A 70-kDa molecular chaperone, DnaK, from the industrial bacterium *Bacillus licheniformis*: gene cloning, purification and molecular characterization of the recombinant protein

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Received: 2 October 2008 / Accepted: 27 November 2008

Abstract The heat shock protein 70 (Hsp70/DnaK) gene of *Bacillus licheniformis* is 1,839 bp in length encoding a polypeptide of 612 amino acid residues. The deduced amino acid sequence of the gene shares high sequence identity with other Hsp70/DnaK proteins. The characteristic domains typical for Hsps/DnaKs are also well conserved in *B. licheniformis* DnaK (*B/DnaK*). *B/DnaK* was overexpressed in *Escherichia coli* using pQE expression system and the recombinant protein was purified to homogeneity by nickel-chelate chromatography. The optimal temperature for ATPase activity of the purified *B/DnaK* was 40°C in the presence of 100 mM KCl. The purified *B/DnaK* had a $V_{\max}$ of 32.5 nmol Pi/min and a K_M of 439 μ M. In vivo, the *dnaK* gene allowed an *E. coli dnaK756-ts* mutant to grow at 44°C, suggesting that *B/DnaK* should be functional for survival of host cells under environmental changes especially higher temperature. We also described the use of circular dichroism to characterize the conformation change induced by ATP binding. Binding of ATP was not accompanied by a net change in secondary structure, but ATP together with Mg^{2+} and K^+ ions had a greater enhancement in the stability of *B/DnaK* at stress temperatures. Simultaneous addition of DnaJ, GrpE, and NR-peptide (NRLLLTG) synergistically stimulates the ATPase activity of *B/DnaK* by 11.7-fold.

Keywords *Bacillus licheniformis* · DnaK · ATPase activity · *Escherichia coli* · Circular dichroism

Introduction

A sudden increase in temperature and other types of environmental stresses induce the synthesis of a specific set of proteins called heat shock proteins (Hsps). The first description of a subset of cellular proteins in *Drosophila* induced by heat shock [1] triggered extensive studies on their function in stress tolerance in a variety of organisms. Hsps are classified into five major families on the basis of their molecular size in kilodaltons (kDa): Hsp110, Hsp90, Hsp70, Hsp60 and the low-molecular weight Hsp family. The Hsp70/DnaK family occurs in diverse organisms and members of this family play an essential role in protein metabolism in both stressed and unstressed organisms. Hsp70/DnaK proteins are involved in de novo protein folding, membrane translocation, formation and disassembly of protein complexes and degradation of misfolded proteins [2–4].

Hsp70s/DnaKs are composed of a highly homologous N-terminal ATPase domain, a substrate-binding domain and a C-terminal variable domain [5]. The molecular chaperone function of Hsp70/DnaK proteins seems to be based on its ATPase activity and this activity is stimulated by Hsp40/DnaJ, GrpE and substrate binding [6]. The chaperone activity of Hsp70/DnaK proteins is dependent on a close interaction between the substrate-binding domain and the ATPase domain. When ATP is bound to the ATPase domain of DnaK, there is poor substrate binding and hence it exhibits a weak chaperone activity. However, there is high affinity for substrate and the increase in the chaperone

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activity when ADP is bound. The ATPase activity is regulated by the co-chaperone protein DnaJ [7, 8]. The DnaJ proteins stimulate the low basal ATP hydrolysis activity of partner Hsp70s/DnaKs [6,9] and in doing so enhance the Hsp70s/DnaKs substrate-binding activity and hence their chaperone activity.

Bacillus licheniformis is a gram-positive, spore-forming bacterium widely distributed as a saprophytic organism in the environment. Unlike most other bacilli, which are predominantly aerobic, *B. licheniformis* is a facultative anaerobe, which may allow it to grow in additional ecological niches. There are numerous commercial and agricultural uses for *B. licheniformis* and its extracellular products. The species has been used for decades in the manufacture of industrial enzymes including several proteases, α -amylase, penicillinase, pentosanase, cycloglucosyltransferase, β -mannanase and several pectinolytic enzymes. The proteases from *B. licheniformis* are used in the detergent industry as well as for dehairing and bating of leather [10, 11]. Additionally, amylases from *B. licheniformis* are deployed for commodity scale production of sugars from starches [12]. Specific *B. licheniformis* strains are also used to produce the peptide antibiotic bacitracin as well as a number of specialized chemicals such as γ -polyglutamate [13–16].

Molecular chaperones have a potential role in biotechnology to enhance the recombinant over-expression of a particular protein of interest [17]. To date, the majority of works that have been done on defined DnaK-DnaJ systems at a prokaryotic level has principally involved in the *E. coli* system [18–22]. A number of works have also been performed on other prokaryotic organisms [23–29]. Given that *B. licheniformis* is an industrial organism used for the manufacture of enzymes, antibiotics, and chemicals, the complete nucleotide sequence of the type strain (ATCC 14580) has been determined [30]. With the release of the genome sequence, it has become straightforward to isolate the interesting genes. In this study, we demonstrate the successful expression, over-production and purification of the *B. licheniformis* DnaK protein (*B/DnaK*). Also, the purified protein was characterized in the aspects of optimal temperature and pH, effects of metal ions and the conformational changes induced by nucleotide binding. The *B/DnaK* protein was shown by in vivo and in vitro means to have the properties of a molecular chaperone.

Materials and methods

Materials

Restriction endonucleases, T4 DNA ligase, *Taq* DNA polymerase, and DNA molecular-mass markers were from

Promega Life Sciences (Madison, WI, USA). Ni^{2+} -nitrilotriacetate (Ni^{2+} -NTA) resin, QIAprep spin miniprep kit and QIA-quick gel extraction kit were obtained from Qiagen (Valencia, CA, USA). *Escherichia coli* BB2362 (*dnaK756 recA::Tc^R pDML1*) was kindly provided by Prof. B. Bukau (University of Heidelberg, Germany). Adenosine 5'-triphosphate (ATP) disodium salt was purchased from Merck Ltd. (Darmstadt, Germany). Reagents for polyacrylamide gel electrophoresis (PAGE) were acquired from Bio-Rad Laboratories (Hercules, CA, USA). All other chemicals were commercial products of analytical grade or molecular grade.

Bacterial strains, plasmids, and growth conditions

B. licheniformis ATCC14580 obtained from the Bioresources Collection and Research Center in Food Industry Research & Development Institute (Hsinchu, Taiwan) was used as a source of chromosomal DNA for the cloning of *dnaK*, *dnaJ*, and *grpE* genes. *E. coli* novablue (Novagen, Madison, WI, USA) was used for the routine preparation and construction of recombinant plasmids. *E. coli* M15 (pRep4) from Qiagen was employed for T5 RNA polymerase-mediated over-expression of recombinant proteins. Plasmids used were pGEM-T Easy vector (Promega) and pQE-30 (Qiagen).

B. licheniformis was cultivated in nutrient broth with rotary shaking (150 rpm) at 37°C for 18 h, whereas *E. coli* strains were grown in LB medium at 20°C during isopropyl- β -D-thiogalactopyranoside (IPTG)-induced gene expression. As required, ampicillin and kanamycin were used at a final concentration of 100 and 25 $\mu\text{g/ml}$, respectively.

General molecular techniques

Chromosomal DNA of *B. licheniformis* was isolated according to the method of Doi et al. [31]. Conventional techniques for DNA manipulations such as restriction enzyme digests and agarose electrophoresis were performed as described by Sambrook and Russell [32]. *E. coli* cells were made competent for transformation by the method of Dagert and Ehrlich [33]. DNA sequencing was performed by Mission Biotechnology (Taipei, Taiwan). Amino acid sequences were analyzed with the programs BLAST-X [34] from the National Center for Biotechnology Information (National Library of Medicine, National Institute of Health, MD) and Alignment from the ExpASY molecular biology server (Swiss Institute of Bioinformatics, Basel, Switzerland).

Expression and purification of recombinant *B/DnaK*, *B/DnaJ* and *B/GrpE*

The open reading frames of *B. licheniformis dnaK* (EMBL AAU24248.2), *dnaJ* (EMBL AAU24247.1), and *grpE*

(EMBLAAU24250) genes were amplified with the following primer pairs: 5'-GGATCCATGAGTAAGCGTG-ATTAC-TA-3' and 5'-GGTACCGTAATCGCCTTTAAAG-GCGC-3', 5'-GGATCCATGAGTAAGCGTGATTACTA-3' and 5'-GGTACCGTAATCGCCTTTAAAGGCGC-3', and 5'-GGATCCATGGCAGAAGAAAAACAAAA-3' and 5'-GGTACCTTATTGATTGACTTTTACCA-3'. The PCR products were cloned into the pGEM-T Easy vector to yield plasmids pGEM-*B/DnaK*, pGEM-*B/DnaJ*, and pGEM-*B/GrpE*. The genes were digested out from these plasmids with the restriction endonucleases *Bam*HI and *Hind*III and cloned into the pQE-30 vector to generate pQE-*B/DnaK*, pQE-*B/DnaJ*, and pQE-*B/GrpE*, respectively. PCR products and plasmids were purified using the Qiagen kits according to the manufacturer instructions. Plasmids pQE-*B/DnaK*, pQE-*B/DnaJ*, and pQE-*B/GrpE* were designed for the heterologous protein expression in *E. coli* M15 (pRep4) to synthesize the N-terminal histidine-tagged recombinant proteins. The correct sequences of these genes cloned into the vector were verified by DNA sequencing.

E. coli M15 cells carrying pQE-*B/DnaK*, pQE-*B/DnaJ* or pQE-*B/GrpE* were grown in LB medium containing ampicillin (100 µg/ml) and kanamycin (25 µg/ml) at 37°C for 16 h. An aliquot (1 ml) of an overnight culture was used to inoculate 100 ml of the same medium and maintained the culture at 37°C with shaking (150 rpm) until the OD₆₀₀ reached 0.6. Expression of the recombinant proteins was induced by the addition of IPTG to a final concentration of 0.05 mM and cultivated at 20°C for 6 h. Cells were harvested by centrifugation at 6,000 × g and 4°C for 10 min, and resuspended in 10 ml of binding buffer (5 mM imidazole, 0.5 M NaCl, and 50 mM Tris-HCl; pH 7.9). The His₆-tagged proteins were purified from cell lysate fraction by affinity chromatography with Ni²⁺-NTA resin according to the manufacturer's protocol. After extensive washing, the bound proteins were eluted with 50 mM Tris-HCl buffer (pH 7.9) containing 0.5 M imidazole and 0.5 M NaCl.

Gel electrophoresis and determination of protein concentration

Sodium dodecyl sulfate-PAGE (SDS-PAGE) was performed with a Bio-Rad Protean III mini-gel system (Bio-Rad). The protein samples were suspended in SDS-PAGE loading buffer (5% 2-mercaptoethanol, 2% SDS, 0.1% bromophenol blue, and 10% glycerol in 50 mM Tris-HCl buffer; pH 6.8) and fractioned with 12% separating gel. After electrophoresis, the gels were stained with 0.25% Coomassie brilliant blue dissolved in 50% methanol–10% acetic acid and then destained in a 30% methanol–10% acetic acid solution. The protein size markers were phosphorylase *b* (97.4 kDa), bovine serum albumin (66.3 kDa), ovalbumin (45.0 kDa),

carbonic anhydrase (31.0 kDa), and trypsin inhibitor (21.5 kDa).

Protein concentration was measured by the method of Bradford [35] using the Bio-Rad protein assay reagent and bovine serum albumin as the standard.

ATPase activity assay

ATPase activity assays were performed with a slight modification according to the malachite green method [36, 37]. Unless stated otherwise, all reactions were performed at 40°C in the following conditions (standard assay conditions). The reaction mixture contained 10 mM Hepes (pH 7.0), 100 mM KCl, 2.5 mM MgCl₂, and an appropriate amount of *B/DnaK* in a total volume of 950 µl. The reaction was started by the addition of ATP (50 µl) at various concentrations (0–500 µM). An aliquot (50 µl) of the reaction was removed at varying time intervals and the reaction was stopped by the addition of 10% SDS (50 µl). Color development was started with the addition of 1.25% ammonium molybdate in 6.5% H₂SO₄ (50 µl), and 9% ascorbic acid (50 µl). The release of free phosphate was recorded by monitoring the corresponding color changes at 660 nm. The assays were corrected for spontaneous ATP degradation. Specific activity was expressed as units of nanomoles phosphate released per min per mg *DnaK*, and activity was defined as nmol phosphate released per min.

Complementation of the *E. coli* mutant temperature-sensitive phenotype

The temperature-sensitive *E. coli* BB2362 was used to determine the ability of *B/DnaK* to functionally replace host cell *DnaK* under heat shock conditions. Firstly, the plasmids pQE-30 and pQE-*B/DnaK* were transformed into the *dnaK756 E. coli* strain. The resultant transformants were grown at 30°C in LB broth containing 100 µg/ml ampicillin and 10 µg/ml tetracycline. Aliquots of 5 µl of this culture and its successive 10-fold dilutions were spread onto the selective LB plates containing the above-mentioned antibiotics and 50 µM IPTG. Test plates were incubated for 18 h at either 30°C or 44°C.

Circular dichroism spectroscopy

Circular dichroism (CD) measurements were carried out on a JASCO J-815 spectropolarimeter equipped with a peltier-type temperature controller and a thermostated cell holder. The far-UV spectrum was recorded in a 0.1-cm path length quartz cell at a protein concentration of 11.6 µg/ml. Ten consecutive scans were accumulated and the average spectra stored. The data were corrected for the baseline con-

tribution of the buffer and the observed ellipticities were converted into the mean residue ellipticities (θ) based on a mean molecular mass per residue of 110 Da. Secondary structure was estimated from fitted far-UV CD spectra using the CDNN software package [38].

Thermal denaturation was explored by monitoring the ellipticity at 222 nm. The temperature was increased with a heating rate of 1°C per min from 20 to 100°C.

Results and discussion

Comparison of *B/DnaK* sequence with microbial and eukaryotic homologues

Based on the available database (<http://tw.expasy.org/Swiss-Prot/TrEMBL>), the DnaK proteins are shown to have an amino acid length ranging from 596 to 656. The sequence analysis of the *B. licheniformis* genome [30] allowed the identification of an open reading frame (ORF) coding for a DnaK protein. The derived amino acid sequence predicted a polypeptide of 612 residues with a calculated molecular mass of 65,892 Da. In this study, the PCR-amplified DNA fragment of the *dnaK* gene was digested with *Bam*HI and *Hind*III, and inserted into the corresponding sites of expression vector pQE-30 to yield pQE-*B/DnaK*. *E. coli* M15 competent cells were transformed with pQE-*B/DnaK* and the cloned gene was confirmed by restriction analysis and DNA sequencing. The sequencing data shows a perfect match with the published gene sequence of *B/DnaK* (EMBL AAU24248.2). The multiple alignment of the Hsp70/DnaK proteins illustrate that *B/DnaK* has an overall primary structure similar to that of the well-described homologues. *B/DnaK* shares 94, 58, 56, and 46% identity with the amino acid sequences of *Bacillus subtilis* DnaK, *E. coli* DnaK, *Homo sapiens* Hsc70, and *Bos Taurus* Hsc70, respectively (Fig. 1), and more than 54% sequence identity with those of other prokaryotic homologues (data not shown). The alignment among *B/DnaK* (TrEMBL Q65H54), *B. subtilis* DnaK (TrEMBL P17820), *E. coli* DnaK (*EcDnaK*) (TrEMBL P0A6Y8), human Hsc70 (TrEMBL P38646), and bovine Hsc70 (TrEMBL A7E3Q2) shows a highly conserved N-terminal ATPase domain. Several regions important for function are shown in Fig. 1. The phosphate 1 and 2 regions are proposed to bind the phosphate tail of ATP. The connect 1 and 2 regions make a helix-helix contact that appears to have the properties of an interdomain linker. The adenosine regions bind to the adenosine molecule of ATP. The amino acid residues for the peptide substrate binding, and lid and hinge regions also show highly conserved. Additionally, a short ${}_{358}^{\text{DVVLLD}}{}_{363}$ segment in the linker region of *B/DnaK* is conserved in the aligned proteins.

Expression and purification of *B/DnaK*, *B/DnaJ* and *B/GrpE*

For high-level expression of *B/DnaK*, *B/DnaJ* and *B/GrpE*, *E. coli* M15 cells harboring pQE-*B/DnaK*, pQE-*B/DnaJ*, or pQE-*B/GrpE* were grown in liquid medium at 20°C for 6 h in the presence of 50 μ M IPTG. One predominant band corresponding to the molecular masses of approximately 70 kDa was observed in the crude extracts of IPTG-induced *E. coli* M15 (pQE-*B/DnaK*). The specific ATPase activity in the crude extract of *E. coli* M15 (pQE-*B/DnaK*) was determined to be 1.12 nmoles/min/mg. The recombinant enzyme in the crude extract was purified by nickel-chelate column chromatography. The purified *B/DnaK* was apparently homogenous as judged by SDS-PAGE (Fig. 2). The specific ATPase activity for the purified *B/DnaK* was 30.2 nmoles/min/mg, indicating that the protein was purified approximately 27-fold by one-step chromatography. As shown in Fig. 2, the *B/DnaJ* and *B/GrpE* proteins were also purified to near homogeneity by Ni^{2+} -NTA.

Properties of *B/DnaK*

The temperature-activity profile of purified *B/DnaK* was determined at values between 20 and 80°C. Maximum ATPase activity occurred at 40°C (Fig. 3A). At this temperature, the optimum pH was determined in sodium-acetic acid, Hepes-NaOH, Tris-HCl, and glycine-NaOH buffers at a concentration of 10 mM and pH values of 3.7–5.5, 6.5–8.9, 7.1–8.9 and 8.6–10.5, respectively. As shown in Fig. 3B, optimal pH to obtain the maximum activity at 40°C was pH 7.0.

The effects of KCl and NaCl on the ATPase activity of *B/DnaK* were tested between 0 and 500 mM. The recombinant protein showed the highest activity at KCl concentrations of 100 mM, with 86% and 79% remaining activities at concentrations of 50 and 500 mM, respectively. When 100 mM KCl was replaced by the same concentration of NaCl, only 18% of the maximal activity was achieved. Moreover, it was striking that 12% of the maximal activity was observed when there was no KCl or NaCl in the buffer. Our results agree with other previous works that suggested KCl is required to maintain a higher ATPase activity of some DnaK proteins [28, 39].

Determination of the kinetic constants of *B/DnaK* was performed using a colorimetric assay to monitor the release of inorganic phosphate (Pi). *B/DnaK* was shown to have a basal ATPase activity with a V_{max} of 32.5 ± 1.9 nmol Pi/min and K_{M} of 439.0 ± 42.5 μ M (Fig. 4). A wide range of values have been reported for the basal ATPase activity of *EcDnaK* with V_{max} values ranging from 0.43 to 3.5 nmol Pi/min and K_{M} values ranging from 20 nM to 20 μ M ATP [6, 40, 41].

Obviously, the $V_{\max}$ and K_M values of *B*/DnaK are higher than those obtained for *Ec*DnaK.

Complementation of the *dnaK756-ts* mutant of *E. coli* with *B*/DnaK

Complementation studies are a well-established method to aid in the determination of the in vivo biological func-

tion of protein. The fact that *E. coli* DnaK is essential for growth at elevated temperatures [42, 43] has facilitated the study of other DnaK homologues using *E. coli dnaK* mutants [26, 28]. The *dnaK756 E. coli* strain produces a mutant DnaK protein that carries three glycine-to-aspartate substitutions [44, 45]. The G32D mutation falls within the GrpE-binding loop of the ATPase domain, whereas G455D and G468D mutations are located in the substrate-binding

Fig. 1 Sequence alignment of some Hsp70/DnaK proteins. The deduced amino-acid sequence for *B*/DnaK, *B. subtilis* DnaK (*Bs*DnaK), *E. coli* DnaK (*Ec*DnaK), human Hsc70 (*Hs*Hsc70), and bovine Hsc70 (*Bt*Hsc70) are shown. Gaps in aligned sequences (dashes) are introduced to maximize similarities. The characteristic domains typical for Hsp70/DnaK proteins are highlighted.

Fig. 2 SDS-PAGE analysis of the purified *B/DnaK*, *B/DnaJ*, and *B/GrpE*. Lanes: M, molecular mass marker; 1, *B/DnaK*; 2, *B/DnaJ*; 3, *B/GrpE*.

domain [44]. To ascertain whether the *B/DnaK* can function in other bacteria, the suppression of the *E. coli dnaK756-ts* mutant by the cloned gene was examined. As shown in Fig. 5, *B/DnaK* rescued the temperature-sensitive growth defect of the *E. coli dnaK756* mutant at 44°C. Complementation of the temperature-sensitive mutant of *E. coli* with *B/DnaK* in vivo indicates a functional similarity between *B/DnaK* and *EcDnaK*.

Secondary structure of *B/DnaK*

The amount of α -helical structure in proteins is established by the presence of two minima at 208 and 222 nm and a maximum at 192 nm in the CD spectrum [46]. CD analysis suggested that the recombinant *B/DnaK* consists mostly of α -helices (data not shown), which is in good agreement with the total amount of the secondary structure content of human Hsc70-NBD (1HJO.pdb) and *E. coli* DnaK-SBD (1DKX.pdb). The CD spectrum of nucleotide-free *B/DnaK* was undistinguishable for that in the presence of ATP. Nucleotide binding and hydrolysis are important steps of the action Hsp70/DnaK proteins. In vitro studies have shown that Hsp70/DnaK proteins bind both denatured proteins and some short peptides, and release these substrates in response to the addition of ATP [47,48]. Hsp70/DnaK proteins have a weak ATPase activity and

Fig. 3 Effects of temperature and pH on the ATPase activity of *B/DnaK*. ATPase activity was measured at various temperatures (A) and pH values (B) under standard assay conditions. All points are the means of three independent experiments.

a peptide binding activity with a mechanism of coupling these two activities such that the addition of ATP induces peptide release [5,49]. The open/closed state of the Hsp70/DnaK substrate-binding domain, which is mediated by the repositioning of a α -helical lid over the substrate-binding pocket, is governed by the nucleotide occupancy and status in the ATPase domain [5, 50]. The conformational change induced by ATP is the switch that releases the bound peptides and denatured proteins in vivo [47, 48]. Accordingly, our results confirm that the secondary structures of Hsp70/DnaK proteins are insensitive to change in the presence of nucleotide.

Fig. 4 Determination of kinetic constants for *B/DnaK* ATPase activity. The ATPase activity of *B/DnaK* (10 μM) was determined with varying concentrations of ATP. The kinetic parameters K_M and V_{max} were determined by a Lineweaver-Burke plot. The inset shows the Michaelis-Menten plot generated with the data. All points are the means of three independent experiments.

Fig. 5 Complementation experiments of the *E. coli* *dnaK*-null mutant with the cloned *dnaK* gene at 30 or 44°C. Overnight cultures of the transformed cells were diluted sequentially 10-fold from 10⁰ to 10⁻⁴ before being spotted onto the selective LB plates supplemented with 50 μM IPTG and evaluated for their abilities to grow at the permissive and non-permissive temperatures.

Nucleotide-induced stability changes

The thermally induced unfolding of *B/DnaK* was completely irreversible as previously showed for bovine and human Hsc70s [51–53], but this is not the case for *EcDnaK* which is capable to recover about 90% of its CD signal at 222 nm after being heated up to 90°C [54]. Differential scanning calorimetry of *EcDnaK* in the absence of nucleotides shows three unfolding transitions with T_m s of 42, 55, and 72°C, respectively [55]. The thermally induced unfolding of *B/DnaK* followed CD showed three temperature transitions in good agreement with that of *EcDnaK* (Fig. 6). Two well-defined transitions, one with T_m of 40.6°C (T_{m1}) and another with T_m of 81.7°C (T_{m3}), and a less well-defined transition with T_m of 57.3°C (T_{m2}) were measured for *B/DnaK*. *B/D*-

naK T_{m1} was in consistent with those of bovine Hsc70 and *EcDnaK* [52, 55], but T_{m3} was higher than that measured for *EcDnaK*.

A study using deletion mutants of *EcDnaK* showed that the first T_m is related to the unfolding of the N-terminus in which the nucleotide-binding site is located [55]. The second T_m is related to the unfolding of the C-terminus and the third to the unfolding of remaining structures at both N- and C-termini [55]. Based on these observations, the first T_m should be raised in the presence of nucleotides due to an increase in stability caused by the binding of these ligands. As shown in Fig. 6, a shift from 40.6 to 45.1–47.2°C was observed for *B/DnaK* T_{m1} upon ATP binding. Thus, *B/DnaK* T_{m1} must have been connected to the partial unfolding of NBD, because nucleotide binding increased its value. This result is in good agreement with the findings of Palleros et al. [52] and Borges and Ramos [53] that showed a temperature shift for the first T_{m1} s of bovine and human Hsc70s upon ATP binding. Other sub-domains of *B/DnaK*-NBD, which are still folded and interacting with ATP, must have unfolded at higher temperatures, since T_{m3} was also increased by nucleotide binding. T_{m2} must have connected

to the unfolding of the SBD, once it was not modified by nucleotide binding. *B/DnaK* stability, as measured by T_{m1} , was further increased to 56.4°C in the presence of 100 mM K⁺ and 2.5 mM Mg²⁺ ions (Fig. 6). This result corroborates that the cation plays a role in mediating contacts between Hsp70 and nucleotide [56, 57].

Effects of co-chaperone and peptide on ATPase activity of *B/DnaK*

As published earlier, the hydrolysis rate of *EcDnaK* towards ATP is stimulated by *EcDnaJ* and other co-chaperones [58]. To analyze the effects of co-chaperone and NR-peptide (NRLLLTG) on the ATPase activity of *B/DnaK* (25 μM), the ATP hydrolysis rate was measured in the absence or the

presence of *B/DnaJ* (50 μ M), *B/GrpE* (100 μ M), and NR-peptide (50 μ M). As shown in Fig. 7, *B/DnaJ* and *B/GrpE* accelerated ATP hydrolysis by a factor of 1.7 and 2.2, respectively. Simultaneous addition of *B/DnaJ* and *B/GrpE* synergistically stimulates the ATPase rate of *B/DnaK* by 9.2-fold and a further enhancement (11.7-fold) was observed by the incorporation of NR-peptide into the reaction mixture. These results strongly confirm that *B/DnaK* acts as a chaperone protein in *B. licheniformis*.

Conclusion

In summary, this study provides evidence that *B/DnaK* has biochemical and molecular chaperone properties of the

Fig. 6 Temperature denaturation of *B/DnaK* in 10 mM Hepes-NaOH (pH 7.0) as monitored by the CD signal at 222 nm. Experiments were done in the absence and in the presence of nucleotide and/or metal ions.

Fig. 7 Effects of co-chaperones and peptide on the basal ATP hydrolysis of *B/DnaK*. ATPase activity was measured under the standard assay conditions in the absence or presence of *B/DnaJ*, *B/GrpE* and NR-peptide.

Hsp70/DnaK family. However, the molecular chaperone DnaK requires the co-chaperone DnaJ to function properly, and the close interaction between DnaK and DnaJ is critical for normal cellular functioning. GrpE is also important for nucleotide exchange during the ATPase cycle. Therefore, our future studies will include the expression and characterization of DnaJ and GrpE proteins from *B. licheniformis*. The ability of *B. licheniformis* DnaK-DnaJ-GrpE system to refold recombinantly produced enzyme will be evaluated.

Acknowledgments The authors are very grateful to Dr. Wen-Hwei Hsu (Institute of Molecular Biology, National Chung Hsing University, Taichung, Taiwan) for technical assistance in some of the analytical experiments. This work was supported in part by the research grants (NSC 95-2313-B-415-012-YM3 and NSC 95-2313-B-415-001) from National Science Council of Taiwan.

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