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Protein Expression and Purification 38 (2004) 161–169
Protein Expression & Purification
www.elsevier.com/locate/yprep

The in vivo and in vitro characterization of DnaK from *Agrobacterium tumefaciens* RUOR

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Received 6 March 2004, and in revised form 10 June 2004
Available online 12 October 2004

Abstract

Molecular chaperones of the heat shock protein 70 family (Hsp70; also called DnaK in prokaryotes) play an important role in the folding and functioning of cellular protein machinery. The *dnaK* gene from the plant pathogen *Agrobacterium tumefaciens* RUOR was amplified using the polymerase chain reaction and the DnaK protein (*Agt* DnaK) was over-produced as a His-tagged protein in *Escherichia coli*. The *Agt* DnaK amino acid sequence was 96% identical to the *A. tumefaciens* C58 DnaK sequence and 65% identical to the *E. coli* DnaK sequence. *Agt* DnaK was shown to be able to functionally replace *E. coli* DnaK in vivo using complementation assays with an *E. coli dnaK756* mutant strain and a *dnaK52* deletion strain. Over-production and purification of *Agt* DnaK was successful, and allowed for further characterization of the protein. Kinetic analysis of the basal ATPase activity of purified *Agt* DnaK revealed a V_{max} of 1.3 nmol phosphate released per minute per milligram DnaK, and a K_m of 62 μM ATP. Thus, this is the first study to provide both in vivo and in vitro evidence that *Agt* DnaK has the properties of a molecular chaperone of the Hsp70 family.

Keywords: *Agrobacterium tumefaciens*; DnaK; ATPase; Complementation assays; Hsp70

The *Escherichia coli* DnaK and DnaJ proteins were initially identified as being important for the replication of λ DNA [1]. DnaK was later classified as a member of the 70 kDa heat shock protein (Hsp70)[1] family of proteins [2]. Hsp70s are present in almost all organisms (reviewed in [3]) and their predominant function is to ensure the correct folding of nascent polypeptides, as well as to prevent the misfolding of proteins due to cellular stress [4,5].

Hsp70 proteins are divided into three main domains, an ATPase domain, a substrate binding domain, and a less characterized C-terminal domain. The structures of the ATPase domain [6,7] and the substrate binding region [8] have been determined. The chaperone activity of Hsp70 proteins is dependent on a close interaction between the substrate binding domain and the ATPase domain. When ATP is bound to the ATPase domain, there is poor substrate binding, and hence there is weak chaperone activity. When ADP is bound however, there is high affinity for substrate, and the chaperone activity is enhanced. The ATPase activity is regulated by the co-chaperone protein DnaJ [9–11]. The DnaJ-like proteins stimulate the low basal ATP hydrolysis activity of partner Hsp70s [12,13], and in doing so enhance the Hsp70s substrate binding activity and hence their chaperone activity.

Agrobacterium tumefaciens is an economically important plant pathogen, whose genome sequence has recently been released [14,15]. *A. tumefaciens* is a Gram-negative plant pathogen instrumental in the formation of Crown Gall disease in plants which causes the formation of invasive tumours in the plant. Certain strains of

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[1] *Abbreviations used: Agt*, *Agrobacterium tumefaciens*; *A. tumefaciens*, *Agrobacterium tumefaciens*; Hsp70, heat shock protein 70; ATP, adenosine triphosphate; His, histidine; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis.

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doi:10.1016/j.pep.2004.06.039

A. tumefaciens, such as *A. tumefaciens* RUOR have been found to be of industrial importance for the over-production of the commercially important hydantoinase and *N*-carbamoyl-*N*-amino acid amidohydrolase (*N*-carbamoylase) enzymes that are involved in the synthesis of optically pure amino acids [16–18]. These enzymes have been recombinantly over-produced in *E. coli*, however protein aggregation was frequently observed [19,20]. The co-expression of molecular chaperones from *E. coli* have been used to enhance the solubility and activity of hydantoinase and *N*-carbamoylase from *Agrobacterium radiobacter* NRRL B11291 and *N*-carbamoylase from *A. tumefaciens* AM10 [19,20]. Consequently molecular chaperones have a potential role in biotechnology to enhance the recombinant over-expression of a particular enzyme of interest [21]. Our aim is to use the molecular chaperones DnaK and DnaJ from *A. tumefaciens* RUOR to improve the yields of recombinantly over-produced *N*-carbamoylase from *A. tumefaciens* RUOR. However, the novel DnaK and DnaJ proteins from *A. tumefaciens* RUOR need to be produced and characterized as molecular chaperones. The majority of work that has been done on defined DnaK–DnaJ systems at a prokaryotic level has involved looking at the *E. coli* system [22–27]. Limited work has been performed on other prokaryotic systems [28–30]. Previous research has shown that the regulation of the heat shock response of *A. tumefaciens* is different to that of *E. coli* and shows a high degree of complexity [31,32]. For this reason it is interesting to look at the properties of molecular chaperones from *A. tumefaciens*. We have therefore focused on the isolation of the gene encoding the chaperone protein DnaK from *A. tumefaciens* RUOR.

The full sequence of the *A. tumefaciens dnaK* gene and a partial sequence of the *dnaJ* gene were released previously [33], and with the release of the genome sequence of *A. tumefaciens* C58 it has become straightforward to design a method for isolating these genes. In this study we report on the successful expression, over-production and purification of the *A. tumefaciens* DnaK protein (*Agt* DnaK). The *Agt* DnaK protein was shown by in vivo and in vitro means to have the properties of a molecular chaperone.

Materials and methods

Materials

All reagents were obtained from Sigma Chemicals (USA), Roche Molecular Biochemicals (USA), or Merck Chemicals (Germany) unless otherwise stated. *A. tumefaciens* RUOR genomic DNA and cells were a kind gift of Ms. M. Jiwaji and Dr. C. Hartley (Rhodes University). *E. coli* strains BB1553 (MC4100 Δ*dnaK52::Cm*^{R} *sidB1*) and BB2362 (*dnaK756 recA::Tc*^{R} *pDMI, 1*) were kindly provided by Prof. B. Bukau (University of Heidelberg, Germany). The pQE60 and pBB46 (*Amp*^{R} and encoding *E. coli* DnaK) plasmids were kindly provided by Dr. W. Burkholder (Stanford University, USA). The pQE30 plasmids were purchased from Qiagen (USA) and the pGEM-T Easy cloning system from Promega (USA). Primers were synthesised by IDT (USA). Polymerase chain reaction (PCR) was performed using the Roche Expand High Fidelity PCR kit (Germany). Nickel-Chelating Sepharose Fast Flow matrix was obtained from Pharmacia Biotech (Sweden). Hybond C-extra nitrocellulose was purchased from Amersham Biosciences (UK).

Isolation of the Agt dnaK coding region

A. tumefaciens RUOR was grown at 30 °C for 5 days and genomic DNA was isolated using standard molecular biology techniques [34]. The primers used for the isolation of the complete *dnaKJ* coding region were AtDnaKJ F (5′GGA TCC ATG GCA AAA GTA ATC GGT ATC 3′) and AtDnaKJ R (5′GTC GAC TCA GCC STC AAA GAA TTY YTY CAT CCG 3′). Primers were designed based on the known *A. tumefaciens* C58 sequence, with some degeneracies incorporated into the reverse primer. The forward primer included a *Bam*HI site, and the reverse primer a *Sal*I site (underlined). The Roche Expand High fidelity PCR kit was used for this reaction. The parameters used for the reaction were as follows: an initial step of 5 min at 95 °C, followed by 25 cycles of 1 min at 95 °C, 1 min at 55 °C, 2 min at 72 °C, followed by a final hold at 72 °C for 10 min. The *dnaKJ* coding region was ligated into pGEM-T Easy, following the manufacturers instructions, and the construct was sequenced on both strands to obtain a complete nucleotide sequence for the gene. The *dnaK* coding region was then inserted into the expression vector pQE30 (Qiagen) for the expression of N-terminally His-tagged protein. Plasmid pQE30 was restricted with *Bam*HI and *Hin*cII, and the pGEM-T-*dnaKJ* was restricted with *Bam*HI and *Nru*I. The *Bam*HI–*Nru*I *dnaK* encoding fragment was then ligated into pQE30 to generate pRK30.

Computational analysis

The amino acid sequences of the *A. tumefaciens* C58 DnaK and *E. coli* DnaK were obtained from GenBank and aligned with the amino acid sequence of *Agt* DnaK using the ClustalW Multiple Sequence Alignment Program [35]. The alignment was run on BoxShade to visualize identical regions.

Production of Agt DnaK

E. coli XL1 Blue was transformed with the expression plasmid pRK30. A single colony was used to inocu-

late 25 mL 2× YT broth containing 100 μg/mL ampicillin. The culture was grown overnight at 37 °C. The culture was diluted into fresh broth containing 100 μg/mL ampicillin to a final volume of 250 mL, and the culture was allowed to grow until mid-log phase (OD_{600} 0.3–0.4). Protein production was then monitored by the collection of hourly and overnight samples both in the absence and the presence of the protein inducer IPTG (1 mM final concentration). The whole-cell extracts were normalized to OD_{600} and the appropriate aliquots were run on 12% SDS–PAGE gels. The protein bands were visualized using Coomassie brilliant blue staining.

Purification of Agt DnaK

An overnight culture of *E. coli* XL1 Blue[pRK30] was diluted into fresh 2× YT broth culture to a final volume of 1 L, and the cultures were allowed to grow until mid-log phase (OD_{600} 0.3–0.4). Protein production was then induced with 1 mM IPTG for 4 h, following which cells were resuspended in 1/20th culture volume lysis buffer (100 mM Tris, pH 8.0, 300 mM NaCl, 10 mM imidazole, and 1 mM PMSF). Cells were frozen overnight, and thawed in the presence of 1 mg/mL lysozyme. Mild sonication was carried out and the cellular debri was removed by centrifugation at 16,000*g* for 20 min in a bench top centrifuge (Eppendorf, Germany). Cell lysate was incubated with 1 mL of a 50% slurry of nickel-charged Sepharose beads overnight at 4 °C. The beads were washed three times in wash buffer (100 mM Tris, pH 8.0, 300 mM NaCl, and 100 mM imidazole). The bound protein was eluted in elution buffer (100 mM Tris, pH 8.0, 300 mM NaCl, and 1 M imidazole), dialyzed overnight (100 mM Tris, pH 8.0, 300 mM NaCl, and 1 mM PMSF), concentrated by dialysis against PEG 20000 (Merck, Germany) and stored at −80 °C. Protein concentration was determined using the Bradford assay, with bovine serum albumin as a standard, and the purity was assessed using SDS–PAGE. Western analysis was performed to confirm production of the *Agt* DnaK protein using anti-His antibodies (Amersham Biosciences, USA). Chemiluminescence-based immunodetection was performed using the BM Chemiluminescence Western blotting kit (Mouse/Rabbit) from Roche, as per the manufacturers instructions.

Complementation assays

The temperature-sensitive *E. coli* strains BB1553 (MC4100 Δ*dnaK52::Cm*^{R} *sidB1*) and BB2362 (*dnaK756 recA::Tc*^{R} *pDMI 1*) were used to determine the ability of *Agt* DnaK to functionally replace *E. coli* DnaK under heat shock conditions. The Δ*dnaK52 E. coli* strain is temperature-sensitive at temperatures below 20 °C and above 35 °C, while *dnaK756* exhibits temperature-sensitive growth above 40 °C. The Δ*dnaK52* mutant strain has an internal fragment of the *dnaK* gene replaced with a *cat* cassette, causing temperature sensitivity [36]. However, the Δ*dnaK52* mutant strain (BB1553) carries the *sidB1* suppressor mutation allowing the cells to grow stably at 30 °C [37]. The *dnaK756* strain produces a mutant DnaK protein that carries three glycine-to-aspartate substitutions [38,39]. The G32D mutation falls within the GrpE-binding loop of the ATPase domain, while G455D and G468D are in the substrate-binding domain [38]. *E. coli* DnaK encoded on pBB46 (a pQE60-based construct) was used as a positive control. The plasmids pQE30, pRK30 (a pQE30-based construct), pQE60, and pBB46 were transformed into *E. coli* BB1553 and BB2362 strains. Overnight cultures were set up at 30 °C using fresh transformants in 2× YT broth containing 100 μg/mL ampicillin for plasmid selection, 50 μg/mL kanamycin and 10 μg/mL tetracycline for *E. coli* BB2362 strain selection and 34 μg/mL chloroamphenicol for *E. coli* BB1553 strain selection. Cultures were serially diluted using 2× YT broth before being spotted onto plates containing 50 μM IPTG for *E. coli* BB1553 strain and 20 μM IPTG for *E. coli* BB2362 strain. The transformed *E. coli* BB1553 strain was grown at 30 and 40 °C, while transformed *E. coli* BB2362 strain was grown at 37 and 44 °C. Western analysis was performed to confirm production of the *Agt* DnaK and *E. coli* DnaK proteins in the *E. coli* BB1553 strain using anti-His antibodies (Amersham Biosciences, UK) and anti-DnaK antibodies (Stressgen, USA) respectively. Chemiluminescence-based immunodetection was performed using the BM Chemiluminescence Western blotting kit (Mouse/Rabbit) from Roche, as per the manufacturers instructions.

Basal ATPase activity assays

ATP hydrolysis assays performed were a modification of the method originally described by Lanzetta, and by Chamberlain and Burgoyne [40,41]. *Agt* DnaK to a final concentration of 0.4 μM was added to the assay buffer (10 mM Hepes, pH 8.0, 100 mM KCl, 2 mM $MgCl_2$, and 0.5 mM DTT) and incubated for 5 min at 37 °C. The reaction was started by the addition of ATP at various concentrations (0–500 μM). An aliquot (50 μL) of the reaction was removed at varying time intervals and the reaction stopped with the addition of 10% SDS (50 μL). Colour development was started by the addition of 1.25% ammonium molybdate in 6.5% H_2SO_4 (50 μL), and 9% ascorbic acid (50 μL). Colour development occurred over a 1 h period, and was read at 660 nm. The assays were corrected for spontaneous ATP degradation. Specific activity was determined in units of nanomoles phosphate released per minute per milligram DnaK, and activity was defined as nmol phosphate released per minute.

Nucleotide sequence accession number

The nucleotide sequence for the complete *A. tumefaciens* RUOR *dnaKJ* coding region has been submitted to GenBank under Accession No. AY494599.

Results

Isolation of the A. tumefaciens RUOR dnaK coding region (Agt dnaK)

The *Agt dnaK* coding region was successfully amplified from genomic DNA using PCR. The *dnaK* coding region was inserted into pQE30 and the size of the predicted *Agt* DnaK protein was theoretically determined to be 68.9 kDa. The amino acid sequence of the *Agt* DnaK protein was compared to the DnaK proteins from *A. tumefaciens* C58 and *E. coli* (Fig. 1). There was 96% amino acid sequence identity between *Agt* DnaK and *A. tumefaciens* C58 DnaK, while there was 65% amino acid sequence identity between *Agt* DnaK and *E. coli* DnaK. A comparison of the *Agt* DnaK and *E. coli* DnaK amino acid residues revealed that *Agt* DnaK consisted of the canonical domains; an ATPase domain (amino acids 1–380), a substrate-binding domain (amino acids 389–532) and a C-terminal domain (amino acids 533–634). There was 70% similarity between the substrate binding domains of *Agt* DnaK and *E. coli* DnaK proteins.

Production and purification of Agt DnaK

The expression plasmid pRK30 resulted in the recombinant over-production of His-tagged *Agt* DnaK protein. SDS–PAGE analysis of whole cell extracts before and after induction with IPTG revealed that the DnaK

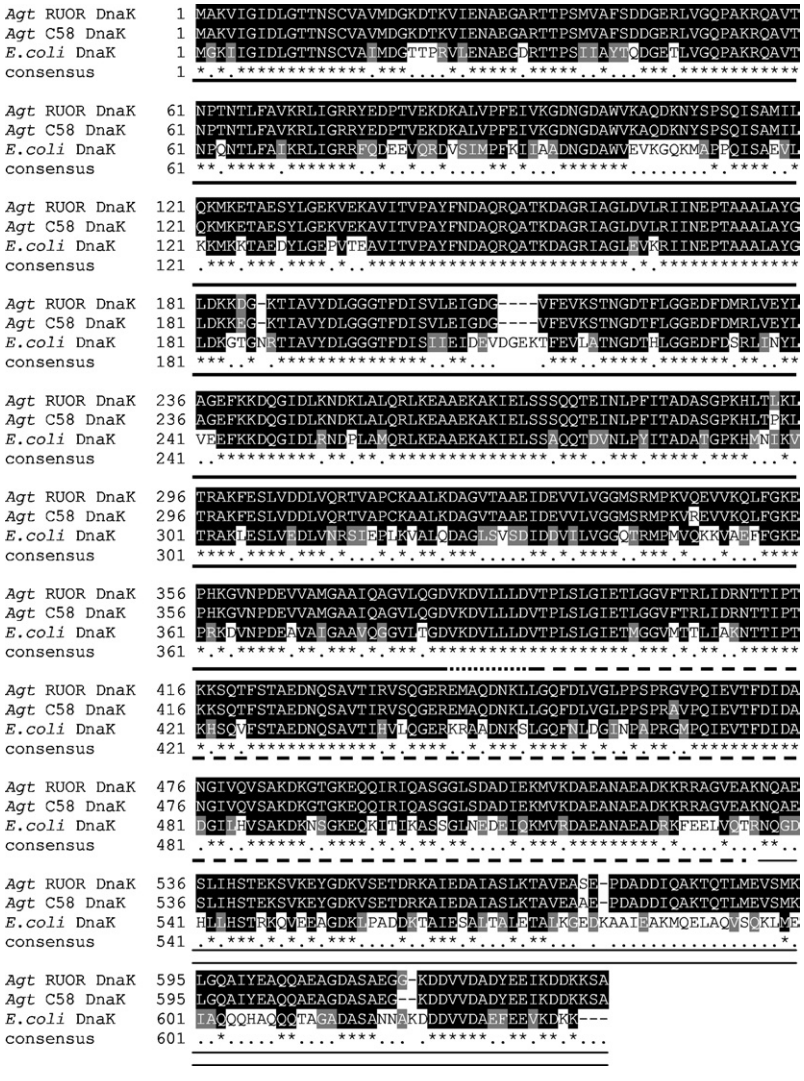

Fig. 1. Comparison of the DnaK proteins from *A. tumefaciens* RUOR, *A. tumefaciens* C58, and *E. coli*. The protein sequences were aligned using ClustalW [35] and shaded using BoxShade. The linker region (dotted) joins the ATPase domain (underlined) and peptide-binding domain (dashed), followed by the C-terminal domain (double underlined). The numbers represent the position of the amino acids within the protein sequence.

protein migrated as a single 70 kDa band and the amount of protein produced before induction with IPTG was very similar to the amount of protein after 5 h of induction, however the protein concentration of *Agt* DnaK decreased significantly overnight (Fig. 2). Protein induction using IPTG was not tightly regulated as the amount of protein before induction was similar to that after 4 h induction (compare lanes 0 and 4 of Fig. 2). Protein production was also monitored in the absence of IPTG and the levels of protein were very similar to those observed in the presence of IPTG, according to SDS–PAGE analysis (data not shown). However, Western analysis revealed an inductive effect when the level of *Agt* DnaK produced before the addition of IPTG (Figs. 3A and B, lane 0) was compared to the level after 4 h of induction with IPTG (Figs. 3A and B, lane 1).

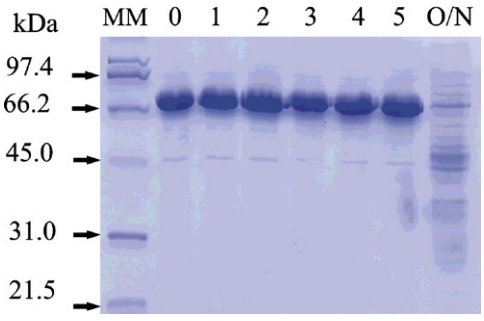

Fig. 2. SDS–PAGE analysis of the over-production of *Agt* DnaK in *E. coli* XL1 Blue[pRK30] cells. The protein represents total cell extract after different times of induction by 1 mM IPTG. MM, molecular mass markers in kDa; lane 0, protein before induction with IPTG; lanes 1–5, protein induced hourly over a period of 5 h using IPTG; and lane O/N, protein produced after overnight induction. The protein bands were visualized using Coomassie brilliant blue staining.

The high over-production of the *Agt* DnaK protein simplified the purification using nickel affinity chromatography. The efficiency of the purification procedure was evaluated by SDS–PAGE analysis. Most *Agt* DnaK was soluble and present in the cell lysate (Fig. 3, lane 2), however protein also remained in the insoluble pellet fraction (Fig. 3, lane 3). The amount of Sepharose beads used to bind the protein was sufficient as very little *Agt* DnaK remained in the cell lysate after binding to the beads (Fig. 3, lane 4). During the subsequent wash steps *Agt* DnaK was also washed off the beads (Fig. 3, lane 5). The protein was eluted from the nickel-charged Sepharose beads in a buffer containing 1 M imidazole (Fig. 3, lane 6) and the imidazole was later removed by dialysis and the purified protein was concentrated against PEG 20000 (Fig. 3, lane 7). *Agt* DnaK was successfully purified as a soluble protein that migrated as a single band on SDS–PAGE. The purity of *Agt* DnaK protein was greater than 95%, and approximately 8 mg of protein was purified per 1 L culture volume.

Complementation for the loss of functional E. coli DnaK with Agt DnaK

Agt DnaK was able to functionally replace the DnaK mutant protein expressed in *E. coli* strain *dnaK756* (BB2362) at 44 °C in the presence of 20 μM IPTG (Fig. 4A). *Agt* DnaK was able to complement fully for the lack of *E. coli* DnaK in the null mutant strain Δ*dnaK52* (BB1553) at 39 °C (data not shown) and partially complement at 40 °C in the presence of 50 μM IPTG (Fig. 4B), when compared to *E. coli* DnaK (expressed on pBB46). The reasons for partial complementation at

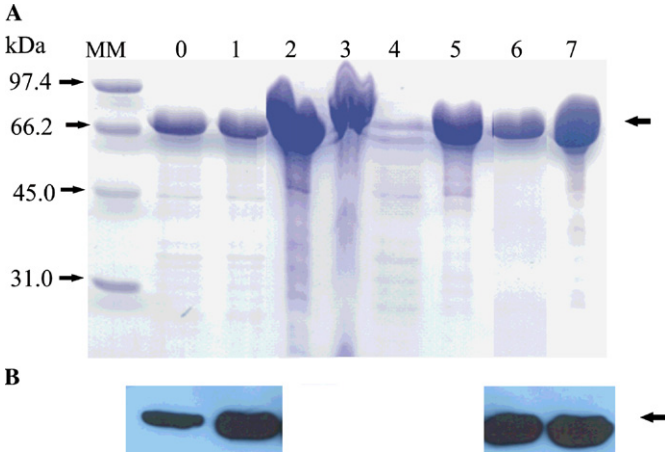

Fig. 3. SDS–PAGE and Western analysis of affinity-purified *Agt* DnaK. MM, molecular mass markers in kDa; lane 0, protein in total cell extract before induction with 1 mM IPTG; lane 1, protein in total cell extract after 4 h of induction with IPTG; lane 2, protein present in the soluble cell lysate; lane 3, protein present in the insoluble pellet fraction; lane 4, protein present in the cell lysate after binding of *Agt* DnaK to nickel-charged Sepharose beads; lane 5, protein present in the first wash of the Sepharose beads; lane 6, elution of protein from the nickel-charged Sepharose beads using 1 M imidazole; and lane 7, purified protein after dialysis and concentration. The protein bands were visualized using Coomassie brilliant blue staining (A). Chemiluminescence-based Western analysis was used to confirm expression of His-tagged *Agt* DnaK protein using anti-His antibodies (B). Molecular mass markers, expressed as kDa, are shown on the left-hand side, and the arrows on the right-hand side indicate the position of DnaK.

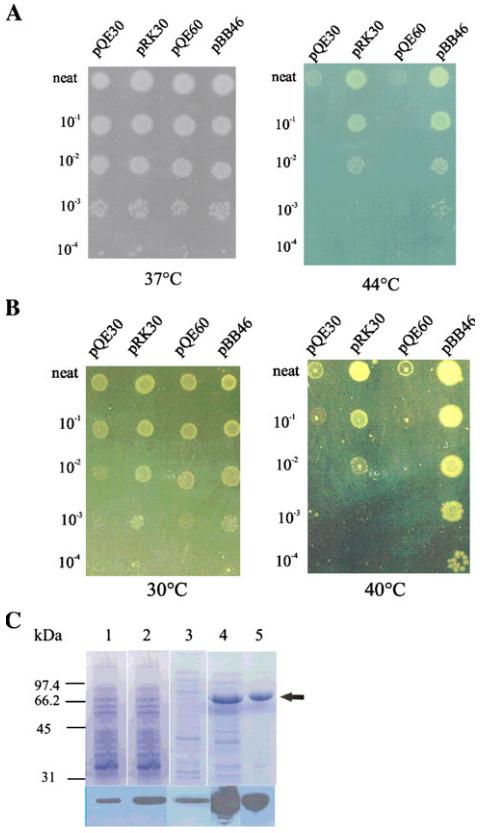

Fig. 4. *Agt* DnaK can successfully complement for the loss of functional *E. coli* DnaK. *E. coli* strains BB2362 and BB1553 were transformed with pQE30, pRK30 (pQE30 vector containing the coding region of *Agt* DnaK), pQE60, and pBB46 (pQE60 vector containing the coding region of *E. coli* DnaK). The cells were diluted 10-fold sequentially from neat ($OD_{600} = 0.2$) to 10^{-4}, spotted on agar plates supplemented with IPTG and evaluated for their abilities to grow at the respective permissive and non-permissive temperatures for each *E. coli* strain. (A) The transformed *E. coli* strain BB2362 grew at the permissive temperature of 37 °C, indicating that the strain was viable. At the non-permissive temperature of 44 °C, the growth of BB2362 transformed with pRK30 (*Agt* DnaK) was similar to that of BB2362 transformed with pBB46 (*E. coli* DnaK) indicating that *Agt* DnaK was able to complement for the lack of a functional DnaK in the *E. coli* strain BB2362. (B) The transformed *E. coli* strain BB1553 grew at the permissive temperature of 30 °C, indicating that the strain was viable. At the non-permissive temperature of 40 °C, the growth of BB1553 transformed with pRK30 (*Agt* DnaK) did not grow as well as BB1553 transformed with pBB46 (*E. coli* DnaK) indicating that *Agt* DnaK was able to partially complement for the lack of DnaK in the *E. coli* strain BB1553. (C) SDS–PAGE (upper panel) and Western analysis (lower panel) of protein levels of *Agt* DnaK and *E. coli* DnaK proteins expressed in *E. coli* BB1553 strain after overnight growth at 40 °C. Lane 1, protein present in total cell extract before induction of BB1553 transformed with pRK30 (*Agt* DnaK); lane 2, protein present in total cell extract after induction with IPTG and overnight growth of BB1553 transformed with pRK30 (*Agt* DnaK); lane 3, protein present in total cell extract before induction of BB1553 transformed with pBB46 (*E. coli* DnaK); lane 4, protein present in total cell extract after induction with IPTG and overnight growth of BB1553 transformed with pBB46 (*E. coli* DnaK); and lane 5, 10 μg of purified *Agt* DnaK. Western analysis of *Agt* DnaK and *E. coli* DnaK proteins was conducted using anti-His and anti-DnaK antibodies respectively. Molecular mass markers, expressed as kDa, are shown on the left-hand side, and the arrow indicates the position of DnaK.

40 °C may be due to the low levels of *Agt* DnaK compared to *E. coli* DnaK proteins produced in the null mutant strain Δ*dnaK52* (BB1553) (compare lanes 2 and 4 of Fig. 4C). The levels of *Agt* DnaK and *E. coli* DnaK proteins were similar before induction with 50 μM IPTG (compare lanes 1 and 3 of Fig. 4C). Both *E. coli dnaK756* and Δ*dnaK52* strains required IPTG to induce *E. coli* DnaK and *Agt* DnaK proteins at high temperatures. The growth of the negative controls (each strain transformed with pQE30 and pQE60) was similar to the growth of the positive control (each strain transformed with pBB46) and test (each strain transformed with pRK30) at permissive temperatures, however under heat shock conditions growth of the negative controls was suppressed (Figs. 4A and B).

ATP hydrolysis

Determination of the kinetic constants of *Agt* DnaK was performed using a colourimetric assay monitoring the release of inorganic phosphate (Pi). *Agt* DnaK was shown to have a basal ATPase activity with a V_{max} of 1.3 nmol phosphate released per minute per milligram DnaK and K_m of 62.0 μM (Fig. 5). The V_{max} and K_m were determined from three independent experiments.

Discussion

The release of the full genome sequence of *A. tumefaciens* C58 facilitated the amplification of the coding region of the *dnaK* operon of *A. tumefaciens* RUOR. Due to the 65% amino acid sequence identity between *Agt* DnaK and *E. coli* DnaK sequences, the chaperone properties of these prokaryotic DnaK proteins were compared in this study.

The *Agt* DnaK protein was successfully over-produced as the major protein in *E. coli* XL1 Blue cells. Relatively high basal expression from the *lac* operator-controlled T5 promoter on pRK30, which is a pQE30-based expression vector, probably accounts for the high level of *Agt* DnaK produced before induction with IPTG. Relatively high levels of basal expression by pQE30-based vectors have been observed during the recombinant production of numerous proteins, including CikA [42] and azoreductase [43]. The co-transformation of the plasmid pREP4 (encoding the *lac* repressor protein) with pQE30-based expression constructs can be used to minimize the basal expression levels [44,45].

The high levels of *Agt* DnaK produced in *E. coli* facilitated the development of a one-step batch purification procedure for the generation of purified *Agt* DnaK. While this procedure had the advantage of limited steps, which in turn limits protein loss, loss of protein did occur during the purification procedure, as approximately a quarter of the protein present in the sonicated fraction

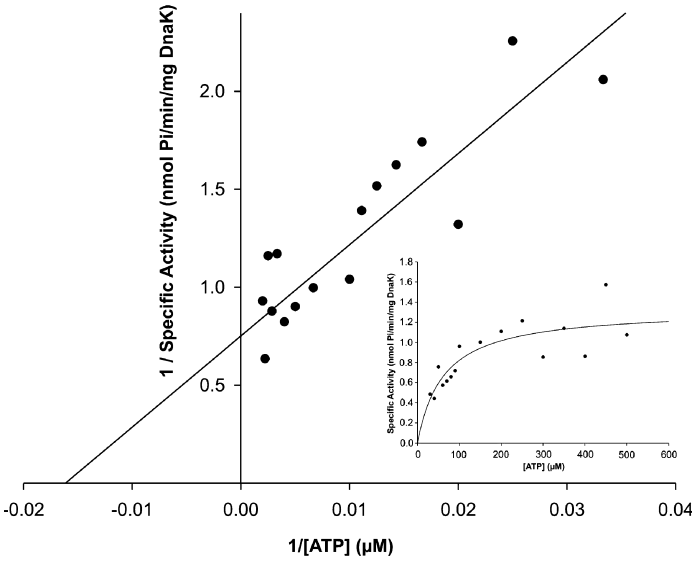

Fig. 5. Determination of kinetic constants for *Agt* DnaK ATPase activity. The ATPase activity of *Agt* DnaK (0.4 μM) was determined using varying concentrations of ATP (0–500 μM). The amount of phosphate released was determined colorimetrically using a modification of the method described by Chamberlain and Burgoyne [41]. The kinetic parameters K_m and V_{max} were determined using a Lineweaver–Burke plot. The inset shows the Michaelis–Menten plot generated with the data. All points are the means of three independent experiments.

was present in the insoluble pellet fraction, and significant amounts of protein were lost during the wash steps. However, the wash steps were important for the removal of proteins that had non-specifically bound to the affinity matrix. The *Agt* DnaK in the insoluble fraction probably represents aggregated protein that resulted from the high rate and level of expression. Numerous enzymes present in the cell, including *E. coli* DnaK possess ATPase activity, thus for subsequent ATPase activity assays on *Agt* DnaK, it was desirable to have protein of high purity. For biotechnological applications, such as protein folding bioreactors, crude cell extracts containing over-produced *Agt* DnaK may be sufficient.

Complementation studies are a well-established method to aid in the determination of the in vivo biological function of proteins. The fact that *E. coli* DnaK is essential for growth at elevated temperatures [36,46] has facilitated the study of other DnaK homologues using *E. coli dnaK* mutants. *Agt* DnaK was able to substitute for *E. coli* DnaK in two different mutant *dnaK* strains of *E. coli* when grown at non-permissive temperatures. The *E. coli* BB1553 strain was characterized by reduced levels of DnaJ proteins, resulting from a polar effect of the chloramphenicol marker upstream of the *dnaJ* gene [47]. This may account for the partial complementation observed for *E. coli* BB1553 transformed with pRK30 at 40 °C. *Bacillus subtilis* DnaK was unable to complement for the lack of *E. coli* DnaK in a Δ*dnaK52* null mutant strain at 40 °C, however co-transformation of a plasmid encoding *E. coli* DnaJ facilitated complementation by this DnaK [48]. Thus increasing the levels of DnaJ proteins could enhance the complementation of *Agt* DnaK in Δ*dnaK52* at 40 °C. However, other possibilities for partial complementation cannot be excluded, such as low levels of *Agt* DnaK or incomplete interaction between *Agt* DnaK and *E. coli* DnaJ proteins in the cell, leading to inefficient chaperone activity. The transformation of a plasmid encoding the *Agt dnaKJ* operon could potentially ensure that the amounts of DnaJ were sufficient in the cell and that the interaction between DnaK and its co-chaperone DnaJ was not compromised.

The high protein yields that were obtained after affinity purification facilitated the biochemical characterization of the basal ATPase activity of *Agt* DnaK. The ATPase activity and nucleotide bound state of Hsp70s are involved in the regulation of assisted refolding of proteins by these chaperones. A wide range of values have been reported for the basal ATPase activity of *E. coli* DnaK, with V_{max} values ranging from 0.43 to 3.5 nmol Pi/min/mg DnaK and K_m values ranging from 20 nM to 20 μM ATP [13,49–51]. The V_{max} of *Agt* DnaK ATPase activity of 1.3 nmol Pi/min/mg determined in this study falls within the range reported in previous studies for the basal ATPase activity of *E. coli* DnaK, however the K_m of 62.0 μM is significantly higher than that obtained for *E. coli* DnaK.

This is the first study that has provided evidence that *Agt* DnaK has biochemical and molecular chaperone properties of the Hsp70 family of proteins. However, the molecular chaperone DnaK requires the co-chaperone DnaJ to function correctly, and the close interaction between DnaK and DnaJ is crucial for normal cellular functioning. GrpE is also important for nucleotide exchange during the ATPase cycle. Thus future studies will include the expression and purification of DnaJ and GrpE proteins from *A. tumefaciens* RUOR. The influ-

ence of *Agt* DnaJ and GrpE on the ATPase activity of *Agt* DnaK will be compared to that of the *E. coli* DnaK–DnaJ–GrpE system. Finally the abilities of both the *Agrobacterium* and *E. coli* DnaKJE chaperone systems to refold recombinantly produced *N*-carbamoylase will be evaluated.

Acknowledgments

This work was funded by a Wellcome Trust Grant (066705, UK), and a National Research Foundation Grant (Gun No: 2053542, NRF, SA) both awarded to G.L.B., and a Joint Research Council Grant (JRC, Rhodes University, SA) awarded to G.L.B. and A.B. The authors gratefully acknowledge the gifts of *A. tumefaciens* RUOR genomic DNA from Ms. M. Jiwaji and Dr. C. Hartley, *E. coli* strains BB1553 and BB2362 from Prof. B. Bukau (University of Heidelberg, Germany) and pQE60 and pBB46 from Dr. W. Burkholder (Stanford University, USA).

References

[1] K. Liberek, C. Georgopoulos, M. Zylicz, Role of the *Escherichia coli* DnaK and DnaJ heat shock proteins in the initiation of bacteriophage DNA replication, Proc. Natl. Acad. Sci. USA 85 (1988) 6632–6636.

[2] J.C.A Bardwell, E.A. Craig, Major heat shock gene of *Drosophila* and the *Escherichia coli* heat inducible *dna*K gene are homologous, Proc. Natl. Acad. Sci. USA 81 (1984) 848–852.

[3] A.J.L Macario, M. Lange, B.K. Ahring, E. Conway De Macario, Stress genes and proteins in the Archaea, Micro. Mol. Biol. Rev. 63 (1999) 923–967.

[4] M. Beissinger, J. Buchner, How chaperones fold proteins, Biol. Chem. 379 (1998) 245–259.

[5] M.P. Mayer, B. Bukau, Hsp70 chaperone systems: diversity of cellular functions and mechanism of action, Biol. Chem. 379 (1998) 261–268.

[6] K.M. Flaherty, S.M. Wilbanks, C. De Luca-Flaherty, D.B. McKay, Three-dimensional structure of the ATPase fragment of a 70 kDa heat shock cognate protein, Nature 346 (1990) 623–628.

[7] C.J. Harrison, M. Hayer-Hartl, M. Di Liberto, F.-U. Hartl, J. Kuriyan, Crystal structure of the nucleotide exchange factor GrpE bound to the ATPase domain of the molecular chaperone DnaK, Science 276 (1997) 431–435.

[8] X. Zhu, X. Zhao, W. Burkholder, A. Gragerov, C.M. Ogata, M.E. Gottesman, W.A. Hendrickson, Structural analysis of substrate binding by the molecular chaperone DnaK, Science 272 (1996) 1606–1614.

[9] M.E. Cheetham, A.J. Caplan, Structure, function and evolution of DnaJ: Conservation and adaption of chaperone function, Cell Stress Chaperones 3 (1998) 28–36.

[10] W.L. Kelley, The J-domain family and the recruitment of chaperone power, Trends Biochem. Sci. 23 (1998) 222–227.

[11] W.L. Kelley, Molecular chaperones: how J domains turn on Hsp70s, Curr. Biol. 9 (1999) R305–R308.

[12] R. Jordan, R. McMacken, Modulation of the ATPase activity of the molecular chaperone DnaK by peptides and the DnaJ and GrpE heat shock proteins, J. Biol. Chem. 270 (1995) 4563–4569.

[13] K. Liberek, J. Marszalek, D. Ang, C. Georgopoulos, M. Zylicz, *Escherichia coli* DnaJ and GrpE heat proteins jointly stimulate ATPase activity of DnaK, Proc. Natl. Acad. Sci. USA 88 (1991) 2874–2878.

[14] B. Goodner, G. Hinkle, S. Gattung, N. Miller, M. Blanchard, B. Qurollo, B.S. Goldman, Y. Cao, M. Askenazi, C. Halling, L. Mullin, K. Houmiel, J. Gordon, M. Vaudin, O. Iartchouk, A. Epp, F. Liu, C. Wollam, M. Allinger, D. Doughty, C. Scott, C. Lappas, B. Markelz, C. Flanagan, C. Crowell, J. Gurson, C. Lomo, C. Sear, G. Strub, C. Cielo, S. Slater, Genome sequence of the plant pathogen and biotechnology agent *Agrobacterium tumefaciens* C58, Science 294 (2001) 2323–2328.

[15] D.W. Wood, J.C. Setubal, R. Kaul, D.E. Monks, J.P. Kitajima, V.K. Okura, Y. Zhou, L. Chen, G.E. Wood, N.F.J. Almeida, L. Woo, Y. Chen, I.T. Paulsen, J.A. Eisen, P.D. Karp, D.S. Bovee, P. Chapman, J. Clendenning, G. Deatherage, W. Gillet, C. Grant, T. Kutyavin, R. Levy, M.J. Li, E. McClelland, A. Palmieri, C. Raymond, G. Rouse, C. Saenphimmachak, Z. Wu, P. Romero, D. Gordon, S. Zhang, H. Yoo, Y. Tao, P. Biddle, M. Jung, W. Krespan, M. Perry, B. Gordon-Kamm, L. Liao, S. Kim, C. Hendrick, Z.Y. Zhao, M. Dolan, F. Chumley, S.V. Tingey, J.F. Tomb, M.P. Gordon, M.V. Olson, E.W. Nester, The genome of the natural genetic engineer *Agrobacterium tumefaciens* C58, Science 294 (2001) 2317–2323.

[16] S. Burton, R. Dorrington, C. Hartley, S. Kirchmann, G. Matcher, V. Phehane, Production of enantiomerically pure amino acids: characterisation of South African hydantoinases and hydantoinase-producing bacteria, J. Mol. Catal. B: Enzymatic 5 (1998) 301–305.

[17] C. Hartley, S. Kirchmann, S. Burton, R. Dorrington, Production of D-amino acids from D,L-substituted hydantoins by an *Agrobacterium tumefaciens* strain and isolation of a mutant with inducer-independent expression of hydantoin hydrolysing activity, Biotechnol. Lett. 20 (1998) 707–711.

[18] C. Hartley, F. Manford, S. Burton, R. Dorrington, Over-production of hydantoinase and *N*-carbomoylamino acids amidohydrolase enzymes by regulatory mutants of *Agrobacterium tumefaciens*, Appl. Microbiol. Biotechnol. 57 (2001) 43–49.

[19] Y.-P. Chao, C.-J. Chiang, T.-E. Lo, H. Fu, Overporduction of D-hydantoinase and carbamoylase in a soluble form in *Escherichia coli*, Appl. Microbiol. Biotechnol. 54 (2000) 348–353.

[20] D. Sareen, R. Sharma, R.M. Vohra, Chaperone-assisted overexpression of an active D-carbamoylase from *Agrobacterium tumefaciens* AM10, Protein Expr. Purif. 23 (2001) 374–379.

[21] C. Schlieker, B. Bukau, A. Mogk, Prevention and reversion of protein aggregation by molecular chaperones in the *E. coli* cytosol: implications for their applicability in biotechnology, J. Biotechnol. 96 (2002) 13–21.

[22] B Banecki, M. Zylicz, Real time kinetics of the DnaK/DnaJ/GrpE molecular chaperone machine action, J. Biol. Chem. 271 (1996) 6137–6143.

[23] J. Gamer, G. Multhaup, T. Tomoyasu, J.S. McCarty, S. Rüdiger, J. Schönfeld, C. Schirra, H. Bujard, B. Bukau, A cycle of binding and release of the DnaK, DnaJ and GrpE chaperones regulates activity of the *Escherichia coli* heat shock transcription factor σ32, EMBO J. 15 (1996) 607–617.

[24] C.S. Gässler, A. Buchberger, T. Laufer, M.P. Mayer, H. Schröder, A. Valencia, B. Bukau, Mutations in the DnaK chaperone affecting interaction with the DnaJ cochaperone, Proc. Natl. Acad. Sci. USA 95 (1998) 15229–15234.

[25] W. Han, P. Christen, Mechanism of the targeting action of DnaJ in the DnaK molecular chaperone system, J. Biol. Chem. 278 (2003) 19038–19043.

[26] T. Langer, C. Lu, H. Echols, J. Flanagan, M.K. Hayer, F.-U. Hartl, Successive action of DnaK, DnaJ and GroEL along the pathway of chaperone-mediated protein folding, Nature 356 (1992) 683–689.

[27] S. Rüdiger, J. Schneider-Mergener, B. Bukau, Its substrate specificity characterises the DnaJ co-chaperone as a scanning factor for the DnaK chaperone, EMBO J. 20 (2001) 1042–1050.

[28] K. Motohashi, M. Yohda, I. Endo, M. Yoshida, A novel factor required for the assembly of the DnaK and DnaJ chaperones of *Thermus thermophilus*, J. Biol. Chem. 271 (1996) 17343–17348.
[29] K. Tilly, R. Hauser, J. Campbell, G.J. Ostheimer, Isolation of *dnaJ*, *dnaK* and *grpE* homologues from *Borrelia burgdorferi* and complementation of *Escherichia coli* mutants, Mol. Microbiol. 7 (1993) 359–369.
[30] M. Zuber, T.A. Hoover, M.T. Dertzbaugh, D.L. Court, Analysis of the DnaK molecular chaperone system of *Francisella tularensis*, Gene 164 (1995) 149–152.
[31] G. Segal, E.Z. Ron, Regulation of heat-shock response in bacteria, Ann. NY Acad. Sci. 851 (1998) 147–151.
[32] R. Rosen, K. Büttner, R. Schmid, M. Hecker, E.Z. Ron, Stress-induced proteins of *Agrobacterium tumefaciens*, FEMS Microbiol. Ecol. 35 (2001) 277–285.
[33] G. Segal, E.Z. Ron, The dnaKJ operon of Agrobacterium tumefaciens: transcriptional analysis and evidence for a new heat shock promoter, J. Bacteriol. 177 (1995) 5952–5958.
[34] J. Sambrook, D.W. Russell, Molecular Cloning: A Laboratory Manual, third ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 2001.
[35] J.D. Thompson, D.G. Higgins, T.J. Gibson, CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice, Nucleic Acids Res. 22 (1994) 4673–4680.
[36] K.H. Paek, G.C. Walker, *Escherichia coli* dnaK null mutants are inviable at high temperature, J. Bacteriol. 169 (1987) 283–290.
[37] T. Hesterkamp, B. Bukau, Role of the DnaK and HscA homologs of Hsp70 chaperones in protein folding in *E. coli*, EMBO J. 17 (1998) 4818–4828.
[38] A. Buchberger, C.S. Gässler, M. Büttner, R. McMacken, B. Bukau, Functional defects of the DnaK756 mutant chaperone of *Escherichia coli* indicate distinct roles for amino- and carboxyl-terminal residues in substrate and co-chaperone interaction and interdomain communication, J. Biol. Chem. 274 (1999) 38017–38026.
[39] A. Buchberger, H. Schröder, M. Büttner, A. Valencia, B. Bukau, A conserved loop in the ATPase domain of the DnaK chaperone is essential for stable binding of GrpE, Nat. Struct. Biol. 1 (1994) 95–101.
[40] P.A. Lanzetta, L.J. Alvarez, P.S. Reinach, O.A. Candia, An improved assay for nanomole amounts of inorganic phosphate, Anal. Biochem. 100 (1979) 95–97.
[41] L.H. Chamberlain, R.D. Burgoyne, Activation of the ATPase activity of heat shock proteins Hsc70 / Hsp70 by cysteine-string protein, Biochem. J. 322 (1997) 853–858.
[42] M. Mutsuda, K.-P. Michel, X. Zhang, B.L. Montgomery, S.S. Golden, Biochemical properties of CikA, an unusual phytochrome-like histidine protein kinase that resets the circadian clock in *Synechococcuc elongatus* PCC 7942, J. Biol. Chem. 278 (2003) 19102–19110.
[43] Y. Suzuki, T. Yoda, A. Ruhul, W. Sugiura, Molecular cloning and characterization of the gene coding for azoreductase from *Bacillus sp.* OY1-2 isolated from soil, J. Biol. Chem. 276 (2001) 9059–9065.
[44] A.C. Martin, G.H. Wadhams, D.S.H. Shah, S.L. Porter, J.C. Mantotta, T.J. Craig, P.H. Verdult, H. Jones, J.P. Armitage, CheR- and CheB-dependent chemosensory adaptation system of *Rhodobacter sphaeroides*, J. Bacteriol. 183 (2001) 7135–7144.
[45] D.S.H. Shah, S.L. Porter, D.C. Harris, G.H. Wadhams, P.A. Hamblin, J.P. Armitage, Identification of a fourth *cheY* gene in *Rhodobacter sphaeroides* and interspecies interaction within the bacterial chemotaxis signal transduction pathway, Mol. Microbiol. 35 (2000) 101–112.
[46] B. Bukau, G.C. Walker, Delta dnaK52 mutants of *Escherichia coli* have defects in chromosome segregation and plasmid maintenance at normal growth temperatures, J. Bacteriol. 171 (1989) 6030–6038.
[47] B. Bukau, G.C. Walker, Mutations altering heat shock specific subunit of RNA polymerase suppress major cellular defects of *E. coli* mutants lacking the DnaK chaperone, EMBO J. 9 (1990) 4027–4036.
[48] A. Mogk, B. Bukau, R. Lutz, W. Schumann, Construction and analysis of hybrid *Escherichia coli–Bacillus subtilis dnaK* genes, J. Bacteriol. 181 (1999) 1971–1974.
[49] W.F. Burkholder, C.A. Panagiotidis, S.J. Silverstein, A. Cegielska, M.E. Gottesman, G.A. Gaitanaris, Isolation and characterization of an *Escherichia coli* DnaK mutant with impaired ATPase activity, J. Biol. Chem. 242 (1994) 364–377.
[50] A. Buchberger, A. Valencia, R. McMacken, C. Sander, B. Bukau, The chaperone function of DnaK requires the coupling of ATPase activity with substrate binding through residue E171, EMBO J. 13 (1994) 1687–1695.
[51] A.S. Kamath-Loeb, C. Lu, W. Suh, M.A. Lonetto, G.A. Gross, Analysis of three DnaK mutant proteins suggests that progression through the ATPase cycle requires conformational changes, J. Biol. Chem. 270 (1995) 30051–30059.