 and 50°C (Figure 6), whereas DnaK lost activity between 50 and 55°C (data not shown; McCarty & Walker, 1991; Panagiotidis et al., 1994). Autophosphorylation was assayed at an ATP concentration above the \(K_s\) for both proteins. The autophosphorylation of both wild-type DnaK and DnaK25 increased with temperature, as previously reported for wild-type DnaK (McCarty & Walker, 1991).

![Figure 5](image_url) Effect of peptides on nucleotide exchange by wild-type DnaK. See the legend to Figure 3 and Materials and Methods for details. Peptides A and C were each added to a final concentration of 500 μM in the chase with unlabeled ADP and inorganic phosphate.

![Figure 6](image_url) Autophosphorylation activities of wild-type DnaK and DnaK25 at various temperatures. The assays were performed using 285 nM DnaK or DnaK25 and 1 μM ATP with 0.8 μCi of [γ-32P]ATP (3000 Ci/mmol) included per reaction. Samples were taken at 15 and 30 min timepoints and added directly to SDS loading buffer. Reaction products were separated on 10% SDS polyacrylamide gels, and the gels were treated and quantified as described under Materials and Methods. Incorporation of [32P]orthophosphate into the DnaK and DnaK25 bands increased linearly between 15 and 30 min in all conditions except DnaK25 at 50°C; the values at the 30 min timepoint are shown.
4. Discussion

We have selected a mutation in the dnaK gene of E. coli that alters a conserved proline residue (proline 143) in the putative ATP-binding cleft of the protein. The dnaK25 mutation greatly impairs the ATPase activity of the enzyme, reduces the affinity of peptides for stimulating the DnaK25 ATPase, and confers a highly pleiotropic phenotype. The dnaK25 mutant is temperature-sensitive for growth, deficient in the propagation of bacteriophage λ, unable to maintain plasmids that require DnaA for replication, and defective in the utilization of mannose and sorbitol.

The mannose and sorbitol utilization defects conferred by dnaK25 are not general properties of dnaK mutants, such as dnaK756 or dnaK7. The Man–phenotype of dnaK25 strains is due, at least in part, to reduced transport of the sugar. The inability of JdnaK to infect mutant cells efficiently is consistent with a defect in mannose permease activity, which is required for phage penetration (Williams et al., 1986).

We also find a partial defect in the adsorption of λ to dnaK25 cells. The failure of dnaK25 strains to support growth of JdnaK may result from improper folding of mannose permease or LamB, from a block in membrane insertion or translocation, or from a more global effect on gene expression.

DnaA activates DnaK by disrupting DnaA–phospholipid aggregates (Hwang et al., 1990). We suggest that the failure of dnaK25 mutants to maintain F plasmids or RK2, all of which require DnaA for replication (Frey et al., 1979; Scott, 1984; Hansen & Yarmolinsky, 1986; Gaylo et al., 1987), is due to sequestration of DnaA in non-functional aggregates with a resulting limitation in the levels of active DnaA. The inability to propagate plasmid λ82, which utilizes λ replication functions (Gaitanaris et al., 1986), may result from the inability of DnaK25 to disassemble the preprimosomal complex at ori-λ (Alfano & McMacken, 1989; Zylitch et al., 1989).

The biochemical characteristics of DnaK25 can best be summarized by referring to a simple four-state model for the cycling of DnaK and the Hsp70s as they interact with nucleotides and peptide or polypeptide substrates (Figure 7). DnaK is bound to either ATP or ADP in each state (Palleros et al., 1991; Gao et al., 1993). Slow hydrolysis converts DnaK–ATP to DnaK–ADP (Figure 7, step a) and nucleotide exchange returns DnaK–ADP to the ATP-bound form (Figure 7, step b; see Gao et al., 1993). Phosphate release may precede the exchange of ADP for ATP, as suggested by the inhibitory effect of inorganic phosphate on exchange (Gao et al., 1993; this work). ATP hydrolysis is stimulated by the binding of peptide and polypeptide substrates (Figure 7, step c; reviewed by Gething & Sambrook, 1982; Hartl & Neupert, 1992; Ang et al., 1991; Craig et al., 1993), but nucleotide exchange is not affected (Figure 7, step d; Figure 5; the rates of steps b and d in Figure 7 are thus equal).

Substrates stimulate ATP hydrolysis by increasing the $k_{cat}$ of ATP hydrolysis without greatly affecting the $K_m$ (Sadis & Hightower, 1992; Table 2). Finally, the ATP bound form of DnaK has a lower affinity for peptide substrate than the ADP bound form (Figure 7, equilibria E and F; Schmid et al., 1994), and the binding of ATP results in substrate dissociation (Polham, 1986; Liberek et al., 1991b; Banek et al., 1992; Palleros et al., 1993b; Gragerov et al., 1994).

We have determined the kinetic parameters of ATP hydrolysis (Table 2) and the rate of nucleotide exchange for wild-type DnaK (Figure 7). The ATPase rate of DnaK (determined at 32°C, pH 7.5, which is in close agreement with the turnover rates for DnaK of 0.06 min⁻¹ (30°C, pH 8.1) reported by McCormy & Walker (1991) and 0.016 (±0.004) min⁻¹ (25°C, pH 7.2) and 0.087 (±0.007) min⁻¹ (37°C, pH 7.2) reported by Palleros et al. (1995a). The DnaK $K_m$ for ATP is very low, 20 (±3) nM. This agrees well with data of R. Jordan and R. McMacken for DnaK, who have determined a $K_m$ of approximately 40 nM (personal communication). These values are close to the estimated $K_m$ of bovine Hsp70 for ATP 10 nM (Gao et al., 1994). However, a $K_m$ of 4 μM has recently been reported for DnaK (Buchberger et al., 1994); we do

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**Figure 7.** Four-state model for the cycle of interactions of DnaK and the Hsp70s with nucleotides and peptide model substrates. DnaK is modeled as always being in a nucleotide-bound form. ATP hydrolysis converts DnaK-ATP to DnaK-ADP (steps a and c) and nucleotide exchange returns DnaK-ADP to the ATP-bound form (steps b and d). Phosphate release may precede the exchange of ADP for ATP as suggested by the inhibitory effect of inorganic phosphate on exchange. ATP hydrolysis is stimulated by the binding of peptide and polypeptide substrates (step c is faster than step a) but nucleotide exchange is not affected (the rates of steps b and d are equal). Both the ATP- and ADP-bound forms of DnaK interact with peptide substrate (equilibria E and F), but the ATP-bound form of DnaK has a lower affinity for peptide substrate than the ADP bound form (equilibrium E is shifted towards dissociation of the DnaK-peptide complex) and the binding of ATP results in substrate dissociation. See text for further discussion and references.
not know the basis of this discrepancy. Estimates of the $K_m$ of ATP hydrolysis for BiP are somewhat higher than ours for DnaK, ranging from 110 nM (Kassenbrock & Kolly, 1989) to 400 nM (Blond-Elguindi et al., 1993), while those for bovine Hsc70 fall between 0.7 and 1.3 μM (Braza et al., 1984; Sadis & Hightower, 1992; O'Brien & McKay, 1993).

Nucleotide exchange by DnaK (step 5 in Figure 7) is very rapid under buffer conditions similar to those used in the ATPase assay. The addition of inorganic phosphate slows the rate of nucleotide exchange for wild-type DnaK eightfold at 30°C (data not shown), but even this rate is tenfold faster than the rate of ATP hydrolysis at 32°C (that is, step b in Figure 7 is approximately tenfold faster than step a; Figure 3 and Table 2). The rate of exchange therefore cannot be the rate limiting step of steady-state ATP hydrolysis under our experimental conditions. Gao et al. (1993) also found, with bovine Hsc70, that nucleotide exchange proceeded much more rapidly than ATP hydrolysis. Sadis & Hightower (1992), however, while finding a similar rate of nucleotide exchange, reported a high rate of ATP hydrolysis, and suggested that nucleotide exchange was rate limiting for the reaction.

DnaJ stimulates ATP hydrolysis by DnaK, whereas the major effect of GrpE is to enhance nucleotide exchange (Liberek et al., 1991a). GrpE added together with DnaJ further stimulates ATP hydrolysis, although the effect in our hands is small (twofold; data not shown). Liberek et al. (1991a) reported stimulation in the range five- to 50-fold, noting that they found variation with different protein preparations (Liberek et al., 1991a).

The biochemical analysis of DnaK25 revealed four differences from the wild-type enzyme:

(1) The rate of ATP hydrolysis by DnaK25 in the absence of peptide is reduced 20-fold relative to wild-type DnaK (Table 2; Figure 7, step a). The $K_m$ for ATP is not significantly affected.

(2) The affinities of several peptides for stimulating the DnaK25 ATPase are reduced 15 to 25-fold. However, the fold-induction of the ATPase by saturating concentrations of peptide is similar to wild-type DnaK (three to ninefold; Table 3 and Figure 4). Thus the rate of DnaK25 ATP hydrolysis in the presence of saturating concentrations of peptide is also reduced roughly 20-fold relative to wild-type (Figure 7, step c). DnaJ and GrpE stimulate the DnaK25 and wild-type ATPases to similar extents (data not shown).

(3) The rate of nucleotide exchange by DnaK25 is fourfold higher than the wild-type rate (Figure 3; step b in Figure 7). Stimulation of nucleotide exchange by GrpE is not affected.

(4) The rate of autophosphorylation by DnaK25 is higher than the wild-type rate at 32°C, but approaches the wild-type rate at higher temperatures (Figure 6).

It is unlikely that the reduced ATPase activity of DnaK25 results from inactivation of the enzyme during purification. Similar kinetic parameters were obtained from three independent purifications of DnaK25. Furthermore, the autophosphorylation activity of the mutant was equal to or greater than that of wild-type, both in the rate and in the absolute levels of [$\gamma$-32P]orthophosphate incorporation (Figure 6). Finally, UV-crosslinking of excess [x-32P]ATP to the wild-type and mutant proteins followed by SDS-PAGE and autoradiography demonstrated equal levels of labeling (data not shown). It should also be noted that the reduced affinities of peptides for stimulating the DnaK25 ATPase can only be explained as a specific defect of the enzyme and not as a mere result of inactivation of the enzyme.

The retention of autophosphorylation activity by DnaK25 despite its greatly reduced ATPase activity implies that ATP hydrolysis and autophosphorylation are independent reactions. Threonine 199, the target of autophosphorylation, does appear to be important for ATP hydrolysis by DnaK (McCarty & Walker, 1991), as does the corresponding residue of BiP (Gaut & Hendershot et al., 1993), either by acting as catalytic residues or by influencing the conformation of the nucleotide binding pocket. However, the corresponding residue of Hsc70 does not appear to be critical for ATP hydrolysis (O'Brien & McKay, 1993).

Our results indicate that a mutation in the N-terminal nucleotide binding domain can alter substrate interactions with the C-terminal substrate binding site. This is consistent with the functionally important coupling between the two domains, linking ATP binding and hydrolysis and polypeptide binding and release. Based on comparison with the Hsc70 ATPase domain crystal structure, proline 143 is predicted to lie near the base of the nucleotide binding cleft in close proximity to glutamate 171. The corresponding residue of bovine Hsc70, glutamate 175, acting via a bridging water molecule, is one of several residues that coordinate the binding of Mg$^2+$ in the nucleotide binding cleft (O'Brien & McKay, 1993). It has been suggested to play a critical role in coupling ATP hydrolysis with substrate binding (Holmes et al., 1993). Mutations of DnaK glutamate 171 have now been reported to couple ATP binding from substrate release and substrate binding from stimulation of ATP hydrolysis (Buchberger et al., 1994). Our proline 143 to serine mutation may reduce the affinities of substrates for stimulating ATP hydrolysis by interfering with glutamate 171 or the water with which it may hydrogen bond. However, since mutation of glutamate 171 is reported not to interfere with basal ATP hydrolysis (Buchberger et al., 1994), mutation of proline 143 must affect other elements of the nucleotide binding pocket as well. It is unclear if the universally conserved proline 143 is only important in determining the structure of the nucleotide binding cleft or if it plays a more direct role in communication with the C-terminal domain. Biochemical analysis of other mutants and more detailed enzymology of the wild-type enzyme will be required to sort out these possibilities.
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