The DnaK chaperones from the archaeon *Methanosarcina mazei* and the bacterium *Escherichia coli* have different substrate specificities

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Hsp70 (DnaK) is a highly conserved molecular chaperone present in bacteria, eukaryotes, and some archaea. In a previous work we demonstrated that DnaK from the archaeon *Methanosarcina mazei* (DnaK\(_{Mm}\)) and the DnaK from the bacterium *Escherichia coli* (DnaK\(_{Ec}\)) were functionally similar when assayed *in vitro* but DnaK\(_{Mm}\) failed to substitute for DnaK\(_{Ec}\) *in vivo*. Searching for the molecular basis of the observed DnaK species specificity we compared substrate binding by DnaK\(_{Mm}\) and DnaK\(_{Ec}\). DnaK\(_{Mm}\) showed a lower affinity for the model peptide (a-CALLQSRLLS) compared to DnaK\(_{Ec}\). Furthermore, it was unable to negatively regulate the *E. coli* σ^32^ transcription factor level under heat shock conditions and poorly bound purified σ^32^, which is a native substrate of DnaK\(_{Ec}\). These observations taken together indicate differences in substrate specificity of archaeal and bacterial DnaKs. Structural modeling of DnaK\(_{Mm}\) showed some structural differences in the substrate-binding domains of DnaK\(_{Mm}\) and DnaK\(_{Ec}\), which may be responsible, at least partially, for the differences in peptide binding. Size-exclusion chromatography and native gel electrophoresis revealed that DnaK\(_{Mm}\) was found preferably in high molecular mass oligomeric forms, contrary to DnaK\(_{Ec}\). Oligomers of DnaK\(_{Mm}\) could be dissociated in the presence of ATP and a substrate (peptide) but not ADP, which may suggest that monomer is the active form of DnaK\(_{Mm}\).

**Keywords:** archaeal Hsp70(DnaK), substrate-binding by archaeal DnaK, archaeal DnaK quaternary structure

**INTRODUCTION**

The Hsp70 proteins form a family of evolutionarily conserved heat shock proteins which act as molecular chaperones, assisting in the folding of nascent polypeptides, in the refolding of partially denatured proteins and in many other cellular processes. They have a weak ATPase activity and function by reversibly binding substrates at the expense of ATP hydrolysis. The Hsp70s of prokaryotic organisms are called the DnaK proteins, after the model protein of *Escherichia coli*, and function as a triad, together with the co-chaperones DnaJ and GrpE (Bukau et al., 2000; 2006; Truscott et al., 2003;
DnaK of *E. coli* is composed of two domains: an approximately 44 kDa amino-terminal ATPase domain and a carboxy-terminal substrate binding domain (SBD) of about 27 kDa. The crystal structures of the ATPase domain (Harrison et al., 1997) and that of the SBD (Zhu et al., 1996) have been solved. The ATPase domain contains a groove for ATP binding; it also binds a dimeric form of the nucleotide-exchange factor GrpE. In the structure of SBD two subdomains exist: the first one, composed mostly of β-sheets, containing a cavity which binds the substrate polypeptide (displaying short stretches of hydrophobic residues on its surface), and the second one, composed mostly of α-helices, which functions as a lid covering the peptide-binding cavity (Zhu et al., 1996).

According to the current model, the α-helical subdomain forming a latch over the substrate-binding cavity is in an open conformation in the ATP-bound state, allowing release of the folded polypeptide and subsequent binding of another molecule of substrate to initiate a second folding cycle. The capture and retention of the polypeptide for folding involves closing of the latch, a conformational change that requires energy from the hydrolysis of the bound ATP, which is converted to ADP. Since the ADP-bound DnaK has a higher affinity for the peptide compared to the ATP-bound form, in order to release the polypeptide, ADP has to be exchanged for ATP. Both the ATPase activity of DnaK and nucleotide exchange are regulated by the co-chaperones DnaJ and GrpE. The amino-terminal DnaJ domain binds to DnaK and stimulates ATP hydrolysis, which promotes substrate binding. Subsequently, GrpE promotes ADP dissociation thus enabling DnaK to bind ATP, which in turn promotes the DnaK-polypeptide complex dissociation and release of the folded polypeptide (Bukau et al., 2000; 2006; Mayer et al., 2000; Deuerling & Bukau, 2004; Erbse et al., 2004; Young et al., 2004).

In *E. coli*, the dnaK, dnaJ, and grpE genes belong to a heat shock regulon positively controlled by the *rpoH* gene product, a promoter-specific σ32 subunit of RNA polymerase. DnaK negatively regulates the level of σ32 by binding and promoting its proteolysis (Yura & Nakahigashi, 1999). The binding of σ32 by DnaK is an example showing that not only unfolded polypeptides but also a native, non-denatured protein may be a substrate for this chaperone (Libereker et al., 1992).

The Hsp70/DnaK proteins are highly conserved in sequence and distribution in Bacteria and Eukarya, but in the Archaea DnaK is present only in some species, one of which is a mesophlic archaean, *Methanosarcina mazei* (Macario et al., 1991). It is hypothesized that the archaeal DnaK system has been obtained by lateral transfer of the dnaK-dnaJ-grpE genes from bacteria. This hypothesis is well documented, mainly on the basis of sequence similarity (Gribaldo et al., 1999; Macario & Conway De Macario, 2001; Macario et al., 2004) and, recently, by showing a functional and structural similarity of purified *M. mazei* and *E. coli* DnaK proteins (Żmijewski et al., 2004; 2007). These similarities notwithstanding, there is a significant functional difference, revealed by the fact that the dnaK gene of *M. mazei* is unable to complement dnaK mutations in *E. coli*. *E. coli* dnaK mutant bacteria are thermosensitive and this phenotype cannot be rescued by DnaK of *M. mazei* (Żmijewski et al., 2004), which indicates DnaK species-specificity.

Generally, chaperones are considered to be promiscuous, since in vitro they interact with a variety of unfolded polypeptides and the presence of exposed hydrophobic groups seems to be the only requirement for Hsp70 binding (Erbse et al., 2004). However, DnaK from one species introduced into another is frequently unable to fully substitute for the DnaK of the host cell (Sussman & Setlow, 1987; Mehlert & Young, 1989; Tilly et al., 1993; Minder et al., 1996; Mogk et al., 1999). Since the species-specificity of DnaK proteins is an unsolved problem and because very little is known so far about function of the archaeal DnaKs, we decided to investigate molecular basis of the observed species-specificity of the *M. mazei* DnaK.

In this work we compared the binding of a typical peptide substrate, representing an unfolded protein, and of a native protein, sigma 32 transcription factor, by DnaK<sub>Mn</sub> and DnaK<sub>Ec</sub>. Searching for structural differences we analyzed the oligomerization status of the purified DnaKs. Our results showed a significantly lower affinity of the DnaK<sub>Mn</sub> for both types of substrates, and also differences in the quaternary structures of the purified DnaKs.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, media.** The *Escherichia coli* strains and plasmids used in this study are listed in Table 1. LB medium (Luria-Bertani broth) and LA (Luria agar) were prepared according to Sambrook and coauthors (1989), and were supplemented with appropriate antibiotics (when necessary): 100 µg ml<sup>−1</sup> ampicillin, 68 µg ml<sup>−1</sup> chloramphenicol, and 30 µg ml<sup>−1</sup> kanamycin.

**Chemicals.** Deuterium oxide (99.9% 2H<sub>2</sub>O), 3HCl, and NaO<sub>2</sub>H were purchased from Aldrich (Sigma-Aldrich S.r.l., Milan, Italy). All other chemicals were commercial products of the purest quality.
Table 1. *Escherichia coli* strains and plasmids used in this study.

<table>
<thead>
<tr>
<th>E. coli strain or plasmid—Relevant genotype</th>
<th>Source/Reference</th>
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<tr>
<td>B178—W3110 galE sup&lt;sup&gt;*&lt;/sup&gt;</td>
<td>BDUG collection*</td>
</tr>
<tr>
<td>MC4100—F&lt;sup&gt;–&lt;/sup&gt; araD (ΔargF-lac)U169 rpsL relA ffbB decC ptsF rbsR</td>
<td>BDUG collection</td>
</tr>
<tr>
<td>BM271—MC4100 ΔdnaK52::Cm&lt;sup&gt;R&lt;/sup&gt;ts</td>
<td>B. Bukau/(Paek &amp; Walker, 1987)</td>
</tr>
<tr>
<td>CG50—B178 dnaK756ts</td>
<td>C. Georgopoulos/(Sell et al., 1990)</td>
</tr>
<tr>
<td>DA259—C600 ΔgrpE::Ω-Cm&lt;sup&gt;R&lt;/sup&gt; thr::Tn10</td>
<td>D. Ang/(Wu et al., 1994)</td>
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Plasmids

<table>
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<tr>
<th>Plasmid</th>
<th>Source/Reference</th>
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<tr>
<td>pMOB45-dnaK&lt;sub&gt;Mm&lt;/sub&gt;—pMOB45-dnaK&lt;sub&gt;Mm&lt;/sub&gt; Cm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>BDUG collection/(Zylicz &amp; Georgopoulos, 1984)</td>
</tr>
<tr>
<td>pI2-Z589—pT7Q9-tpcdnaK&lt;sub&gt;E&lt;/sub&gt; colidna</td>
<td>E. coli Amp&lt;sup&gt;R&lt;/sup&gt;</td>
</tr>
<tr>
<td>pAM1—pBS-dnaK&lt;sub&gt;Mm&lt;/sub&gt; Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>A.J.L. Macario/R. Kim</td>
</tr>
<tr>
<td>pAM4—pBS-dnaK&lt;sub&gt;Mm&lt;/sub&gt; Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>A.J.L. Macario/R. Kim</td>
</tr>
<tr>
<td>pBS—pBS (pBlueScript) Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>BDUG collection/Stratagene</td>
</tr>
<tr>
<td>pAK1—pT7-5-rpoH Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>BDUG collection/(Kotlarz et al., 1998)</td>
</tr>
<tr>
<td>pGPI1-2—T7pol Kan&lt;sup&gt;R&lt;/sup&gt;</td>
<td>BDUG collection/(Tabor &amp; Richardson, 1985)</td>
</tr>
</tbody>
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*BDUG, Biochemistry Department, University of Gdańsk*

Purchased from Sigma (Poznań, Poland), or were obtained as indicated in the text.

**Proteins, peptides, electrophoresis, and immunoblotting.** DnaK proteins from *M. mazei* and *E. coli* were purified as described previously (Zmijewski et al., 2004). The proteins were dialyzed against 25 mM Hepes, 100 mM KCl, 10% glycerol, pH 7.2. Protein (>95% pure) concentrations were determined by the Bradford method (Bradford, 1976) and were confirmed by densitometry of Coomassie-stained sodium dodecyl sulphate polyacrylamide-gel electrophoresis (SDS/PAGE) gels, using BSA as a standard protein. DnaK preparations were free of ATP, as tested by the malachite green form infrared spectroscopy. Preparation of protein samples and Fourier-transform infrared spectroscopy was carried out as described previously (Zmijewski et al., 2007).

**Fourier-transform infrared spectroscopy.** Preparation of protein samples and Fourier-transform infrared spectroscopy was carried out as described previously (Zmijewski et al., 2007).

**Size exclusion chromatography.** Size-exclusion chromatography was performed using an Agilent HPLC 1100 system equipped with a diode array detector and a Zorbax GF-250 column (Agilent Biotechnologies). In all experiments Zorbax GF-250 was equilibrated with 50 mM Tris, pH 7.5, 100 mM KCl buffer. DnaK proteins (100 µg/100 µl, or as stated in figure legends) were incubated in the presence (3 mM ATP or 3 mM ADP+Pi) or in the absence of nucleotide, or in the presence of 200 nM peptide NR (NRLLLGTG) or in the absence of nucleotide, or in the presence of 200 nM peptide NR (NRLLLGTG) as indicated in figure legends. Immunoblotting was performed as described before (Zmijewski et al., 2004), using anti-σ<sup>32</sup> polyclonal rabbit antibodies raised by immunization with purified σ<sup>32</sup> protein (Szewczyk & Harper, 1994).

**Protein modeling.** Modeling of *M. mazei* chaperone proteins was done by use of the Swiss Model server and Deep View/Swiss-PdbViewer (Peitsch 1996; Guex & Peitsch, 1997). Model of the ATPase domain of the DnaK<sub>Mm</sub> protein was done with the *E. coli* structure (1DKGD) used as a template. After modeling, the structures of the *M. mazei* proteins were minimized with the GROMOS96 force field implementation of Swiss-PdbViewer.

**Substrate binding by archaeal DnaK.** Peptides were stored in 30% (v/v) acetonitrile at 100 µM concentration, at −70 °C. Proteins were analysed by SDS/PAGE according to Laemmli (1970), using 10 or 12.5% (w/v) acrylamide. Native gel electrophoresis was performed using the Laemmli system without SDS and without stacking gel, in 10% resolving gels. Before the native electrophoresis, proteins (3 µM) were incubated for 30 min in 50 mM Hepes, pH 7.5, 50 mM KCl, and 10 mM MgCl<sub>2</sub> buffer, in the presence of either 3 mM ATP or 3 mM ADP+Pi, or in the absence of nucleotide; or in the presence of 200 nM peptide NR (NRLLLGTG) as indicated in figure legends. Immunoblotting was performed as described before (Zmijewski et al., 2004), using anti-σ<sup>32</sup> polyclonal rabbit antibodies raised by immunization with purified σ<sup>32</sup> protein (Szewczyk & Harper, 1994).
Determination of fluorescence spectra. A Perkin-Elmer LS55 spectrophotometer, equipped with a thermostated cuvette holder with stirrer and a Julabo thermostat, was used to record fluorescence emission spectra. All experiments were performed in 0.5×0.5 cm quartz cuvettes at 25°C, in a buffer containing 25 mM HEPES pH 7.0, 100 mM KCl, and 10 mM MgCl₂. Kinetics of complex formation of acrylodan-labeled peptide a-p4 with DnaK was measured with the excitation wavelength set at 370 nm (bandpass 4.6 nm, with excitation slits set at 4.6 nm), and the spectra were recorded at 510 nm (bandpass 18.6 nm, with emission slits set at 18.6 nm) for 900 s at 25°C. The reaction was started by addition of DnaK at final concentrations ranging from 0.1 to 5 µM. The concentration of the acrylodan-labeled peptide was held constant in all experiments (200 nM), and the final volume of the reaction mixture was 400 µl.

Determination of  \( k_{\text{obs}} \) kinetic constants \( (k_{\text{on}}, k_{\text{off}}) \) and evaluation of kinetic measurements for the DnaK–a-p4 peptide binding. To determine the rate constants \( k_{\text{on}} \) and \( k_{\text{off}} \) for complex formation between DnaK\(_{Ec}\) or DnaK\(_{Mm}\) and acrylodan-labelled peptide a-p4, the apparent rate constants \( k_{\text{obs}} \) were calculated for 200 nM peptide a-p4 and increasing concentrations (0.1 to 5 µM) of DnaK. Collected data were fitted to a single exponential equation (1):

\[
F(t) = \Delta F[1-\exp(-k_{\text{on}}t)] + F_0
\]

where \( k_{\text{on}} \) represents first-order rate constant, \( \Delta F \) amplitude and \( F_0 \) initial fluorescence.

For all tested concentrations of DnaK the error of fitting for \( k_{\text{obs}} \) was lower than 2% and fitting to a double-exponential equation resulted in an increase of fitting error. The \( k_{\text{obs}} \) values were plotted against concentration of DnaK protein and data were fitted to the linear function. The association (\( k_{\text{on}} \)) and dissociation (\( k_{\text{off}} \)) rate constants were calculated according to equation (2):

\[
K_{\text{obs}} = k_{\text{on}}[\text{DnaK}]+k_{\text{off}}
\]

The dissociation equilibrium constants \( (K_d) \) of DnaK\(_{Mm}\)–a-p4 and DnaK\(_{Ec}\)–a-p4 complexes were calculated according to equation (3):

\[
K_d = k_{\text{off}}/k_{\text{on}}
\]

All calculations were performed with Origin 7.5 software (similar results for DnaK\(_{Ec}\) were obtained with the Dynafit program).

Assay of \( \sigma^{32} \) levels. E. coli strains were transformed with appropriate plasmids according to (Sambrook et al., 1989). The transformed bacteria were grown in LB medium with aeration at 30°C to an OD\(_{600}\) of 0.6, and then were heat-shocked at 45°C for 1 h. The heat shock was followed by a 1-h recovery period at 30°C. Equivalent amounts of each culture were harvested before and after heat shock, and after the recovery period, and were analyzed by 12.5% SDS-PAGE followed by Western blotting with anti-\( \sigma^{32} \) antibodies.

Enzyme-linked immunosorbent assay (ELISA). The ELISA assays were performed as described (Krzewski et al., 2003), with some modification. Briefly, microtiter multiwell assay plates (Costar 3590) were coated with 0.5 µg of \( \sigma^{32} \) or BSA per well in 50 µl of PBS buffer, and were allowed to incubate overnight. Wells were washed four times with PBS buffer (0.17 M NaCl, 5 mM KCl, 10 mM Na\(_2\)HPO\(_4\); 7 mM KH\(_2\)PO\(_4\)) containing 0.1% BSA (PBS/BSA), and were then washed with binding buffer (25 mM HEPES/KOH, pH 7.5, 150 mM KCl, 10 mM MgCl\(_2\), 0.1 mM EDTA, 2 mM DTT, 5% (v/v) glycerol, 0.05% Triton X-100, and 0.2% BSA). They were then incubated with serial dilutions of DnaK\(_{Ec}\) or DnaK\(_{Mm}\) Protein (0.2–0.003125 µg/ml) in the binding buffer for 1 h. The glutaraldehyde cross-linking step was omitted. The wells were washed with binding buffer and four times with PBS/BSA. Anti-DnaK\(_{Ec}\) (Wawrzynow & Zyliec, 1995) or anti-DnaK\(_{Mm}\) (Clarens et al., 1995) rabbit polyclonal antibodies were added (at 1:5000 dilution) in PBS/BSA for 2 h. Wells were washed four times with PBS/BSA, and then secondary antibodies (goat anti-rabbit IgG coupled with horseradish peroxidase) were added. Bound conjugates were detected colorimetrically, after 60 min incubation, by use of the horseradish peroxidase substrate tetramethylbenzidine (TMB). Each assay was repeated at least three times.

Surface plasmon resonance spectroscopy. Studies on interaction of DnaK proteins with \( \sigma^{32} \) were carried out by use of the BIAcore 2000 biosensor (Pharmacia Biosensor AB, Uppsala, Sweden) and determination of SPR changes. Research-grade CM5 chips were coated with \( \sigma^{32} \) to about 2000 resonance units (RU) using the EDC/NHS coupling kit (N-ethyl-N-(dimethylaminopropyl)carbodiimide, N-hydroxysuccinimide; Pharmacia Biosense AB). The cell temperature was 25°C, and the running buffer was 50 mM HEPES (pH 7.5), 100 mM KCl, 10 mM MgCl\(_2\), 0.005% (v/v) Tween 20. The DnaK\(_{Ec}\) or DnaK\(_{Mm}\) protein was passed over the \( \sigma^{32} \)-coated chip and an empty control chip with a flow rate of 10 µM/min at 1 µM concentrations. As a control, BSA was used with the same concentration and conditions. ATP (12.5 µM) was added at least 5 min before injection. The signal obtained from the non-coated chip was subtracted from the signal obtained from the chip with coupled \( \sigma^{32} \). The \( \sigma^{32} \)-coupled chip was regenerated by the addition of 20 µl of 1 M urea at a flow
rate of 10 µl/min. Spectra were evaluated with the software supplied with the BIAcore instrument.

RESULTS

Binding of substrates by DnaK<sub>Mm</sub> and DnaK<sub>Ec</sub>

In our previous work we demonstrated that DnaK<sub>Mm</sub> has a chaperoning capacity comparable to that of DnaK<sub>Ec</sub>; however, the substrate (polypeptide)-binding properties of the archaeal protein were not investigated (Żmijewski et al., 2004). In this work, substrate binding was assessed to determine whether or not DnaK<sub>Mm</sub> and DnaK<sub>Ec</sub> differ in specificity. For this purpose, we compared the abilities of the two proteins to bind the acrylodan-labeled peptide a-p4 (a-CALLQSRLLS), a known DnaK<sub>Ec</sub> substrate successfully used for kinetic measurements (Gisler et al., 1998). We measured by spectrofluorometry the kinetics of the binding of the a-p4 peptide by the two DnaKs, and then used the data to calculate the kinetic constants, as described under Materials and Methods. The results are shown in Fig. 1 (k<sub>diss</sub> values plotted against DnaK concentrations); Table 2 shows the calculated kinetic constants. The k<sub>1</sub> values were significantly different for the two proteins, indicating that the chaperone–a-p4 peptide complex formation was considerably slower (by approx. 8-fold, in the experiment shown) for DnaK<sub>Mm</sub> than for DnaK<sub>Ec</sub>. The dissociation equilibrium constants (K<sub>d</sub>) differed between the two chaperones to a smaller extent, showing nonetheless that binding of the peptide to DnaK<sub>Mm</sub> was at least 2-fold weaker (e.g., 0.91 µM for DnaK<sub>Mm</sub> vs. 2.57 µM for DnaK<sub>Ec</sub>). The differences in the substrate-binding properties between the archaeal and bacterial chaperones must be due to fine structural differences, most importantly those occurring in the substrate-binding domain (SBD). Our modeling of DnaK<sub>Mm</sub> showed a high degree of similarity between the SBDs of the <i>M. mazei</i> and <i>E. coli</i> proteins (Żmijewski et al., 2007). However, close examination of the substrate-binding cavities of the two DnaKs revealed that the highly conserved methionine 404 in DnaK<sub>Ec</sub> forming an arch over the cavity, is replaced in DnaK<sub>Mm</sub> by leucine 378. Also, in the latch region, DnaK<sub>Ec</sub> histidine 544 is replaced in DnaK<sub>Mm</sub> by asparagine 518, and DnaK<sub>Mm</sub> aspartic acid 540 is replaced in DnaK<sub>Mm</sub> by glutamic acid 514 (Fig. 2A, B).

DnaK<sub>Mm</sub> is unable to down-regulate the <i>E. coli</i> heat-shock transcription factor σ<sub>32</sub> in vivo

In <i>E. coli</i>, DnaK<sub>Ec</sub> participates in its own regulation, by interacting with the transcription factor σ<sub>32</sub> and steering it toward degradation, which results in down-regulation of the dnaK gene when the cellular need for this chaperone diminishes. To find out whether DnaK<sub>Mm</sub> is able to interact with σ<sub>32</sub> in the cell, we investigated whether DnaK<sub>Mm</sub> can down-regulate σ<sub>32</sub> in <i>E. coli</i>, as DnaK<sub>Ec</sub> does. We used <i>E. coli</i> strains lacking DnaK (<i>E. coliΔdnaK</i>) and strains that produce a mutant DnaK protein that lacks the self-regulatory ability (<i>E. coli dnaK<sub>32</sub></i>); both of these strains are unable to shut off the heat-shock response, in contrast to the wild-type. These strains were transformed with plasmids bearing the gene coding for DnaK<sub>Mm</sub> or the genes encoding DnaK<sub>Mm</sub> and DnaJ<sub>Mm</sub>. We carried out the experiment with both the dnaK<sub>Mm</sub> and dnaJ<sub>Mm</sub> genes to rule out the possibility that a lack of DnaK<sub>Mm</sub> function was caused by a poor cooperation of DnaK<sub>Mm</sub> with DnaJ<sub>Mm</sub>. The level of σ<sub>32</sub> protein in cell lysates from the transformants was assessed by Western blotting (Fig. 3A). We also confirmed the presence of DnaK<sub>Mm</sub> in the transformants by Western blotting (not shown). Neither the presence of the dnaK<sub>Mm</sub> gene alone nor the presence of both genes from <i>M. mazei</i>, dnaK<sub>Mm</sub> and dnaJ<sub>Mm</sub>, in the transformed mutant <i>E. coli</i> cells, caused a decrease in the levels of σ<sub>32</sub> under heat shock conditions (when σ<sub>32</sub> is elevated). In mutant <i>E. coli</i> cells transformed with a plasmid carrying dnaK<sub>Ec</sub> alone, or transformed with plasmids carrying both <i>E. coli</i> genes, dnaK<sub>Ec</sub> and dnaJ<sub>Ec</sub>, the σ<sub>32</sub> protein was barely detectable or undetectable, as expected. Likewise, in

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**Figure 1. Kinetics of peptide binding by DnaK proteins.**

To follow the binding of the test polypeptide to the chaperone, we added DnaK<sub>Mm</sub> or DnaK<sub>Ec</sub> to the assay buffer containing acrylodan-labeled a-p4 (a-CALLQSRLLS) and measured the increase of acrylodan fluorescence with time, as described under Materials and Methods. Final concentrations were: a-p4, 0.2 µM; DnaK, 0.01–5 µM. The binding curves obtained were fitted to a single exponential function, and the k<sub>diss</sub> values were derived for DnaK<sub>Ec</sub> (circles) and DnaK<sub>Mm</sub> (squares). The k<sub>diss</sub> values were plotted as a function of DnaK concentration; the plots were obtained from least-squares fitting of the data to an equation for a straight line.
the control, wild-type bacterium with its own dnaK gene (E. coli B178), the σ32 protein was undetectable. These results taken together indicate that DnaK<sub>Mm</sub> did not negatively regulate the σ32 transcription factor in E. coli, presumably because the chaperone was unable to bind the transcription factor, contrary to what happens with DnaK<sub>Ec</sub>.

**DnaK<sub>Mm</sub> poorly interacts with E. coli heat shock transcription factor σ32 in vitro**

In order to check whether DnaK<sub>Mm</sub> is unable to physically interact with σ32 as efficiently as DnaK<sub>Ec</sub> does, we compared the binding capacities of DnaK<sub>Mm</sub> and DnaK<sub>Ec</sub> to E. coli σ32, by applying an enzyme-linked immunosorbent assay (ELISA) and surface plasmon resonance (SPR) spectroscopy. In ELISA, we determined the amount of DnaK protein bound to σ32 immobilized on the plate wells, using anti-DnaK antibodies. The amount of DnaK<sub>Mm</sub> bound to σ32 was significantly lower than that of DnaK<sub>Ec</sub> (Fig. 3B). Reverse experiments were also performed. In these, the DnaK proteins were immobilized onto the ELISA-plate wells first, and then they were incubated with serial dilutions of σ32; this was followed by detection of bound transcription factor with anti-σ32 antibodies. The results, not shown, were essentially the same as those obtained with immobilized σ32, displayed in Fig. 3B.

The kinetics of interaction of either DnaK with immobilized σ32 protein was measured by SPR spectroscopy. DnaK<sub>Mm</sub> interacted with σ32 poorly as compared with DnaK<sub>Ec</sub> (Fig. 3C). In conclusion, the ELISA and SPR spectroscopy results showed that the DnaK<sub>Mm</sub> interaction with E. coli σ32 in vitro was considerably weaker than the interaction of DnaK<sub>Ec</sub> with the same factor. This finding is in agreement with the observed inabil-
ity of DnaK\textsubscript{Mm} to negatively regulate the σ\textsuperscript{32} levels in vivo.

**Purified DnaK\textsubscript{Mm} exists predominantly in oligomeric forms**

It is known that oligomerization status modulates chaperoning ability, e.g. in small heat-shock proteins (Narberhaus, 2002; Franzmann \textit{et al.}, 2005), in eukaryotic Hsp70 (Blond-Elguindi \textit{et al.}, 1993), and in some bacterial DnaKs (Motohashi \textit{et al.}, 1994; Schonfeld \textit{et al.}, 1995). Searching for differences in quaternary structure which might contribute to the differences in substrate binding by DnaK\textsubscript{Mm} and DnaK\textsubscript{Ec}, we performed experiments to elucidate whether the archaeal chaperone oligomerizes under conditions resembling those in vivo.

Size-exclusion chromatography showed that DnaK\textsubscript{Mm} formed oligomers over a broad range of concentrations (0.1–5 mg/ml). The high-molecular mass oligomeric forms of DnaK\textsubscript{Mm} were predominant under all tested conditions, even at the lowest

\[\begin{array}{c}
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\text{Substrate binding by archaeal DnaK}\end{array}\]
protein concentration, as shown by high-pressure liquid chromatography (HPLC) (Fig. 4A). DnaK$_{Mm}$ showed the coexistence of three major forms, eluting as proteins with molecular masses of 440, 300 and 135 kDa; the ratio of these forms changed very little with concentration (these molecular masses represent mean values calculated from the results of five independent experiments; molecular-mass standards were run in each experiment). DnaK$_{Ec}$ subjected to HPLC under similar conditions showed substantial aggregation only at high (non-physiological) concentrations (5–10 mg/ml), and the lowest-molecular-mass form (eluting as a 140–150 kDa protein) was predominant within the range of 0.1–5 mg/ml (Fig. 4B). SDS/PAGE of the fractions containing a mixture of all the various DnaK forms did not resolve these forms, but showed the bands for DnaK$_{Mm}$ and DnaK$_{Ec}$ monomers (not shown).

The smallest DnaK$_{Ec}$ and DnaK$_{Mm}$ forms eluted (by HPLC) as species larger than 100 kDa. We assume that the 130–140 kDa form of DnaK$_{Mm}$ is the monomer, since it eluted like the smallest DnaK$_{Ec}$ form, and did not change its position upon ATP addition, when high-molecular-mass forms dissociated (Fig. 5A). Other investigators have also noticed that DnaK$_{Ec}$ does not behave like a 70 kDa globular protein should (Żylicz & Georgopoulos, 1984; A. Żylicz, personal communication). The presence of at least three forms with different molecular masses and the predominance of the high molecular-mass forms in the DnaK$_{Mm}$ preparations were confirmed by native gel electrophoresis (Fig. 5C, lanes 3, 8). In contrast, under the same experimental conditions, the low molecular-mass forms predominated in the DnaK$_{Ec}$ preparations (Fig. 5C, lanes 1, 7).

In order to investigate whether the DnaK$_{Mm}$ oligomerization has a functional significance with regard to the ATP cycle and substrate binding, we preincubated DnaK$_{Mm}$ with ATP, ADP+Pi, or the peptide NRLLLTG (NR), and then carried out size-exclusion chromatography. In these experiments we used the NR peptide as a model substrate, since it has previously been shown to efficiently bind DnaK (Gragerov et al., 1994; Buczynski et al., 2001).
Substrate binding by archaeal DnaK

In the presence of ATP or NR, the high-molecular-mass oligomers dissociated, and the majority of DnaK$_{Mm}$ eluted as about 140 kDa protein (Fig. 5A). In a similar experiment with DnaK$_{Ec}$, the major peak eluted as an about 130 kDa protein. Also, the small shoulder on the ascending portion of the curve, representing oligomers, decreased in magnitude in the presence of ATP (Fig. 5B), as expected from previous knowledge on dissociation of DnaK$_{Ec}$ (Palleros et al., 1993). The elution profile of DnaK$_{Mm}$ preincubated with ATP was identical in the absence (Fig. 5A) and in the presence (not shown) of ATP in the elution buffer, which reflects the stability of the DnaK$_{Mm}$−ATP complex. Dissociation of DnaK$_{Mm}$ in the presence of ATP or NR was confirmed by native electrophoresis (Fig. 5C). The observed dissociation in the presence of ATP and a substrate suggests that the monomeric form of DnaK$_{Mm}$ may be the active form, participating in peptide binding. ADP, in contrast to ATP and NR, did not cause dissociation of the DnaK$_{Mm}$ oligomers (Fig. 5A and 5C, lane 10), which resembled the situation described for DnaK$_{Ec}$ (Palleros et al., 1993).

Protein oligomerization may theoretically lead to a restricted accessibility of certain region(s) of a molecule to a solvent, which could result in a decreased access of the substrate to the protein. Our previous analysis of DnaK$_{Mm}$ by Fourier-transform infrared spectroscopy showed that the $^1$H/$^2$H exchange of the amide hydrogens of the polypeptide chain was less complete than in the case of DnaK$_{Ec}$ (Zmijewski et al., 2007). The extent of the $^1$H/$^2$H exchange can be measured by monitoring the intensity of the residual amide II band (encompassing the 1600–1500 cm$^{-1}$ interval) absorption, i.e., the absorption of the amide II band after the $^1$H/$^2$H exchange. In $^1$H$_2$O medium, the amide II band intensity was about 2/3 of the intensity of the amide I band (not shown). In $^2$H$_2$O medium, the intensity of the amide II band decreases as a consequence of the ex-

Figure 5. Effects of ATP, ADP, and substrate on DnaK oligomerization state.

(A, B) size-exclusion chromatography. DnaK$_{Mm}$ (A) or DnaK$_{Ec}$ (B) was incubated in the presence or absence of ATP, or ADP+Pi, or in the presence or absence of NR peptide (NRLLLTTG), as indicated. The incubated mixtures were resolved by size-exclusion chromatography and the UV spectra at 230 nm of the effluents are shown. For clarity, the spectra for DnaK$_{Ec}$ are displayed at different heights; otherwise, they would be indistinguishable, due to their great similarity. In contrast, the spectra for the various mixtures including DnaK$_{Mm}$ differ considerably from one another and are displayed at the same level. (C) PAGE. DnaK$_{Mm}$ or DnaK$_{Ec}$ was incubated for 30 min in the presence or absence (the latter indicated by a minus sign above the lane) of ATP, ADP+Pi or peptide NR (NRLLLTTG), as shown. The incubated mixtures were analyzed by 10% PAGE under non-denaturing conditions. Lanes 1–4 and 5–10 are from two separate gels in which the controls DnaK$_{Mm}$ and DnaK$_{Ec}$ without additives were repeated (lanes 1, 3, 7, and 8).
change of amide hydrogens with deuterium (Osborne & Nabedryk-Viala, 1982; D’Auria et al., 2004). The larger the decrease in intensity of the amide II band, the larger the $^{1}H/^{2}H$ exchange and, in turn, the greater the accessibility of the solvent ($^{2}H_{2}O$) to the protein. Fig. 6A, B shows the temperature-dependent changes of the DnaK$_{Mm}$ and DnaK$_{Ec}$ infrared absorbance spectra in the 1600–1500 cm$^{-1}$ interval. At 20°C the absorbance of DnaK$_{Mm}$ close to 1550 cm$^{-1}$ is higher than in the DnaK$_{Ec}$ spectrum, indicating a lower accessibility of $^{2}H_{2}O$ to DnaK$_{Mm}$. A temperature increase causes a decrease of absorption close to 1550 cm$^{-1}$ (residual amide II band) due to a further $^{1}H/^{2}H$ exchange, a consequence of temperature-dependent molecular dynamics and/or protein denaturation. A more detailed comparison was possible when the $^{1}H/^{2}H$ exchange as a function of temperature was calculated, Fig. 6C, as done previously (D’Auria et al., 2004). Full $^{1}H/^{2}H$ exchange was reached by DnaK$_{Mm}$ at higher temperatures compared to DnaK$_{Ec}$ and the temperature at which a full exchange occurred can be estimated to be 55–60°C for DnaK$_{Ec}$ and about 65°C for DnaK$_{Mm}$ (Fig. 6C), the temperatures corresponding approximately to the melting points (Tms) previously calculated for DnaK$_{Ec}$ and DnaK$_{Mm}$ (Żmijewski et al., 2007). On the other hand, the maximum rate of exchange (midpoint of the $^{1}H/^{2}H$ exchange curves) was

Table 2. Rates of formation, and dissociation equilibrium constants, of DnaK$_{Mm}$ and DnaK$_{Ec}$ complexes with a-p4 (a-CALLQSRLLS) peptide

<table>
<thead>
<tr>
<th>Protein</th>
<th>$k_{obs}$ (s$^{-1}$)$^{a}$</th>
<th>$k_{+1}$ (M$^{-1}$s$^{-1}$)$^{b}$</th>
<th>$k_{-1}$ (s$^{-1}$)$^{b}$</th>
<th>$K_d$ (µM)$^{c}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>DnaK$_{Mm}$</td>
<td>0.00176 (± 0.0000016)</td>
<td>494 (±56)</td>
<td>0.00118 (± 0.000137)</td>
<td>2.57 (± 0.39)</td>
</tr>
<tr>
<td>DnaK$_{Ec}$</td>
<td>0.00786 (± 0.00001)</td>
<td>3871 (±304)</td>
<td>0.00353 (± 0.0001)</td>
<td>0.91 (± 0.077)</td>
</tr>
</tbody>
</table>

$^{a}$For 200 nM peptide a-p4 and 1 µM DnaK protein. $^{b}$The rate constants $k_{+1}$ and $k_{-1}$ were determined by titration of acrylodan-labeled peptide a-p4 with increasing concentrations of DnaK (see Fig. 1). $^{c}$Dissociation equilibrium constant $K_d$ was calculated from ratio $k_{+1}/k_{-1}$. For details on the determination of $k_{obs}$ and the kinetic constants, see Materials and Methods.

Figure 6. Fourier-transform infrared spectroscopy results. (A, B) Temperature-dependent changes of DnaK$_{Mm}$ (B) and DnaK$_{Ec}$ (A) absorbance spectra in the range encompassing amide II band. The graphs display the absorbance spectra of DnaK$_{Mm}$ and DnaK$_{Ec}$ at 20, 30, 40, 50, 60, 70, 80, and 95°C. The vertical dotted lines indicate position of the peak at 1547 cm$^{-1}$. These spectra were used to calculate the proteins’ $^{1}H/^{2}H$ exchanges, shown in (C) (O) $^{1}H/^{2}H$ exchange curves of DnaK$_{Mm}$ (circles) and DnaK$_{Ec}$ (squares), respectively. The maximum rate of $^{1}H/^{2}H$ exchange (midpoint of the $^{1}H/^{2}H$ exchange curves) was calculated from the curves as described (Meersman et al., 2002). The maximum rate of $^{1}H/^{2}H$ exchange is at 54.8 for DnaK$_{Mm}$ and at 46.3 °C for DnaK$_{Ec}$. All determinations and the calculation of arbitrary units (a.u.) were done as described in Materials and Methods.
with differing affinities (Fourie et al., 1994), a fact which could be attributed to amino-acid substitutions within the DnaK substrate-binding cavity and in the lid region (Mayer et al., 2000). It is possible that different affinity of DnaK<sub>Mm</sub> for unfolded protein substrates could be one of the reasons why it is unable to substitute for DnaK<sub>Ec</sub> in a cell.

DnaK<sub>Mm</sub> interacted poorly, if at all, with <i>E. coli</i> σ<sup>32</sup>, a natural ligand for DnaK<sub>Ec</sub>, and it did not negatively regulate the σ<sup>32</sup> level in <i>E. coli</i> cells (Fig. 3). The observed lack of σ<sup>32</sup> down-regulation is not likely to be the reason why DnaK<sub>Mm</sub> does not suppress the thermosensitivity of the <i>E. coli</i> dnaK mutants. High levels of σ<sup>32</sup> promote transcription of heat shock genes which should help bacterium survive at high temperatures (Yura & Nakahigashi, 1999). However, there may be other native proteins, as yet unidentified, which are substrates for DnaK<sub>Ec</sub>, and whose binding is essential for bacterial survival at elevated temperatures. The failure to recognize such proteins by DnaK<sub>Mm</sub> would result in its inability to suppress the thermosensitivity of the <i>E. coli</i> bacteria without a functional DnaK<sub>Ec</sub>. Mogk and coworkers (1999) showed that DnaK from the Gram-positive bacterium <i>Bacillus subtilis</i>, a protein that is very close to DnaK<sub>Mm</sub> in phylogenetic trees and shows a high percentage of identical amino acids, did not have the ability to cause degradation of σ<sup>32</sup>. They found that the degradation-promoting activity depends on the integrity of a carboxy-terminal DnaK<sub>Ec</sub> region (about 10 kDa, encompassing amino acids 543–637). While the primary sequence of this small region is the least-conserved portion of the DnaK proteins (about 35% identity), the putative three-dimensional structures of this region do not differ appreciably between DnaK<sub>Mm</sub> and DnaK<sub>Ec</sub> (Zmijewski et al., 2007). It remains to be determined which amino acids in the carboxy-terminal region of DnaK<sub>Ec</sub> are directly involved in σ<sup>32</sup> binding and unfolding, the processes that precede degradation of the transcription factor.

Searching for differences in the quaternary structure which might contribute to the differences in substrate binding, we performed size-exclusion chromatography and native gel electrophoresis, and found that DnaK<sub>Mm</sub> occurred in solution mainly as high-molecular-mass oligomeric forms (Fig. 4), and that these forms dissociated upon ATP or peptide binding (Fig. 5). Under the same conditions, DnaK<sub>Ec</sub> formed smaller oligomers, in smaller quantities. It is possible that dissociation of the DnaK<sub>Mm</sub> oligomers is necessary for full activation of the chaperone, as has been postulated for DnaK from the bacterium <i>Thermus thermophilus</i> (Watanabe & Yoshida, 2004). Moreover, it has been shown that the monomeric form of DnaK<sub>Ec</sub> is fully active
(Palleros et al., 1993). Furthermore, the ATP-driven monomerization has been shown to occur for the eukaryotic DnaK homologs Hsp70 and Hsc70, and it has been suggested that the non-aggregated forms exert the chaperone activity (Angelidis et al., 1999). It is possible that formation of highly oligomeric forms by DnaK_Mm decreases the efficiency of substrate binding/releasing and thus may be one of the reasons why DnaK_Mm functioning in E. coli cell is impaired. The FTIR analysis of the 1H/2H exchange of the amide hydrogens of the polypeptide chain indicated that DnaK_Mm is less accessible to the solvent than is DnaK_Ec (Fig. 6). This might be caused by DnaK_Mm oligomerization and could theoretically lead to a decreased access of the substrate to the protein. It can also be speculated that oligomerization of DnaK_Mm may be part of an additional regulatory mechanism of DnaK function in M. mazei, and lack of this regulation in E. coli may contribute to the inefficient functioning of DnaK_Mm in E. coli cells.

It is worth pointing out that this is the first study showing substrate binding by a purified archaeal DnaK and the first attempt to solve the species-specificity of an archaeal Hsp70. Furthermore, this work shows a new aspect of the archaeal DnaK protein, namely its highly oligomeric structure.

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REFERENCES


