BIOCHEMISTRY
including biophysical chemistry & molecular biology
Biochemistry, 1998, 37(2), 596-607, DOI:10.1021/bi972025p

Supporting Information for Microfilm Edition

MATERIALS AND METHODS

Materials. Materials and their sources were: restriction enzymes, polynucleotide kinase, and T4 DNA ligase (New England Biolabs); T7 DNA polymerase (Sequenase, U.S. Biochemicals); heparin-Sepharose resin, MonoQ and Superose 12 columns (Pharmacia Biotech); heparin-agarose and ATP-agarose (C-8 linkage; Sigma); stirred cell concentrator, PM30 membranes, and Centricon-30 concentration units (Amicon). All other biochemicals were from Sigma.

Buffers and Media. Tris-sucrose buffer is 50 mM Tris/HCl, pH 7.5, 10% (w/v) sucrose. Lysis buffer is 20 mM Tris/HCl, pH 8.0, 0.1 M NaCl, 2 mM $MgCl_2$, and 2 mM DTT. Buffer I is 10 mM imidazole/HCl, pH 6.8, 50 mM KCl, 1 mM DTT, 1 mM EDTA,and 10% (v/v) glycerol. Buffer B is 10 mM Imidazole/HCl, pH 6.8, 10 mM $MgSO_4$, 1 mM DTT, 1 mM EDTA, and 10% (v/v) glycerol. Buffer T is 25 mM Hepes/KOH, pH 7.6, 1 mM DTT, 1 mM EDTA, and 10% (v/v) glycerol. AZ broth and Terrific broth (TB) growth media were prepared as described (*1,2*).

Strains and Plasmids. Two expression systems were employed for the production of DnaK used in these experiments. In one, DnaK was overexpressed from the plasmid pMOB45*dnaK*$^+$ in the *E. coli* strain RLM893 [equivalent to B178/pMOB45*dnaK*$^+$ (*3*)]. Some experiments used DnaK protein overexpressed from a plasmid, pRLM163, that is

derived from the vector pRLM156. Plasmid pRLM156 was constructed from pRLM76 (*1*) by inserting a new multiple cloning site downstream from the phage λ p_L promoter to replace the smaller polycloning site present in pRLM76. To accomplish this, pRLM76 DNA was digested to completion with *Bam*HI and *Pst* I, which excises the existing multiple cloning site. The two fragments were treated with calf intestinal phosphatase and the large DNA fragment was purified by agarose gel electrophoresis. Synthetic oligonucleotides were used to create a new multiple cloning site that had recognition sites for *Bam*HI, *Ase* I, *Nde* I, *Nco* I, *Kpn* I, *Sma* I, *Sal* I, *Acc* I, *Hinc* II, *Bgl* II, *Cla* I, *Eco*RI, *Xho* I, *Xba* I, and *Pst* I (in order of increasing distance from the p_L promoter). The sequences were: oligonucleotide A, 5'-GATCCATTAATCATATGCCATGGTACCCGGGGTCGACAGATCTATCGATGAATTCC TCGAGTCTAGACTGCA-3' and oligonucleotide B, 5'-GTCTAGACTCGAGGAATTCATCGATAGATCTGTCGACCCCGGGTACCATGGCATA TGATTAATG-3'. These complementary oligonucleotides were annealed, phosphorylated using polynucleotide kinase, and ligated to the pRLM76 backbone fragment, generating pRLM156.

Plasmid pRLM163, which carries the *dnaK* gene, was constructed in a two-step process. The nucleotide sequence coding for the N-terminal domain of DnaK was PCR amplified from pMOB45*dnaK*$^+$ using synthetic oligonucleotide primers C, 5'-CCACCGGATCCAGGAGGTAAAAATTAATGGGTAAAATAATTGGT-3', and D, 5'-CCACCTCTAGAGGTACCCTCGAGCCGCGGCACCGATTGCTACAGCTTC-3'. Oligonucleotide C contained a *Bam*HI restriction site and a consensus ribosome binding site

that were juxtaposed to an ATG initiation codon and *dnaK* codons 2–6 (underlined). Oligonucleotide D contained a *Kpn* I restriction site to be used for cloning, and additionally contained sequences that created a *Sac* II restriction site in the *dnaK* coding sequence. This latter change did not alter the amino acid sequence of DnaK. Oligonucleotide D also contained the complement of *dnaK* codons 369–374 (underlined). Polymerase chain reaction (PCR) amplification was performed in a reaction mixture (100 μL) containing 15 ng of pMOB45*dnaK*$^+$ plasmid DNA, 20 pmol each of primer C and primer D, 125 μM of each of the four dNTPs, 20 mM Tris/HCl, pH 8.55, 16 mM ammonium sulfate, 150 μg/mL BSA, 3.5 mM $MgCl_2$, 10% (v/v) glycerol, and 1 μL of Klentaq-LA DNA polymerase (a generous gift from Dr. Wayne Barnes, Washington U., St. Louis). The amplified DNA product (1.2 kb) was digested to completion with *Bam*HI and *Kpn* I, purified by agarose gel electrophoresis, and ligated to plasmid pRLM156 DNA that had been similarly digested with *Bam*HI and *Kpn* I. The ligation products were transformed into *E. coli* strain RLM569, and ampicillin-resistant clones were screened for 5.0 kb plasmids that yielded a 1.2 kb DNA fragment upon digestion with *Bam*HI and *Kpn* I. One such plasmid was named pRLM161.

The 3' portion of the *dnaK* gene, encoding the C-terminal portion of DnaK, was subsequently inserted into pRLM161. This *dnaK*-coding segment was PCR amplified from pMOB45*dnaK*$^+$ using oligonucleotide primers E (5'-CCACCGAATTCGGATCCCCGCGGTTCAGGGTGGTGTTCTGACT-3') and F (5'-CCGCACTCTAGAGGTACCCCCGGGCTATTATTTTTTGTCTTTG-3'). Primer E contained a *Sac* II recognition sequence followed by *dnaK* coding sequence (codons 377–383,

underlined), and primer F contained a *Kpn* I recognition sequence, the complement of two consecutive termination codons, and sequence complementary to the 3' terminus of the *dnaK* gene (codons 635–638, underlined). The amplified DNA fragment was digested to completion with *Sac* II and *Kpn* I, and ligated to pRLM161 DNA, which had been similarly digested. Ligation products were transformed into RLM569 and ampicillin-resistant clones selected at 30 °C were screened for the capacity to overproduce a polypeptide the size of DnaK when thermally induced at 42 °C. One such strain, named RLM1200, harbored a plasmid of 5703 bp which was designated pRLM163. The *dnaK*-coding region of plasmid pRLM161, the additional *dnaK*-coding segment of pRLM163, and the multiple cloning site of pRLM156 were each sequenced on both strands and found to contain the expected nucleotide sequences.

DNA Sequencing. DNA sequencing was performed with the dideoxynucleotide chain termination method (*4*) using modified T7 DNA polymerase (Sequenase) as described by the manufacturer.

Expression and Purification of DnaK. Overexpression of DnaK in strain RLM893 was accomplished by growing cells in AZ broth in a 150 L New Brunswick FM250 fermenter at 30 °C to an optical density of 0.08 at 600 nm. The temperature was quickly raised to 37 °C to induce plasmid overreplication and the culture was grown for an additional 6 h at this temperature to a final optical density of 1.1. The cells (354 g of paste) were collected by centrifugation, resuspended in 175 mL of Tris-sucrose buffer, quick-frozen in liquid nitrogen, and stored at -80 °C.

For DnaK expression in strain RLM1200, cultures were grown aerobically at 30 °C in 750 mL of Terrific Broth in a Fernbach flask to an optical density of 2 to 3 at 600 nm. Thermal induction of DnaK expression was accomplished by adding 250 mL of Terrific Broth that had been pre-warmed to 70 °C and continuing cell aeration for 2 h at 42 °C. Subsequently, the cells (4.5 g) were collected by centrifugation, resuspended in 10 mL of lysis buffer, quick-frozen in liquid nitrogen, and stored at -80 °C.

DnaK protein was purified as previously described (*5,6*) with the following modifications. The ammonium sulfate pellet (~ 160 mg of protein from 4 - 5 g of cells) was resuspended in 10–20 mL of buffer I and dialyzed overnight against 4 L of buffer I. A precipitate that formed during dialysis was removed by centrifugation, The supernatant was applied to a heparin-Sepharose column (150 mL) at 1.5 – 2.0 mL/min and the column was subsequently washed with buffer I. The flow-through fractions (typically ~ 70 mg of protein in ~ 100 mL) were pooled and concentrated to ~ 15 mL using a stirred-cell concentrator fitted with a PM30 membrane. The concentrated sample was repeatedly (~ 3 times) diluted with 100 mL of buffer B and reconcentrated. This protein fraction was then applied to an ATP-agarose column (2.4 × 7 cm) at a flow rate of ~ 2 mL/min. After washing the column with 2 column volumes of buffer B containing 2 M KCl, bound protein was eluted with 2 column volumes of buffer B containing 5 mM ATP. Fractions containing significant amounts of DnaK were pooled (typically 15 – 20 mg of protein in ~ 40 mL) and concentrated to ~ 15 mL using a stirred cell concentrator. This sample was repeatedly (3–4 times) diluted with 50 mL of buffer T and reconcentrated, ultimately achieving a ~ 100-fold

dilution of ATP as monitored spectrophotometrically. The protein sample (typically 15 to 20 mg of protein) was applied to a MonoQ 10/10 FPLC column equilibrated in buffer T and eluted (at 1.0 mL/min) with a 160 mL linear gradient of NaCl (from 0 to 0.6 M) in buffer T. DnaK eluted in three distinct peaks. Only material from the first peak of DnaK (which eluted at ~ 0.2 M NaCl and represented ~ 50 % of the total DnaK recovered from the Mono Q column) was used in the experiments described in this report. Peak-I DnaK was found to have the lowest k_{cat} in ATPase assays as well as the lowest level of contaminating polypeptide. DnaK was estimated to represent > 98 % of the total protein in this sample, which typically contained ~ 10 mg of protein.

DnaK prepared by this method was found to contain, depending on the preparation, 0.15 – 0.4 moles of nucleotide per mole of protein. The DnaK-bound nucleotide was predominantly ADP as assayed by the method of Gao et al. (*7*). In certain preparations, the ATP-agarose column run was performed in the absence of Mg^{2+}. This variation in protocol did not dramatically alter the total nucleotide content of the final purified protein; however, the residual nucleotide was primarily ATP.

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