BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **250,** 115–118 (1998)
ARTICLE NO. RC989255

Hsc62, a New DnaK Homologue of *Escherichia coli*

Kazuaki Yoshimune, Tohru Yoshimura, and Nobuyoshi Esaki[1]
Institute for Chemical Research, Kyoto University, Uji, Kyoto 611, Japan

Received July 21, 1998

We have cloned and expressed the ORF o170#1 of *Escherichia coli,* which encodes a 62-kDa protein sharing 33% homology in primary structure with DnaK and Hsc66, Hsp70 homologues of *E. coli.* The purified gene product, which we named Hsc62, clearly showed ATPase activity and was bound to a gelatin–agarose gel, from which it was specifically eluted with ATP magnesium salt. Thus, Hsc62 is similar to DnaK in this respect and probably functions as a molecular chaperon in *E. coli.* However, Hsc62 differs markedly from DnaK and also from Hsc66 in response to temperature: the optimum temperature for ATPase activity was increased stepwise in the order of Hsc62, Hsc66, and DnaK. Hsc66 is activated by DnaJ of *E. coli* in the same manner as DnaK, the natural partner protein of DnaJ. However, Hsc62 is distinct from the others: the ATPase activity of Hsc62 was not elevated by DnaJ.

Hsp70 and its homologues are ubiquitously distributed in cells, where they function as molecular chaperones to facilitate various processes, including protein folding, association and dissociation of oligomeric proteins, protein translocation across membranes, and regulation of heat shock response (1-5). McCarty and Walker showed that the autophosphorylation and ATPase activities of DnaK, an Hsp70 homologue of *E. coli*, were greatly changed in response to temperatures *in vitro.* Accordingly, they proposed that DnaK functions as a cellular thermometer that directly senses the environmental temperatures (6). Recently, Schilke et al. found that deletion of the gene for SSH1, an Hsp70 homologue in the mitochondria of *Saccharomyces cerevisiae,* extremely retarded the growth of this yeast at lower temperatures (23°C) without affecting the growth rate at higher temperatures (37°C) (7). Similarly, mutation of the genes for cytosolic Hsp70 homologues Ssb1 and Ssb2 made the yeast cold-sensitive. James et al. found, however, that introduction of the gene for a chimeric protein derived from the ATPase domain of Ssb2 and Ssa1, another cytosolic Hsp70 homologue of this yeast, diminished the cold-sensitivity in the mutant (8). As a result of these findings, it is thought that Hsp70 homologues participate in the vital response to environmental temperatures by using their ATPase domains as sensors.

Hsc66 is a homologue of DnaK with 41% identity in primary structure, and exhibits ATPase activity in the same manner as DnaK and other Hsp70 homologues (9). DnaK functions as a molecular chaperon in cooperation with GrpE and DnaJ; the latter stimulates the ATPase activity of DnaK (10, 11). The Hsc66 gene (*hscA*) lies in the same operon as the *hscB* gene encoding Hsc20, which contains a region similar to the J-domain of DnaJ and elevates by 3.8-fold the ATPase activity of Hsc66 (12). Thus, Hsc66 and Hsc20 are considered to operate synergistically as a molecular chaperone in the same manner as DnaK and DnaJ (9). However, Hsc66 is markedly different from DnaK in that the former protein is expressed constitutively in *E. coli* and is not a heat-shock protein.

We have found that an ORF registered as o170#1 in the *E. coli* genome encodes a putative protein of 61,992 Da with 33% sequence identity to that of DnaK. We have purified and characterized this protein, which we named Hsc62, with the aim of examining whether its ATPase activity, if any, shows a unique temperature dependence distinct from those of DnaK and Hsc66: this turned out to be the case. Moreover, we also found that Hsc62 is bound to gelatin in the same manner as DnaK. These findings have led us to propose that Hsc62 is a new homologue of HSP70 acting as a chaperon by sensing a different temperature range from those detected by DnaK and Hsc66.

MATERIALS AND METHODS

Materials. Plasmid pNRK416 encoding the *E. coli* DnaK and the DnaK null mutant of *E. coli* MC4100 were kindly given by Professor Y. Akiyama and Dr. C. Wada of the Institute for Virus Research, Kyoto University, respectively. The *E. coli* DnaJ was obtained from Stressgen Biotechnologies, Victoria, BC, Canada. Oligonucleotides used for polymerase chain reaction (PCR) were synthesized by means of phosphoamidite chemistry. Enzymes used for DNA manipulation were from Ta-

[1] To whom correspondence should be addressed. Fax: +81-774-38-3248. E-mail: esaki@scl.kyoto-u.ac.jp.

0006-291X/98 $25.00

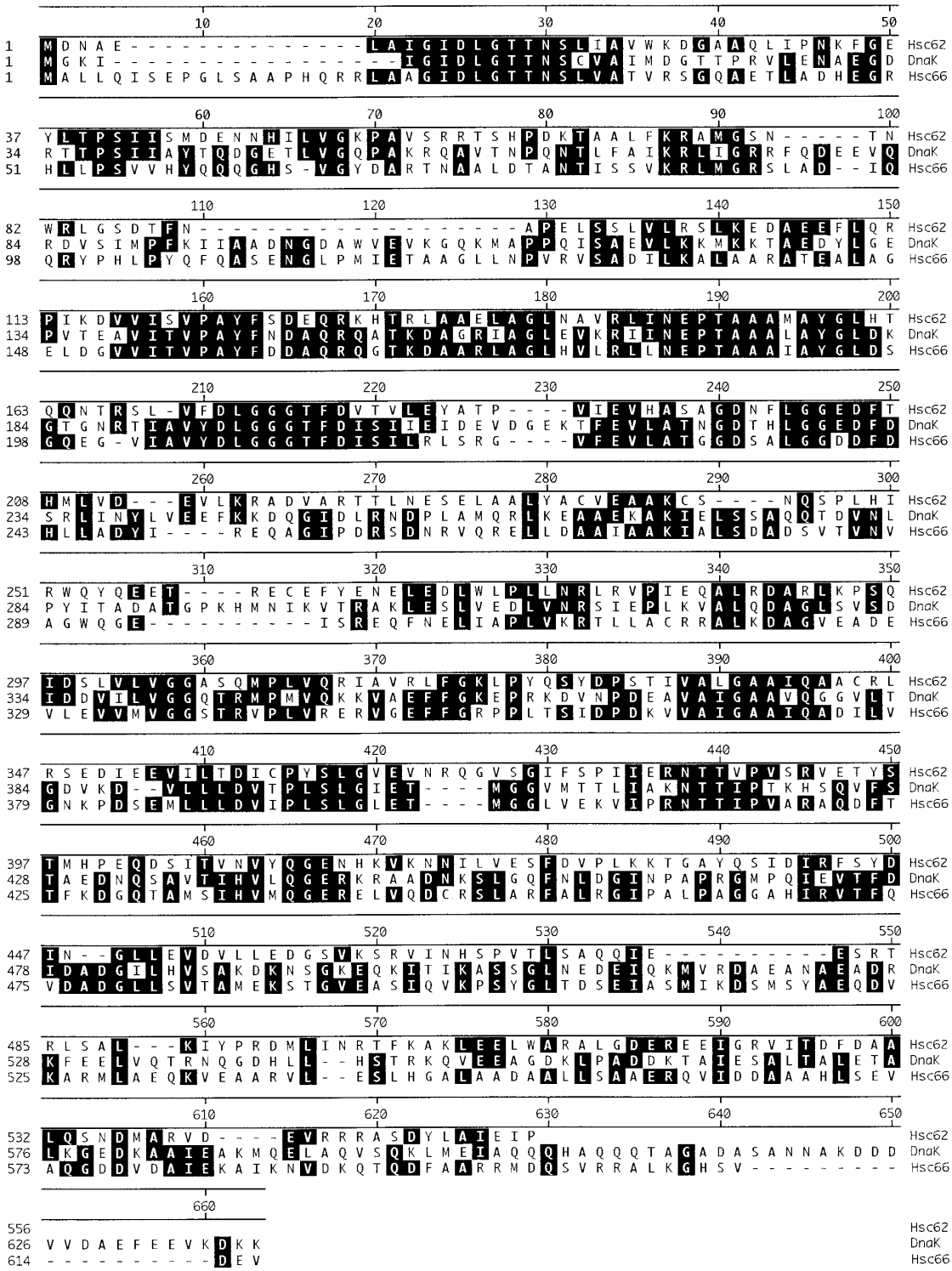

FIG. 1. Linear alignment of the deduced amino acid sequences of Hsc62, DnaK, and Hsc62. Alignment was carried out with the CLUSTAL V program of the DNASTAR software (DNASTAR, Madison, Wisconsin). Conserved residues are boxed.

kara Shuzo, Kyoto, Japan. Gelatin-agarose gel was obtained from Sigma, St. Louis, MO. All other reagents and chemicals were of analytical grade.

Preparation of the E. coli DnaK. *E. coli* JM109 cells transformed with plasmid pNRK416 were cultured for 12 h at 37°C in 5 ml of the Luria-Bertani's broth supplemented with ampicillin (50 μg/ml). The culture was diluted 100-fold with the fresh medium, and incubated for 12 h after the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The harvested cells were suspended in an appropriate volume of Buffer A consisting of 20 mM Tris HCl (pH 7.5), 20 mM NaCl, 0.1 mM EDTA, and 15 mM 2-mercaptoethanol, and disrupted by sonication. The supernatant solution obtained by centrifugation

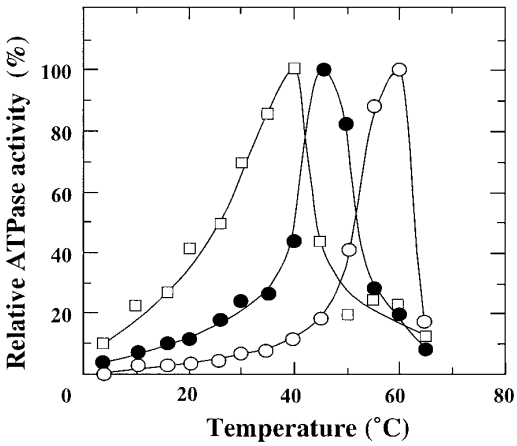

FIG. 2. Temperature dependence of the ATPase activities of Hsc62, Hsc66, and DnaK. The activities of Hsc62 (□), Hsc66 (●), and DnaK (○) at different temperatures are shown in terms of the values relative to the corresponding maximum value taken as 100%.

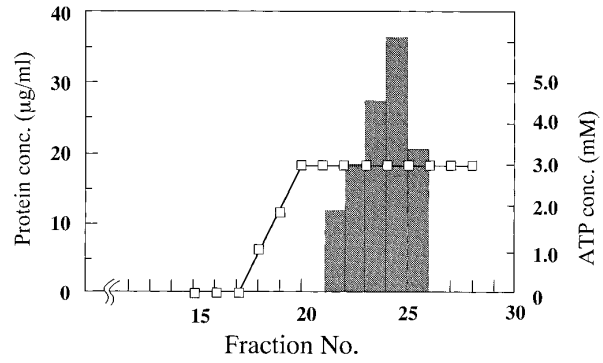

FIG. 3. Elution profile of Hsc62 from a gelatin-agarose column. Protein (shown by bar) and ATP (square) concentrations were determined with a Bio-Rad protein assay kit and by measurement of the absorbance at 254 nm, respectively.

was used as the cell extract (total protein, 70 mg). The cell extract dialyzed against Buffer A was applied to a gelatin-agarose column (25 ml) equilibrated with the same buffer. The column was washed with Buffer A containing 0.5 M NaCl, and the DnaK was eluted with Buffer A containing 3 mM ATP magnesium salt. The amount of the purified DnaK was 2 mg.

Construction of plasmid pHSC62, an expression vector for Hsc62. The ORF o170#1 coding for Hsc62 was amplified with the genomic DNA of *E. coli* K-12 as a template by PCR with *Ex Taq* polymerase and the following two primers: forward, 5′-GAGGAATTCATGAAAAATTGCTGGAAGATCC (*Eco*RI site, underlined); reverse, 5′-ATTAACGTCTGCAGTCACCCGAACAGATGG (*Pst*I site, underlined). The PCR product was digested with *Eco*RI and *Pst*I, and ligated into pKK223-3 which had been digested with the same enzymes. The DNA sequence of the cloned gene was confirmed by dideoxy chain-termination method with an applied Biosystems Model 370A DNA sequencer (Perkin-Elmer). The resultant plasmid, pHSC62, expressed the Hsc62 under the control of *tac* promoter.

Production and purification of Hsc62. *E. coli* JM109 cells carrying pHSC62 were cultured overnight in 5 ml of the Luria-Bertani's broth supplemented with ampicillin (50 μg/ml). The cultures were diluted 100-fold with the fresh medium, and incubated at 37°C for 10 h. Hsc62 was produced by addition of IPTG 2 h after inoculation. The cells were harvested and suspended in an appropriate volume of Buffer A, followed by sonication. After centrifugation, the supernatant solution was used as the cell extract. The cell extract dialyzed against Buffer A was applied to a DEAE-TOYOPEARL column (Tosoh, Tokyo) equilibrated with the same buffer. The column was washed with Buffer A containing 0.1 M NaCl, and Hsc62 was eluted with a linear gradient of 100–300 mM NaCl in Buffer A. The fractions containing Hsc62 (total protein, 8 mg) were pooled, and 0.2 volume of the Buffer A containing 100%-saturated ammonium sulfate was added slowly. The solution was applied to a BUTYL-TOYOPEARL column (Tosoh) equilibrated with the Buffer A containing 20%-saturated ammonium sulfate. After the column was washed with the same buffer, Hsc62 was eluted with a linear gradient of 20 - 0% saturated ammonium sulfate in Buffer A. The homogeneity of Hsc62 (at least 90%) was confirmed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (data not shown).

Preparation of Hsc66. The Hsc66 gene was cloned by PCR in the same manner as described above but with a forward primer of 5′-AAGGAATTCATGGCCTTATTACAAATTAGTGAACC (*Eco*RI site, underlined) and a reverse primer of 5′-ATCTTTCTGCAGTTAAACCTCGTCCACGG (*Pst*I site, underlined). Hsc66 was purified from the *E. coli* JM109 cells containing pHSC66 essentially by the same procedure as for Hsc62.

Interaction of Hsc62 with a gelatin-agarose column. Purified Hsc62 (0.2 mg) was applied to a gelatin-agarose column (5 ml) equilibrated with Buffer B consisting of 20 mM Tris HCl buffer (pH 7.5), and 0.1 mM EDTA. The column was washed with 15 ml of Buffer B containing 20 mM NaCl, followed by washing with Buffer B containing 3 mM ATP magnesium salt. The eluate was collected in a fraction of 1 ml, and the concentrations of protein and ATP of each fraction were determined.

ATPase assay. ATPase activities of Hsc62, DnaK, and Hsc66 were determined by measuring ADP formed from ATP by a reverse phase HPLC (13). Reaction mixtures (49 μl) containing 0.1 M HEPES-NaOH buffer (pH 7.5), 150 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, and 2 μM protein (calculated as a monomer) were pre-incubated for 3 min before addition of ATP. The reaction, which was started by addition of ATP (1 μl) at a final concentration of 1 mM, was carried out at the same temperature for 30 min, and then stopped by addition of 5 μl of 20% perchloric acid. Perchloric acid was added before addition of ATP as a control. The mixtures were centrifuged at 12,000 rpm for 10 min at 4°C, and the supernatant solutions were diluted 200-fold with 100 mM potassium phosphate buffer (pH 7.0). A 100-μl aliquot of the diluted solution was subjected to the HPLC analysis with a COSMOSIL 5C18-MS column (4.6 mm × 150 mm, Nacalai Tesque, Kyoto, Japan). Buffer C consisting of 1 volume of methanol

TABLE 1

Effect of *E. coli* DnaJ on the ATPase Activities of DnaK, Hsc66, and Hsc62

Protein[a]	ATPase activity (nmol/mg/min)[b] − DnaJ (A)	+ DnaJ (B)	Ratio (B/A)
DnaK	10.7	35.9	3.3
Hsc66	53.5	200	3.6
Hsc62	30.8	32.8	1.1

[a] Final protein concentration was 2 μM as a monomer.
[b] ATPase activity was determined at 40°C as described in text.

and 4 volumes of 20 mM potassium phosphate buffer containing 5 mM tetra-*n*-butyl ammonium phosphate (pH 7.0) was used in the mobile phase at a flow rate of 1.0 ml/min.

Protein assay. Protein was assayed with a Bio-Rad protein assay kit using bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Primary structure of Hsc62. The deduced amino acid sequence of Hsc62 was compared with those of DnaK and Hsc66 (Fig. 1). Hsc62 showed 33% sequence identity with each of DnaK and Hsc66. Hsc62 contains a threonine residue corresponding to Thr199 of DnaK and which is conserved among all Hsp70 family proteins: this is the site essential for the ATPase activity and the site targeted for autophosphorylation (6).

ATPase activity of Hsc62. Hsc62 was overproduced in the *E. coli* JM109 cells. About 2 mg of Hsc62 was obtained from 0.8 g (wet weight) of JM109 cells containing pHSC62. ATPase activity is the intrinsic function of the Hsp70 family proteins. We found a low but distinct ATPase activity of Hsc62. This activity increased with increase in temperature up to 40°C, but decreased inversely at temperatures higher than 45°C (Fig. 2). Its optimum temperature was the lowest of the three homologues, followed by those of Hsc66 and DnaK. These results suggest that the three proteins function in different temperature regions.

Interaction of Hsc62 with gelatin-agarose. Hsp47 is a molecular chaperon specifically acting on collagen, and is tightly bound with gelatin (17). DnaK of *E. coli* is also bound with gelatin and released from it specifically by ATP magnesium salt; this characteristic of DnaK has enabled its affinity chromatography by means of a gelatin column (18). Since DnaK is a different type of molecular chaperon from Hsp47, one can speculate that various other kinds of molecular chaperons may be bound with gelatin. Here we found that the behavior of Hsc62 in a gelatin column is similar to that of DnaK (FIG. 3). The specific desorption of Hsc62 from the column with ATP magnesium is probably due to its ATPase activity. Thus, Hsc62 probably functions as a molecular chaperon in *E. coli.*

Effect of E. coli DnaJ on the ATPase activities of Hsc62 and Hsc66. DnaJ serves as a co-chaperon of DnaK and stimulates its ATPase activity. We examined whether the ATPase activities of Hsc66 and Hsc62 are also stimulated by DnaJ. Although the natural co-chaperon of Hsc66 is Hsc20, DnaJ also activated Hsc66 (Table 1). The degree of activation by DnaJ was similar to that observed for DnaK. However, the ATPase activity of Hsc62 was not influenced by DnaJ under the same conditions (Table 1). We are currently investigating the function of Hsc62 by preparing a deletion mutant of its gene in *E. coli.*

ACKNOWLEDGMENT

We are grateful to Professor Hirotada Mori of Nara Institute of Science and Technology for his helpful suggestion.

REFERENCES

1. Gething, M. J., and Sambrook, J. (1992) *Nature* **355,** 33–45.
2. McKay, D. B. (1993) *Adv. Prot. Chem.* **44,** 67–98.
3. Becker, J., and Craig, E. A. (1994) *Eur. J. Biochem.* **219,** 11–23.
4. Frydman, J., and Hartl, F.-U. (1996) *Science* **272,** 1497–1502.
5. Hartl, F.-U. (1996) *Nature* **381,** 571–580.
6. McCarty, J. S., and Walker, G. C. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 9513–9517.
7. Schilke, B., Forster, J., Davis, J., James, P., Walker, W., Laloraya, L., Johnson, J., Miao, B., and Craig, E. (1996) *J. Cell. Biol.* **134,** 603–613.
8. James, P., Pfund, C., and Craig, E. A. (1997) *Science* **275,** 387–389.
9. Vickery, L. E., and Silberg, J. J., and Dennis, T. TA. (1997) *Protein Science* **6,** 1047–1056.
10. Zylicz, M., Ang, D., and Georgeopoulos, C. (1987) *J. Biol. Chem.* **262,** 117437–17442.
11. Liberek, K., Georgeopoulos, C., and Zylicz, M. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 6632–6636.
12. Lelivelt, M. J., and Kawula, T. H. (1995) *J. Bacteriol.* **177,** 4900–4907.
13. Fleischmann, R. D., Adams, M. D., White, O., Clayton, R. A., et al. (1995) *Science* **269,** 496–512.
14. Cry, D. M., and Douglase, N. G. (1994) *J. Biol. Chem.* **269,** 9798–9804.
15. Ueguchi, C., Kakeda, M., Yamada, H., and Mizuno, T. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 1054–1058.
16. Wall, D., Zylicz, M., and Georgopoulos, C. (1994) *J. Biol. Chem.* **269,** 5446–5451.
17. Sauk, J. J., Smith, T., and Ferreira, L. (1995) *J. Biol. Chem.* **269,** 3941–3946.
18. Shigenobu, H. (1997) *Seikagaku* **69,** 663.