A Bipartite Signaling Mechanism Involved in DnaJ-mediated Activation of the Escherichia coli DnaK Protein*

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The DnaK and DnaJ heat shock proteins function as the primary Hsp70 and Hsp40 homologues, respectively, of Escherichia coli. Intensive studies of various Hsp70 and DnaJ-like proteins over the past decade have led to the suggestion that interactions between specific pairs of these two types of proteins permit them to serve as molecular chaperones in a diverse array of protein metabolic events, including protein folding, protein trafficking, and assembly and disassembly of multienzyme protein complexes. To further our understanding of the nature of Hsp70-DnaJ interactions, we have sought to define the minimal sequence elements of DnaJ required for stimulation of the intrinsic ATPase activity of DnaK. As judged by proteolysis sensitivity, DnaJ is composed of three separate regions, a 9-kDa NH₂-terminal domain, a 30-kDa COOH-terminal domain, and a protease-sensitive glycine- and phenylalanine-rich (G/F-rich) segment of 30 amino acids that serves as a flexible linker between the two domains. The stable 9-kDa proteolytic fragment was identified as the highly conserved J-region found in all DnaJ homologues. Using this structural information as a guide, we constructed, expressed, purified, and characterized several mutant DnaJ proteins that contained either NH₂-terminal or COOH-terminal deletions. At variance with current models of DnaJ action, DnaJ 1-75, a polypeptide containing an intact J-region, was found to be incapable of stimulating ATP hydrolysis by DnaK protein. We found, instead, that two sequence elements of DnaJ, the J-region and the G/F-rich linker segment, are each required for activation of DnaK-mediated ATP hydrolysis and for minimal DnaJ function in the initiation of bacteriophage λ DNA replication. Further analysis indicated that maximal activation of ATP hydrolysis by DnaK requires two independent but simultaneous protein-protein interactions: (i) interaction of DnaK with the J-region of DnaJ and (ii) binding of a peptide or polypeptide to the polypeptide-binding site associated with the COOH-terminal domain of DnaJ. This dual signaling process required for activation of DnaJ function has mechanistic implications for those protein metabolic events, such as polypeptide translocation into the endoplasmic reticulum in eukaryotic cells, that are dependent on interactions between Hsp70-like and DnaJ-like proteins.

The DnaJ, DnaK, and GrpE proteins of Escherichia coli were first identified via genetic studies of E. coli mutants that are incapable of supporting the replication of bacteriophage λ DNA (1–3). Later, it was found that DnaJ, DnaK, and GrpE are all prominent bacterial heat shock proteins, which comprise a set of about 30 proteins whose expression is transiently induced when cells are grown at elevated temperatures (reviewed in Refs. 4 and 5). In recent years it has become apparent that eukaryotic cells contain families of proteins that are homologous to each DnaJ and DnaK (6, 7). Intensive investigations in numerous laboratories have demonstrated that these universally conserved proteins participate in a wide variety of protein metabolic events in both normal and stressed cells, including protein folding (8, 9), protein trafficking across intracellular membranes (10, 11), proteolysis, protein assembly, as well as disassembly of protein aggregates and multienzyme structures (reviewed in Refs. 12 and 13). Because several of these DnaJ and DnaK family members have the capacity to modulate polypeptide folding and unfolding, they have been classified as molecular chaperones (14).

The available evidence indicates that DnaJ, DnaK, and GrpE of E. coli often cooperate as a chaperone team to carry out their physiological roles. Each of these three proteins functions in (i) regulation of the bacterial heat shock response (15, 16); (ii) general intracellular proteolysis (17); (iii) folding of nascent polypeptide chains, maintaining proteins destined for secretion in a translocation-competent state, and disassembly and refolding of aggregated proteins (reviewed in Ref. 18); (iv) flagellar synthesis (19); (v) replication of coliphages λ and P1 and replication of the F episome (20). DnaK, the primary Hsp70 homologue of E. coli (21), is believed to play a central role in these processes. Like other members of the Hsp70 family, DnaK possesses a weak ATPase activity (22). The DnaK ATPase activity is stimulated by the DnaJ and GrpE heat shock proteins (23, 24), as well as by many small peptides that are at least 6 amino acids in length (24, 25). Peptide interactions with DnaK may be representative of the binding of Hsp70 proteins to unfolded or partially folded polypeptides.

In contrast to the situation for DnaK and Hsp70 proteins, relatively few investigations have focused on DnaJ or other members of the Hsp40 family. It is known from in vitro studies of λ DNA replication that DnaJ participates along with DnaK in the assembly and disassembly of nucleoprotein structures that form at the viral replication origin (26–30). DnaJ may play a dual role in this and other Hsp-mediated processes. In addition to activating ATP hydrolysis by DnaK, DnaJ, by binding first to multiprotein assemblies or nascent polypeptides, may also assist DnaK by facilitating its interaction with polypeptide substrates (26, 30–34).

Comparisons of the amino acid sequences of DnaJ family members has led to the identification of three conserved sequence domains in E. coli DnaJ (35). These sequence domains,
proceeding from the amino terminus, are: 1) a highly conserved 70-amino acid region, termed the J-region, that is found in all DnaJ homologues; 2) a 30-amino acid sequence that is unusually rich in glycine and phenylalanine residues; and 3) a cysteine-rich region that contains four copies of the sequence Cys-X-Cys-Ser-Gly-X, where X generally represents a charged or polar amino acid residue. The COOH-terminal portion of E. coli DnaJ, comprising residues 210–376, is not well conserved.

To investigate the functional roles of the conserved sequence domains of DnaJ, we constructed a series of recombinant plasmids that express truncated DnaJ proteins, each of which carries a deletion of one or more of the conserved sequence elements. Following purification, each deletion mutant protein was examined for its capacity to activate ATP hydrolysis by DnaK. Our results indicate that the J-region and the Gly/Phex-rich segment of DnaJ must both be present in cis in the DnaJ deletion mutant protein to achieve stimulation of DnaK-mediated ATP hydrolysis. We have, however, discovered that the J-region alone is capable of activating ATP hydrolysis by DnaK if it is supplemented in trans with small peptides that have high affinity for the polypeptide-binding site on DnaK. We discuss the relevance of these findings for protein metabolic events mediated in part by cooperative action of Hsp70 homologues and DnaJ-like proteins.

**EXPERIMENTAL PROCEDURES**

**Reagents and Materials**—Reagents and materials and their sources were: papain (Worthington Biochemical); Hepes (Research Organics); ATP, ADP, DEAE-Sepharose, butyl-Sepharose 4B, and Mono-Q HR FPLC columns (Pharmacia Biotech); restriction enzymes (New England Biolabs); AmpliTaq DNA polymerase (Perkin-Elmer); polyethyleneimine-cellulose thin layer chromatography sheets (EM Industries); Bio-Rex 70 (100–200 mesh) and hydroxyapatite (Bio-Rad); P-11 Cellulose Phosphate (Whatman Lab Sales); [3H]ATP (3000 Ci/mmol) (Amersham); staphylococcal protein A and PM-20 and YM-3 membranes (Amicon). All other biochemicals were from Sigma.

**Buffers and Media**—Buffer A is 50 mM Hepes/NaOH, pH 7.6, 2 mM DTT, 0.15 mM NaCl, 10% (v/v) glycerol; buffer B is 50 mM Hepes/NaOH, pH 7.6, 2 mM DTT, 0.15 mM NaCl, 10% (v/v) glycerol; buffer C is 50 mM potassium phosphate, pH 6.8, 2 mM DTT, 0.15 mM KCl, and 10% (v/v) glycerol; buffer D is 50 mM Hepes/NaOH, pH 7.6, 2 mM DTT, 0.15 mM NaCl, 1 mM ammonium sulfate, and 10% (v/v) glycerol; buffer E is 25 mM Hepes/NaOH, pH 7.6, 2 mM DTT, 0.1 mM NaCl, and 10% (v/v) glycerol; buffer F is 50 mM Hepes/NaOH, pH 7.6, 2 mM DTT, 25 mM NaCl, and 10% (v/v) glycerol; SDS-PAGE sample buffer is 0.18 M Tris-HCl, pH 6.8, 2.0 M β-mercaptoethanol, 0.01% (v/v) bromophenol blue, 30% (v/v) glycerol. Lysis buffer is 50 mM Hepes/NaOH, pH 7.6, 2 mM DTT, 1 mM NaCl, and 2 mM MgCl2. Denaturation buffer is 50 mM potassium phosphate, pH 6.8, 0.15 mM KCl, 10% (v/v) glycerol, 1 mM DTT, and 7 mM guanidinium hydrochloride. Terrific broth (TB) medium is prepared by mixing at room temperature 900 ml of sterile broth (12 g of Difco BactoTryptone, 24 g of Difco BactoYeast Extract, 4 ml of glycerol, 900 ml of H2O) with 100 ml of sterile phosphate buffer (0.17 M KH2PO4 and 0.72 M K2HPO4) (37).

**Determination of Protein Concentration**—The protein concentrations of samples containing partially purified proteins were determined by the method of Bradford (38), using bovine γ-globulin as a standard. The concentrations of purified DnaJ and DnaJ deletion mutant proteins were determined in denaturation buffer, using their individual molar extinction coefficients (εmax) as determined by the method of Gill and von Hippel (39). The concentration of GrpE was determined by a modification of the method of Lowry et al. (40) using bovine serum albumin as a standard. The concentration of DnaJ was determined using the calculated molar extinction coefficient of the native protein, 15,800 M–1 cm–1 (24).

**Strains and Plasmids**—Two E. coli strains were used for the expression of DnaJ and DnaJ deletion mutant proteins; RLM569 (C600, recA, hsdR, tanA, lac, pro, leu, thr, dnaJ−) and PK102 (admA15), which carries a deletion of the primary portion of the dnaJ coding sequence resulting in a truncated pRLM76 was used as the expression vector for E. coli DnaJ deletion mutant proteins. Plasmid pRLM76 is a derivative of plasmid pH6E (42) that is deleted for the DNA sequence that encodes the amino-terminal portion of the λ N gene. Plasmid pRLM76 contains a polylinker downstream from a phage λ p romoter. Thermosensitive Δ857 repressor protein, which represses transcription from λ at 30°C, is constitutively expressed from a mutant λ d gene present in pRLM76. Expression of genes cloned into the polylinker of pRLM76 can be greatly induced by shifting the growth temperature of cells harboring the plasmid to 42°C. Incubation at this temperature results in a rapid inactivation of 857 repressor protein and leads to an enormous increase in transcription from the strong p romoter. Plasmid pRLM76 was constructed as follows: plasmid pH6E DNA was digested to completion with EcoRI and the 575-bp fragment carrying both the λ p romoter and a portion of the λ N gene was isolated. This fragment was further digested with HindII to produce two fragments of 148 and 227 bp. The 148-bp fragment carrying the p romoter was isolated and ligated to a 3573-bp fragment isolated from pH6E DNA that had been digested with SmaI and partially digested with HindIII. This ligated mixture was transformed into E. coli strain RLM76 and ampicillin-resistant clones that carried a 3.7-kilobase plasmid were identified. A plasmid having the p romoter in the desired orientation (i.e. directing transcription across the polylinker sequence) was identified and named pRLM76 (3721 bp).

Plasmids carrying the wild type dnaJ gene or a dnaJ deletion mutation were constructed by cloning a DNA fragment produced by polymerase chain reaction (PCR)-mediated amplification of E. coli genomic DNA with the aid of synthetic oligonucleotide primers. The sequences of the forward primers used were: oligonucleotide A (5'-CCACCGATCCAGGAGGATAAAGCCATTGCAGGAAGGATTTAC-3'), oligonucleotide B (5'-CCACCGATCCAGGAGGTTAAAAATTAATGGCTGCGTTTGAGCAAGGT-3'), and oligonucleotide C (5'-CCACCGATCCAGGAGGTTAAAAATTAATGGCTGCGTTTGAGCAAGGT-3'). Each reverse primer contained a BamHI restriction site, a consensus ribosome binding site, and an ATG initiation codon juxtaposed to dnaJ coding sequence (underlined in the primer sequences listed above). These coding sequences correspond to dnaJ nucleotides 1–21 for primer A; dnaJ nucleotides 217–234 for oligonucleotide B; and dnaJ nucleotides 397–414 for oligonucleotide C. The sequences of the reverse primers were: oligonucleotide D (5'-CCACCTCTAGATCGACGCAGTGCACTATCCGGGTTAGCTCGTCT-3'), oligonucleotide E (5'-CCACCTCTAGATCGACGCAGTGCACTATCCGGGTTAGCTCGTCT-3'), oligonucleotide F (5'-CCACCTCTAGATCGACGCAGTGCACTATCCGGGTTAGCTCGTCT-3'), and oligonucleotide G (5'-CCACCTCTAGATCGACGCAGTGCACTATCCGGGTTAGCTCGTCT-3'). Each reverse primer contained a PstI recognition site, the complement of two tandem translation stop codons, and the complement of dnaJ coding sequence (underlined). The sequences complementary to dnaJ coding sequence were: oligonucleotide D, complement of dnaJ nucleotides 1131–1114; primer E, complement of dnaJ nucleotides 225–211; and oligonucleotide F, complement of dnaJ nucleotides 318–334. PCR amplification was performed in a reaction mixture (100 μl) containing 120 ng of DNA template and 1000 pmol each of one forward and one reverse primer, 50 μM of each of the four dNTPs, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl2, 0.01% gelatin, and 2.5 units of AmpliTaq DNA polymerase. Plasmid pRLM232 was constructed by PCR amplification of the entire E. coli dnaJ coding sequence, using primers A and D. Following amplification, the PCR product was digested with SmaI and ligated to pRLM76 DNA which had been similarly digested at the unique BamHI site present in the polylinker carried by this vector. The DNA in the ligation mixture was transformed into E. coli strains RLM569 and PK102. Ampicillin-resistant colonies were selected at 30°C and screened for their capacity to overproduce a polypeptide of the size of full-length DnaJ protein (i.e. 43 kDa) when grown at 42°C. Plasmid pRLM232 (a pRLM76 derivative that expresses DnaJ-1–75) was constructed and identified as above, except that the primers A and E were used for the initial PCR amplification and that the amplification...
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Resistant transformants were screened for their capacity to thermally induce overproduction of a protein of 9 kDa. Plasmids pRLM234, pRLM238, and pRLM239, i.e. pRLM76 derivatives for expression of DnaJ 1–106, DnaJ 73–376, and DnaJ 106–376, respectively, were constructed and identified by similar procedures. Several plasmids of each type were selected for DNA sequence analysis.

Expression and Purification of DnaJ 1–75—RLM1340 (RLM569/pRLM233) cells were grown, thermally induced, harvested, and lysed as described above for wild type DnaJ. A cell lysate from 8 g of cells was centrifuged at 120,000 × g for 1 h. The supernatant (Fraction I, 70 ml) was supplemented with ammonium sulfate to 75% saturation (0.476 g of ammonium sulfate per ml of supernatant), stirred at 4°C for 30 min, and centrifuged at 30,000 × g for 1 h. The supernatant was concentrated to 25 ml, using an Amicon stirred cell concentrator fitted with a YM3 membrane, and dialyzed against 2 liters of buffer F (Fraction II, 80 mg, 30 ml). Fraction II was applied to a Bio-Rex 70 column (3.4 × 11 cm) and fractionated into DnaJ 1–75 deletion mutant fractions eluted at approximately 0.12M potassium phosphate. Fractions containing DnaJ 1–75 at greater than 95% purity were pooled (25 ml) and concentrated to 50 ml using an Amicon stirred cell concentrator fitted with a YM3 membrane, and dialyzed against 2 liters of buffer F (Fraction III, 30 mg). Fraction III protein was applied to a Bio-Rad hydroxyapatite column (2.4 × 11 cm) that had been equilibrated with buffer C and had a conductivity equivalent to that of buffer D. This fraction was dialyzed against 2 liters of buffer C (Fraction IV, 30 mg, 10 ml). Fraction IV protein was applied to a Bio-Rex 70 column (3.4 × 11 cm) equilibrated with buffer C and had a conductivity equivalent to that of buffer D. The fractions containing the highest concentration of DnaJ 1–106 were pooled (90 ml) and concentrated to 40 ml in an Amicon stirred cell concentrator fitted with a YM3 membrane (Fraction V, 35 mg, 40 ml). Fraction V protein was applied to a hydroxyapatite column (2.4 × 11 cm) equilibrated with buffer C. The column was washed with 150 ml of buffer C and bound proteins were eluted with a 250-ml linear gradient of 0.15–1.0 M NaCl in buffer C at a flow rate of 1.4 column volumes per h. DnaJ eluted at approximately 0.35 M NaCl. The primary DnaJ-containing fractions were pooled (80 ml) and concentrated to 40 ml with an Amicon concentrator as described above. This sample was dialyzed against 2 liters of buffer F to produce Fraction V (35 mg, 40 ml). Fraction V protein was applied to a hydroxyapatite column (2.4 × 11 cm) equilibrated with buffer C. The column was washed with 150 ml of buffer C and bound proteins were eluted with a 250-ml linear gradient of 120–500 mM potassium phosphate in buffer C at a flow rate of 2.4 column volumes/h. DnaJ eluted at approximately 0.4 M potassium phosphate. Fractions containing DnaJ 1–106 were subsequently pooled (150 ml) and concentrated to 10 ml in an Amicon apparatus, and dialyzed extensively against buffer A. The dialyzed sample (Fraction V, 20 mg, 10 ml) was diluted with an equal volume of buffer E, containing 2 M ammonium sulfate, to produce a conductivity equivalent to that of buffer D. This sample was applied to a Pharmacia-BioTech Butyl-Sepharose 4B column equilibrated with buffer B, followed by a 200-ml linear gradient of 100% buffer D to 100% buffer E, followed by 50 ml of buffer E, at a flow rate of 1 column volume/h. DnaJ eluted at approximately 95–100% buffer E. Fractions containing DnaJ at greater than 90% purity, as analyzed by SDS-PAGE, were pooled (30 ml) and concentrated to 10 ml using an Amicon apparatus (Fraction VI, 10 mg, 10 ml). This protocol routinely produces approximately 1.2 mg of DnaJ at greater than 90% purity per gram of cell paste.

The physical properties of DnaJ 73–376 and DnaJ 106–376 are similar to those of wild type DnaJ. Consequently, these DnaJ deletion mutant proteins could be purified by using a slightly modified version of the purification protocol used for DnaJ. Frozen cells were resuspended in lysis buffer and lysed as described above, except that the lysis buffer also contained 0.1% octyl-β-D-glucoside and 1 mM phenylmethylsulfonyl fluoride and that the cell lysate was mixed gently for 12 h on a shaker at 4°C. The lysate was centrifuged at 120,000 × g for 1 h and the supernatant was supplemented with ammonium sulfate to 40% saturation (0.226 g/ml of supernatant). The precipitated protein was collected by centrifugation at 30,000 × g for 1 h. The protein pellet was dissolved in 50 ml of buffer A and dialyzed extensively against buffer B. All of the remaining purification steps were identical to those used for purifying wild type DnaJ. The final preparations of DnaJ 73–376 and DnaJ 106–376 deletion mutant proteins were shown to be greater than 90% pure. They were quick-frozen in liquid nitrogen and stored at −80°C.

Expression and Purification of DnaJ 1–106—RLM1341 (RLM569/pRLM234) cells were grown, thermally induced, harvested, and lysed as described above for wild type DnaJ. A cell lysate from 8 g of cells was centrifuged at 120,000 × g for 1 h. The supernatant (Fraction I, 70 ml) was supplemented with ammonium sulfate to 75% saturation (0.476 g of ammonium sulfate per ml of supernatant), stirred at 4°C for 30 min, and centrifuged at 30,000 × g for 1 h. The supernatant was concentrated to 25 ml, using an Amicon stirred cell concentrator fitted with a YM3 membrane, and dialyzed against 2 liters of buffer F (Fraction II, 80 mg, 30 ml). Fraction II was applied to a Bio-Rex 70 column (3.4 × 11 cm) and fractionated into DnaJ 73–376 deletion mutant fractions eluted at approximately 0.35 M NaCl. Fractions containing DnaJ 73–376 at greater than 95% purity were pooled (80 ml) and concentrated to 30 ml in an Amicon apparatus (Fraction III, 30 mg). Fraction III protein was applied to a Bio-Rad hydroxyapatite column (2.4 × 11 cm) that had been equilibrated with buffer C. The column was washed with 150 ml of buffer C and eluted with a 250-ml linear gradient of 0.05–0.5 M potassium phosphate at a flow rate of 1.4 column volumes per h. DnaJ 1–75 eluted at approximately 0.9 M potassium phosphate. Fractions containing DnaJ 1–75 at greater than 95% purity were pooled (50 ml) and concentrated to 15 ml in an Amicon apparatus. This sample (Fraction IV, 20 mg, 15 ml) was quick-frozen in liquid nitrogen and stored at −80°C.

Expression and Purification of DnaJ 1–106—RLM1341 (RLM569/pRLM234) cells were grown, thermally induced, harvested, and lysed as described above for wild type DnaJ. A cell lysate from 8 g of cells was centrifuged at 120,000 × g for 1 h. The supernatant (Fraction I, 70 ml) was supplemented with ammonium sulfate to 75% saturation (0.476 g of ammonium sulfate per ml of supernatant), stirred at 4°C for 30 min, and centrifuged at 30,000 × g for 1 h. The supernatant was concentrated to 25 ml, using an Amicon stirred cell concentrator fitted with a YM3 membrane, and dialyzed against 2 liters of buffer F (Fraction II, 80 mg, 30 ml). Fraction II was applied to a Bio-Rex 70 column (3.4 × 11 cm) and fractionated into DnaJ 73–376 deletion mutant fractions eluted at approximately 0.35 M NaCl. Fractions containing DnaJ 73–376 at greater than 95% purity were pooled (80 ml) and concentrated to 30 ml in an Amicon apparatus (Fraction III, 30 mg). Fraction III protein was applied to a Bio-Rad hydroxyapatite column (2.4 × 11 cm) that had been equilibrated with buffer C. The column was washed with 150 ml of buffer C and eluted with a 250-ml linear gradient of 0.05–0.5 M potassium phosphate at a flow rate of 1.4 column volumes per h. DnaJ 1–75 eluted at approximately 0.9 M potassium phosphate. Fractions containing DnaJ 1–75 at greater than 95% purity were pooled (50 ml) and concentrated to 15 ml in an Amicon apparatus. This sample (Fraction IV, 20 mg, 15 ml) was quick-frozen in liquid nitrogen and stored at −80°C.

Expression and Purification of DnaJ 1–106—RLM1341 (RLM569/pRLM234) cells were grown, thermally induced, harvested, and lysed as described above for wild type DnaJ. A cell lysate from 8 g of cells was centrifuged at 120,000 × g for 1 h. The supernatant (Fraction I, 70 ml) was supplemented with ammonium sulfate to 75% saturation (0.476 g of ammonium sulfate per ml of supernatant), stirred at 4°C for 30 min, and centrifuged at 30,000 × g for 1 h. The supernatant was concentrated to 25 ml, using an Amicon stirred cell concentrator fitted with a YM3 membrane, and dialyzed against 2 liters of buffer F (Fraction II, 80 mg, 30 ml). Fraction II was applied to a Bio-Rex 70 column (3.4 × 11 cm) and fractionated into DnaJ 73–376 deletion mutant fractions eluted at approximately 0.35 M NaCl. Fractions containing DnaJ 73–376 at greater than 95% purity were pooled (80 ml) and concentrated to 30 ml in an Amicon apparatus (Fraction III, 30 mg). Fraction III protein was applied to a Bio-Rad hydroxyapatite column (2.4 × 11 cm) that had been equilibrated with buffer C. The column was washed with 150 ml of buffer C and eluted with a 250-ml linear gradient of 0.05–0.5 M potassium phosphate at a flow rate of 1.4 column volumes per h. DnaJ 1–75 eluted at approximately 0.9 M potassium phosphate. Fractions containing DnaJ 1–75 at greater than 95% purity were pooled (50 ml) and concentrated to 15 ml in an Amicon apparatus. This sample (Fraction IV, 20 mg, 15 ml) was quick-frozen in liquid nitrogen and stored at −80°C.
29,659. Prior to the addition of ATP as the final component, all reaction mixtures were preincubated at 25°C for 2 min. The reaction was initiated by the addition of ATP and incubated at 25°C. At each time point 15-μl portions were removed to tubes containing 2 μl of 1 N HCl. This treatment lowered the pH to between 3 and 4 and quenched the ATPase reaction (control experiments indicated that little or no additional ATP hydrolysis occurred after subsequent to the addition of HCl). Portions (4 μl) from each quenched reaction mixture were applied to polyethyleneimine-cellulose thin layer chromatography plates that had been prespotted with 1 μl of a mixture containing ATP and ADP (each at 20 mM). The plates were developed in 1 N formic acid and 0.5 M LiCl. The migration positions of ATP and ADP were visualized by short wave UV light. The amount of ATP on the plate was determined by scintillation counting. The kinetic data obtained from the single turnover ATPase reactions were fit to a first-order rate equation using the nonlinear regression program, "Enzfitter" (Biosoft, Cambridge, UK). Kₐ values, for activation under single turnover conditions of the ATP hydrolysis step in the DnaK ATPase reaction cycle by DnaJ and DnaJ deletion mutant proteins, were determined by a replot of kₐ values versus concentration of DnaJ or DnaJ deletion mutant protein. For each activator protein, at least five different activator concentrations (over a 100-fold range of concentration) were examined along with the accumulation of data collected from 45 laser shots.

Matrix-assisted Laser Desorption/Ionization Mass Spectral Analysis (MALDI-MS) of Proteins and Polypeptides—MALDI-MS analysis of DnaJ was performed by the Middle Atlantic Mass Spectroscopy Facility (Johns Hopkins University School of Medicine) with a Kratos Kompact III linear time-of-flight mass spectrometer equipped with a nitrogen laser (337 nm). Protein samples (20 μg, consisting of DnaJ or papain-resistant fragment of DnaJ), were prepared for mass spectral analysis using Bond-Elute disposable, solid phase extraction, C8 columns, according to the manufacturer’s specifications (Varian Inc.). Briefly, 100 μl (20 μg) of protein sample was loaded onto a 0.5-ml Bond Elute C8 column equilibrated in buffer I (0.1% (v/v) trifluoroacetic acid in deionized H₂O). The column was washed with 2.0 ml of buffer I and the protein sample was eluted with 500 ml of buffer I containing 5% aqueous acetonitrile. The eluted protein sample was dried in a Speed-Vac centrifugal concentrator and redissolved in 20 μl of buffer I containing 20% aqueous acetonitrile. The sample, or analyte (0.3 μl), was deposited on a sample site of a 20-site stainless steel slide that contained 0.3 μl of a saturated solution of the matrix (3,5-dimethoxy-4-hydroxycinnamic acid; 207.8 Da) in 50:50 (v/v) ethanol/water. The analyte/matrix solution was air-dried and the slide was subsequently inserted into the mass spectrometer. The spectra acquired represent the accumulation of data collected from 45 laser shots.

RESULTS

Partial Proteolysis of DnaJ—Analysis of the amino acid sequence of the DnaJ heat shock protein family indicates that there are two large regions that are conserved in multiple members of this family. The most highly conserved region is the 70 amino acid "J"-region, which is found in all members of the DnaJ family. The second region, which is present in several, but not all, DnaJ homologues, contains multiple Cys-rich motifs. We wished to determine if these conserved regions represent stable structural domains of DnaJ. A nonspecific protease, such as papain, can be useful for delimiting structural domains in proteins, since its enzymatic activity is generally ineffective on stable secondary and tertiary structures in substrate proteins. DnaJ was digested with papain and the resulting polypeptide products were subjected to analysis by SDS-PAGE. Proteolysis of DnaJ with papain produced two stable fragments of approximately Mₑ = 9,000 and 30,000 (Fig. 1, J 1–376). Edman analysis of the NH₂-terminal amino acid sequences of these fragments yielded the amino acid sequences AKODDY for the 9-kDa fragment and GGGRGQ for the 30-kDa fragment, which correspond, respectively, to amino acids 2–7 and 104–109 of DnaJ. This demonstrates that the 9-kDa polypeptide encompasses the highly conserved "J"-region, whereas the 30-kDa fragment includes the cysteine-rich motifs of full-length DnaJ, but does not contain most of the Gly/Phe-rich segment of the native molecular chaperone.

To obtain a more refined estimate of the positions of the COOH termini in each papain-resistant fragment of DnaJ, we digested DnaJ with papain again, purified each polypeptide by reverse-phase chromatography, and subjected each fragment to MALDI. This analysis revealed that the small NH₂-terminal J-region fragment was actually a series of fragments ranging in mass from approximately 8770 to about 9900 Da. Comparison to the known sequence of DnaJ indicates that the smaller protease-resistant fragments consist of polypeptides containing DnaJ amino acid residues 2-75 through 2-89. More prolonged digestion of a related polypeptide (DnaJ 2-106, see below) with papain yielded polypeptides whose masses were approximately equivalent to DnaJ 2-75 and DnaJ 2-78. Thus, extensive papain treatment of DnaJ results in nearly complete digestion of the Gly/Phe-rich segment (amino acids 77–106). We conclude that the papain-resistant structural domain encoded by the J-region corresponds to DnaJ 2-75 (Fig. 2). The removal of the NH₂-terminal methionine of DnaJ, however, is not the result of papain action, but rather seems to be due to post-translational processing in vivo, since our sequence analysis indicated that alanine 2 is the NH₂-terminal amino acid of purified DnaJ.
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The larger COOH-terminal polypeptide produced by the repeat papain digestion of DnaJ was found by MALDI analysis to have a mass centered about 30,115 Da (data not shown). If it is assumed that the COOH terminus of DnaJ is resistant to proteolytic cleavage by papain, then this preparation of the COOH-terminal papain fragment seemingly includes DnaJ residues 99–376 (M, = 30,130). More prolonged digestion of DnaJ with papain results in a COOH-terminal polypeptide of approximately 29 kDa that has DnaJ residue 112 at its amino terminus, as revealed by NH2-terminal sequence analysis. We conclude that the initial papain cleavages of DnaJ occur between amino acid residues 80 and 100 and that the remainder of the Gly/Phe-rich segment of this heat shock protein is excised following more extensive papain treatment (Fig. 2).

Expression and Purification of DnaJ Deletion Mutant Proteins—Based on the identification of stable protease-resistant domains in DnaJ and on the location of sequence motifs that are conserved in multiple DnaJ homologues, we designed a series of DnaJ deletion mutant proteins to be used in structure-function studies. These mutant proteins, depicted schematically in Fig. 3, consist of the NH2-terminal J-region (DnaJ1–75) and the COOH-terminal, 30-kDa papain-resistant domain (DnaJ106–376) as well as derivatives of each that also contain the Gly/Phe-rich segment (DnaJ1–106 and DnaJ73–376, respectively).

DnaJ1–75 was designed for investigations of the functional significance of the highly conserved J-region. PCR was used to amplify the sequence for dnaJ codons 1–75. In this amplification, as well as in other amplifications of segments of the dnaJ gene by PCR, one of the primers included a “hang-off” sequence encoding a consensus Escherichia coli ribosome binding site and an ATG initiator codon. Similarly, the second primer used in the PCR amplification included a hang-off sequence encoding the complement of two tandem stop codons. These “3′” primers were designed such that tandem stop codons were juxtaposed in the proper reading frame, to the 3′ terminus of dnaJ coding sequences in the amplified DNA. The PCR products were inserted into the polylinker site on pRLM76, an expression vector that provides thermoinducible expression of genes cloned downstream from a lac promoter present on the plasmid. The resulting plasmid, pRLM233, was transformed into strain PK102, a dnaJ deletion mutant of E. coli (41). Induction of expression of the cloned gene fragment by aeration at 42°C of cells harboring pRLM233 resulted in production of DnaJ1–75 (J1–75) to amounts greater than 10% of the total cellular protein (data not shown).

The DnaJ1–106 (J1–106) deletion mutant protein was designed for investigations of the functional significance of the Gly/Phe-rich sequence distal to the J-region. As for J1–75, a pRLM76-derivative that expresses J1–106 was constructed (pRLM234). Overexpressed J1–106 protein, like J1–75, was highly soluble and constituted approximately 10% of the total cellular protein following induction. Both J1–75 and J1–106 were purified to greater than 95% homogeneity as described under “Experimental Procedures” (Fig. 1). Although J1–75 and J1–106 have the same relative electrophoretic mobility in a 10–20% gradient SDS-polyacrylamide gel (Fig. 1), these two polypeptides can be readily resolved by electrophoresis in a
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We obtained evidence that the J73–376 and J106–376 deletion mutant proteins retained any of the functional activities characteristic of wild type DnaJ, for example, its capacity to stimulate the weak intrinsic ATPase activity of DnaK (23, 24). Because DnaJ stimulates the DnaK ATPase specifically at the hydrolytic step in the ATPase reaction cycle, the DnaK ATPase activity is especially sensitive to the presence of DnaJ when the ATPase assay is performed using single turnover conditions (i.e. when the concentration of DnaJ greatly exceeds that of ATP). Under these conditions, DnaJ strongly stimulates ATP hydrolysis by DnaK (Fig. 5). The rate constant for ATP hydrolysis by DnaK is increased at least 200-fold at saturating levels of DnaJ, from 0.04 min⁻¹ to more than 8.5 min⁻¹. These data yielded an apparent Kₐ of 0.2–0.3 µM DnaJ for activation of the DnaK ATPase (Fig. 5 and Table I).

It has been suggested (48) that the highly conserved J₁-region, which we have shown is essentially equivalent to the NH₂-terminal structural domain of DnaJ, interacts with DnaK. We used the single turnover ATPase assay to determine if the J₁-region is both necessary and sufficient for stimulation of DnaK ATPase activity. Purified J₁–106 failed, even at very high concentrations, to produce any detectable activation of the DnaK ATPase activity (Fig. 5). We next examined whether J₁–106, which contains both the J₁-region and the Gly/Phe-rich segment, had any capacity to stimulate ATP hydrolysis by DnaK. In striking contrast to J₁–75, J₁–106 is capable of stimulating DnaK’s intrinsic ATPase activity (Fig. 5). However, the interaction of DnaJ₁–106 with DnaK is clearly deficient in...
The peptide sequence and can be as much as 30-fold (24). For DNAK, the level of stimulation depends on the intrinsic ATPase activity of DNAK as well as the intrinsic ATPase activities of its eukaryotic counterparts in the Hsp70 family.

Extended conformation (49) and that many short peptides of 7-15 residues for DNAJ’s capacity to activate the DNAK ATPase. It is never, it was still possible that the Gly/Phe-rich segment alone is responsible for DNAJ’s capacity to activate DNAK ATPase.

These results suggested to us that two conserved DNAJ motifs, i.e. the J-region and the Gly/Phe-rich segment, participate jointly in the activation of the ATPase activity of DNAK. However, it was still possible that the Gly/Phe-rich segment alone is responsible for DNAJ’s capacity to activate DNAK ATPase.

It is known in this regard that peptide C binds to DNAK in an extended conformation (49) and that many short peptides of 7 to 9 amino acids or longer are capable of stimulating the intrinsic ATPase activity of DNAK as well as the intrinsic ATPase activities of its eukaryotic counterparts in the Hsp70 family (24, 25, 46, 47). For DNAK, the level of stimulation depends on the sequence and can be as much as 30-fold (24). To examine the potential role of the Gly/Phe-rich segment in the DNAJ-mediated activation of the DNAK ATPase, we synthesized a set of five overlapping peptides, each 15 amino acids in length, that span the entirety of the Gly/Phe-rich segment and tested each for its capacity to serve as an effector of the DNAK ATPase.

Two additional DNAJ deletion mutant proteins, J106–376 and J106–376 (Fig. 3), were studied to examine whether the cysteine-rich motifs and the COOH-terminal portion of DNAJ play any independent role in activation of DNAK’s ATPase activity. Neither mutant protein contains the J-region, but J73–376 does contain the flexible Gly/Phe-rich segment in addition to the COOH-terminal structural domain of DNAJ. Our results indicate that neither J73–376 nor J106–376 (in the range between 0.1 and 20 μM) was capable of providing detectable stimulation of the intrinsic ATPase of DNAK (Table I and data not shown). The inability of J73–376 to activate the DNAK ATPase provides additional evidence that the Gly/Phe-rich segment and the J-region must be simultaneously present in the DNAJ polypeptide in order to achieve significant stimulation of ATP hydrolysis by the DNAK heat shock protein.

Activation of the DNAK ATPase by a Combination of DNAJ 1–75 and Peptide—Polypeptides, such as full-length DNAJ and J1–106, that have a covalent linkage between the J-region and the Gly/Phe-rich segment, have the capacity to stimulate ATP hydrolysis by DNAK. The apparently unstructured nature of the Gly/Phe-rich segment, as judged by its sensitivity to proteolysis by papain, suggested the possibility that it interacts with the peptide-binding site on DNAK during DNAJ-mediated activation of the DNAK ATPase activity. We therefore sought to determine if a free peptide could replace the Gly/Phe-rich segment and complement the J-region for activation of DNAK. Incubation of DNAK with both J1–75 and any of the five synthetic peptides derived from the Gly/Phe-rich region produced no detectable stimulation of DNAK’s ATPase activity under single turnover conditions (data not shown). However, when we used peptides, such as peptide C (24) or peptide NR (47), that are capable of stimulating DNAK’s ATPase activity, a significant further increase in the rate constant for ATP hydrolysis was observed in the presence of both J1–75 and peptide (Fig. 6). At saturating levels of J1–75 and peptide, the rate of ATP hydrolysis by DNAK was roughly equivalent to the enhanced DNAK ATPase rate elicited by the presence of wild type DNAJ. The maximal rate constant obtained in the presence of peptide and J1–75 was more than 200-fold greater than that for DNAK alone under similar conditions and more than 15-fold higher than that obtained when DNAK was supplemented with just peptide C or peptide NR. The concentration of J1–75 which produced half-maximal stimulation of DNAK’s ATPase activity (i.e. apparent Kₐ) in the presence of added peptide was determined to be 1.3 μM (Table I). Under similar conditions, those DNAJ deletion mutant proteins that lack the 70-amino acid J-region, J73–376 and J106–376, were unable to activate DNAK.

### Table I

| Protein | Kₐ (μM) | λ DNA replication |
|---------|---------|--------------------|
| DNAJ | 0.2 | 100.0 |
| DNAJ 1–75 | None | 0.0 |
| DNAJ 1–75 + peptide C | 1.3 | ND |
| DNAJ 1–75 + peptide NR | 1.3 | ND |
| DNAJ 1–106 | 4.0 | 0.1 |
| DNAJ 173–376 | None | 0.0 |
| DNAJ 106–376 | None | 0.0 |

- a DNAK ATPase assays were performed under single-turnover conditions (2.3 μM DNAK; 30 nM [α-32P]ATP) as described under “Experimental Procedures.”
- b The relative molar specific activities of DNAJ protein or DNAJ deletion mutant proteins in the in vitro λ DNA replication system are listed.
- c ND, not done. Free peptide, at the level (500 μM) required for maximal stimulation of ATP hydrolysis by DNAK, acts as a potent inhibitor of DNA replication in vitro (25).

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DNA replication assays were performed as described under “Experimental Procedures,” except that the standard reaction mixture was supplemented with DnaJ or with a DnaJ deletion mutant protein as indicated. The titration of wild type DnaJ in this assay is incomplete due to the fact that the presence of high levels (i.e. 1–2 μg) of wild type DnaJ protein in the reaction mixture severely inhibited λ DNA replication (data not shown). Filled circles, DnaJ protein; filled squares, DnaJ 1–106 protein; open squares, DnaJ 1–75 protein.

DnaK molecular chaperone has identified two regions of DnaJ that mediate this activation. The first region consists of the highly conserved J-region, located at the amino terminus of DnaJ. This 70-amino acid region, which is the signature sequence of each member of the ubiquitous DnaJ (Hsp40) family of molecular chaperones, forms a stable structural domain in DnaJ (50, 51). The Gly/Phe-rich region of DnaJ, a polypeptide segment that links the NH2- and COOH-terminal domains of DnaJ, also plays a central role in the activation of ATP hydrolysis by DnaK. The hypersensitivity of this segment to proteolysis, as well as the preponderance of glycine residues in this region, suggest that this polypeptide linker segment is both relatively unstructured and highly flexible. This conclusion is consistent with the results of a recent NMR structure determination of an amino-terminal DnaJ fragment (DnaJ 2–108) which found that the Gly/Phe-rich region was flexibly disordered in solution (50). Mutant DnaJ proteins that contain either the J-region or the linker region alone are incapable of stimulating ATP hydrolysis by DnaK (Table I). Furthermore, in preliminary experiments, we have not observed any capacity of these two regions of DnaJ to complement one another and stimulate DnaK under conditions where the required regions are located in trans on separate polypeptides (e.g. when DnaJ 1–75 and DnaJ 73–376 (Fig. 3) are mixed together with DnaJ). A truncated DnaJ polypeptide produced detectable activation of DnaK’s ATPase activity only when both the J-region and the Gly/Phe-rich linker region were present in cis on the same DnaJ deletion mutant polypeptide, as with, for example, DnaJ 1–106.

We sought to localize more definitively the amino acid sequence or sequences present in the Gly/Phe-rich linker region of DnaJ that contribute to the activation of ATP hydrolysis by DnaK. However, none of a series of overlapping 15-amino acid synthetic peptides corresponding to subsections of this linker region were found to provide significant activation of DnaK, whether or not the J-region domain (i.e. DnaJ 1–75) was also present in the incubation mixture. This result was interesting, especially in view of the fact that previous studies have established that random peptides with as few as 6–9 amino acid residues are capable of stimulating the intrinsic ATPase activity of DnaK (24, 46, 47). We have not determined if the synthetic DnaJ linker peptides simply fail to bind stably to DnaK or if, on the other hand, they bind to DnaK but fail to provoke

**DISCUSSION**

Our investigation of the capacities of various DnaJ deletion mutant proteins to stimulate ATP hydrolysis by the E. coli DnaK molecular chaperone identified two regions of DnaJ, the J-region and the Gly/Phe-rich sequence, respectively. We have previously demonstrated that the DnaJ- and DnaK molecular chaperones are absolutely required for the initiation of phage λ DNA replication in a system that is reconstituted with 10 highly purified λ and E. coli proteins (26, 36). We examined various DnaJ deletion mutant proteins for their capacity to support λ DNA replication in the reconstituted multiprotein system. We wished to determine if there was a direct correlation between the capacity of a particular mutant protein to stimulate ATP hydrolysis by DnaK and its ability to support the initiation of bacteriophage λ DNA replication. Only a few nanograms of wild type DnaJ is sufficient to support maximal λ DNA replication in vitro (Fig. 7). In contrast, the DnaJ 1–75 deletion mutant protein was inactive in this replication assay, even at very high protein concentrations (Fig. 7). DnaJ 1–106, which contains both the J-region and the Gly/Phe-rich segment, supported limited DNA synthesis in the λ replication assay (Fig. 7). This response was extremely weak, however, requiring on a molar basis ~1000-fold more J 1–106 than full-length DnaJ to attain a similar level of replication. In related studies, we found that both J 73–376 and J 106–376 were inactive in the λ replication assay (Fig. 8). These data indicate that linkage of the J-region to the Gly/Phe-rich segment produces the minimal combination of DnaJ sequence elements that is capable of both activating ATP hydrolysis by DnaK as well as supporting initiation of λ DNA replication in vitro.
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A necessary response, e.g. a conformational change in DnaK needed to potentiate ATP hydrolysis.

Further exploration of the factors that influence ATP hydrolysis by DnaK led us to the conclusion that DnaK must simultaneously undergo two separate interactions to acquire optimal activation. One such interaction is with the J-domain of its partner chaperone, DnaJ. But, as discussed above, the presence of the J domain alone has no discernible impact on ATP hydrolysis by DnaK. Thus, our results are not in agreement with a previous study that concluded that the J-domain of DnaJ is both necessary and sufficient to stimulate ATP hydrolysis by DnaK (52). We have shown that a second stimulatory interaction is required, one that involves binding of a peptide or protein substrate to the polypeptide-binding site on DnaJ. Proteins, such as wild type E. coli DnaJ or DnaJ deletion mutant DnaJ 1–106, that carry both the J-domain and the Gly/Phε-rich linker segment can individually furnish in cis both interactions needed for activation of the DnaK ATPase (52). We have demonstrated, however, that the requisite stimulatory interactions with DnaK can also occur in trans. A combination of the J-domain (DnaJ 1–75) and any short peptide that has a high affinity for the polypeptide-binding site of DnaK produced an activation of the DnaK ATPase comparable to wild type DnaJ alone (e.g. compare the data in Figs. 5 and 6). While peptide C alone can stimulate ATP hydrolysis by DnaK as much as 20-fold (24), addition of the J-domain to a reaction mixture containing peptide C and DnaK resulted in a 15–20-fold further enhancement of the rate constant for ATP hydrolysis.

Considerable genetic and biochemical evidence has been accumulated in support of the idea that proteins of the DnaJ family functionally cooperate with specific Hsp70 proteins in all organisms to mediate protein folding, protein assembly, and disassembly events, and translocation of polypeptides across intracellular membranes. Although direct physical evidence for a stable protein-protein interaction between these two ubiquitous chaperone types has been observed only in a thermophilic bacterium thus far (53), recently it was demonstrated that the primary E. coli Hsp70 protein, DnaK, does bind to DnaJ when ATP is present (54).

Genetic suppression studies in the yeast Saccharomyces cerevisiae (55), as well as subsequent biochemical and cell biological analysis (56, 57), provide additional support for the occurrence of both functional and direct physical interactions in the endoplasmic reticulum (ER) between chaperone-like proteins of the Hsp70 and Hsp40 families, i.e. between Kar2p, a DnaK and BiP homologue, and Sec63p, a member of the DnaJ family, respectively. These two proteins are thought to play a central role in the translocation of polypeptides from the yeast cytosol into the ER (10, 11, 58, 59). While the precise molecular role of ER-localized Hsp70 proteins in protein translocation remains to be defined, it is reasonable to assume that this translocation process takes advantage of the capacity of such molecular chaperones to couple ATP hydrolysis and binding to polypeptide binding and release. Although Sec63p is an integral membrane protein associated with the polypeptide translocation complex in the ER, it does contain a 70-amino acid J-domain that faces the ER lumen (60). Interestingly, Sec63p does not contain a segment that is homologous to the Gly/Phε-rich linker polypeptide of DnaJ. Thus, it is highly probable that Sec63p itself, like the J-domain (DnaJ 1–75), is capable of contributing only one of the two signals required for activation of ATP hydrolysis by the Kar2 Hsp70 protein. In reaching this conclusion, we make the presumption that the dual signal requirement for maximal ATP hydrolysis we identified for DnaK has been conserved during evolution in all primary Hsp70 family members.

If Sec63p indeed only contributes a J-domain to the activation process for Kar2p, then what is the source of the second signal? Since the missing signal involves interaction of a peptide or polypeptide with the polypeptide-binding site on the COOH-terminal domain of Kar2p (BiP), we suggest that it is the translocating polypeptide itself that supplies the other required signal for activation of the Kar2p ATPase. This proposal is consistent with the polypeptide binding specificity of the Hsp70 COOH-terminal domain as well as the presumed structure of translocating polypeptide chains. The available evidence indicates that Hsp70 proteins prefer to bind to extended polypeptide chains containing substantial hydrophobic character (25, 47, 49, 61–63). Accordingly, translocating polypeptides associated with Sec63p and the ER translocation apparatus would be expected to be in an unfolded or partially folded state as they emerge from the lipid bilayer of the ER. In our proposal, simultaneous interaction of a molecule of the ATP-bound form of BiP (Kar2p) with both the translocating polypeptide and Sec63p would activate ATP hydrolysis by BiP. Recent findings suggest that such ATP hydrolysis by BiP would effectively lock the polypeptide substrate onto a BiP-ADP enzyme complex (34, 64). Moreover, this stable Hsp70-polypeptide interaction, mediated in part by the J-domain of Sec63p, may render polypeptide translocation into the ER irreversible, a role that has also been suggested for Hsp70-polypeptide interactions that occur during protein translocation into mitochondria (65, 66). The translocating polypeptide, presumably still in an unfolded or partially folded conformation, would be anticipated to remain firmly bound to BiP until the ADP present on the enzyme is exchanged for ATP (64). Since no GrpE homologue in the ER lumen has yet been identified, this nucleotide exchange step may be slow (23, 64).

A number of instances have been described where it is DnaJ, rather than DnaK, that first binds to a protein substrate of this chaperone system. This situation was initially found for binding of DnaJ to a nucleoprotein preinitiation complex formed at the bacteriophage λ replication origin (26, 27, 30) and for binding of DnaJ to the P1 phage-encoded RepA replication initiator protein (67). DnaJ also has high affinity for the E. coli r32° heat shock transcription factor (31, 68) and there is experimental support for the idea that DnaJ may bind to nascent polypeptides as an early step in protein folding in vivo (33, 69, 70). In each of these cases, it seems likely that DnaJ may play roles both in recruiting one or more molecules of DnaK to the locale of the protein substrate and in subsequently facilitating the action of DnaK on the substrate.

While our data and that of others (52, 59, 71) provide clear biochemical evidence that the J-domain is critical to the process of Hsp70 recruitment and activation, the potential involvement of the Gly/Phε-rich segment of DnaJ in Hsp70 recruitment, suggested by the findings in this report, draws attention to a possible mechanistic problem. For example, we have concluded here that the Gly/Phε-rich segment of DnaJ provides one of the signals for DnaK activation by binding to the polypeptide binding site of DnaK. Thus, DnaK recruited to close spatial proximity of a protein substrate via interactions with DnaJ apparently would first have to release the Gly/Phε-rich segment of DnaJ before it could bind to its protein substrate. Our biochemical studies are consistent with this pathway for DnaK action. The inability of any of the synthetic peptides derived from the DnaJ Gly/Phε-rich region to stimulate ATP hydrolysis by DnaK to a significant extent suggests that the interaction of the DnaJ Gly/Phε-rich region with DnaK must be both weak and transient. Perhaps the interaction between DnaJ and the J-domain present in the DnaJ-substrate complex is sufficiently strong to keep DnaK from disso-
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in initiation of a DNA replication may well reflect the inability of this truncated DnaJ mutant to bind specifically to preinitiation nucleoprotein structures; as a consequence, DnaJ 1-106, unlike wild type DnaJ, is not capable of directing DnaK to act at precise sites on specific substrate molecules. A recent characterization of the properties of a similarly truncated DnaJ polypeptide lends additional support to this interpretation (71).

An amino-terminal fragment of DnaJ, DnaJ 12 (equivalent to DnaJ 2-108), was found to be capable of activating DnaK to bind to one of its physiological substrates, the χ2 heat shock transcription factor. Furthermore, in contrast to the behavior of wild type DnaJ, the DnaJ 12 mutant protein was reported to be capable of activating DnaK to bind to the χ2 polypeptide in the absence of any prior interaction of the DnaJ 12 protein itself with this heat shock factor.

There are two discrepancies between the findings reported here and previously published data that merit further discussion. First, in contradiction to this report, it was previously concluded that the DnaJ J-domain alone was both necessary and sufficient to activate ATP hydrolysis by DnaK (52). This inference was based on the properties of a DnaJ deletion mutant protein, DnaJ 12, composed of the first 108 amino acids of DnaJ. The properties of the DnaJ 12 mutant protein appear to be nearly identical to those of the DnaJ 1-106 protein described here. It is evident that the mutant DnaJ protein used to reach the earlier conclusion in fact contained not only the J-domain, but also the essential Gly/Phe-rich region as well. Second, Wall et al. (68) have recently described a DnaJ deletion mutant protein, DnaJ Δ77-107, that is missing 31 amino acids covering the entire Gly/Phe-rich region. These authors demonstrated that this mutant protein, nevertheless, is still capable of activating the ATPase activity of DnaJ. One possible explanation for the difference between our findings is that, as alluded to earlier, DnaJ may contain multiple sequence elements capable of interacting with the polypeptide-binding site of DnaK. In addition to the element reported here in the Gly/Phe-rich segment, other potential DnaK interaction sites could reside in the COOH-terminal structural domain of DnaJ. A second possibility is that the random six amino acid linker, HMGSNM, that replaced the Gly/Phe-rich segment as a consequence of the construction of the DnaJ Δ77-107 deletion mutant protein (52), can itself serve as a polypeptide binding element for DnaJ. Perhaps almost any unstructured and flexible polypeptide chain of sufficient length (i.e. greater than 5 amino acids (24)) covalently linked to the J-domain will support productive interactions between DnaJ and DnaK.

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