# $K^+$ is an indispensable cofactor for GrpE stimulation of ATPase activity of DnaK·DnaJ complex from *Thermus thermophilus*

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**Abstract** **$K^+$ is an indispensable cofactor for ATPase activity of eukaryotic cytosolic Hsp70 chaperone systems which lack a GrpE homolog. In the case of the bacterial Hsp70 (DnaK) system, GrpE, a nucleotide exchange factor, stimulates ATPase activity but little is known about the effect of $K^+$. Here, we have cloned a *grpE* gene from a thermophile, *Thermus thermophilus*, and purified a homodimeric GrpE protein. Using proteins of this bacterium, we found that the GrpE stimulation of ATPase activity of DnaK·DnaJ complex was absolutely dependent on the presence of $K^+$.**



*Key words:* DnaK; DnaJ; GrpE; Heat-shock protein; Hsp70; Molecular chaperone

## 1. Introduction

The ubiquitous and highly conserved Hsp70 proteins are major members of molecular chaperone and are involved in folding and degradation of proteins [1–5]. A key feature of their chaperone function is the ability of Hsp70 to bind non-native protein substrates. Binding and release of substrate proteins are regulated by ATPase activity of Hsp70 which is under tight control with cofactors of Hsp70 [6,7].

In the bacterial Hsp70 chaperone system, the weak ATPase activity of DnaK (Hsp70 homolog) is stimulated by DnaJ (41 kDa), which accelerates the rate of ATP hydrolysis, and by GrpE (22 kDa), which promotes nucleotides exchange of ATP and ADP [8]. DnaJ also possesses a molecular chaperone activity of its own, as revealed by its capacity of binding to denatured proteins to prevent aggregation [9,10]. DnaJ and GrpE are essential for chaperone activity of DnaK in vivo and in vitro and these three proteins constitute the DnaK chaperone system in *Escherichia coli* [3,4,11]. In contrast to bacterial Hsp70 chaperone system, a GrpE homolog has not been found in eukaryotic cytosolic Hsp70 chaperone system [5]. Eukaryotic cytosolic Hsp70 members require $K^+$ for its optimal ATPase activity [12,13], and X-ray crystallography of the ATPase domain of Hsc70, which is a member of eukaryotic Hsp70, shows that Hsc70 has two bound $K^+$ ions in the catalytic site [14]. Whereas the role of $K^+$ for ATPase activity of eukaryotic cytosolic Hsp70 system has been well established, little is known about the effect of $K^+$ on ATPase activity of the bacterial DnaK. Feifel et al. observed $K^+$ stimulation of ATPase of DnaK protein alone [15], but the effect of $K^+$ on ATPase of the DnaK chaperone system has not yet been investigated. We previously reported the isolation of a stable, functional DnaK·DnaJ hexamer complex (DnaK: DnaJ = 3:3) from a thermophilic bacterium, *Thermus thermophilus* [16]. Later, it was found that this complex contained additional three copies of small peptide, DafA [17]. Here, we report cloning of *T.grpE* (*T.* represents *Thermus*) gene from *Thermus thermophilus*, purification of recombinant *T.*GrpE protein which is a homodimer, and effect of $K^+$ on the ATPase activity of the DnaK chaperone system. Only when GrpE and $K^+$ were present simultaneously, but not each alone, ATPase activity of *T.*DnaK·DnaJ complex is stimulated about 4-fold.

## 2. Materials and methods

### 2.1. Expression and purification of T.GrpE

In order to construct *T.*GrpE expression system, pMKJ1 [17] was digested with *Nco*I–*Hin*cII and the fragment was ligated into *Nco*I–*Hin*cII sites of the pET23d. BL21 (DE3) was used for gene expression of thus constructed plasmid, pMGE3, which carried T7 promoter [18]. *T.*GrpE expressed in *E. coli* was purified with the following procedures. *E. coli* cells were suspended in Buffer A (25 mM Tris-HCl, pH 7.5, 3 mM $MgCl_2$), disrupted by a French press (5501-M, Ohtake Works) at 4°C. The disrupt cells were centrifuged at 100 000 × *g* for 40 min at 4°C. The supernatant (crude extract) was applied to a DE-52 cellulose column (Whatman) equilibrated with Buffer A. The column was washed with Buffer A and was eluted with a 0–250 mM linear gradient of NaCl in Buffer A. The peak fractions containing *T.*GrpE were pooled and solid ammonium sulfate was added to 600 mM. The solution was applied to a Butyl-Toyopearl column (Tosoh) equilibrated with Buffer A containing 600 mM ammonium sulfate. The column was washed with the same buffer and was eluted with a 600–200 mM linear reverse gradient of ammonium sulfate. The peak fractions containing *T.*GrpE were pooled and concentrated by ammonium sulfate precipitation. The concentrated solution was applied on a gel filtration column (Sepharose CL-6B, Pharmacia) equilibrated with Buffer A containing 100 mM $Na_2SO_4$. The peak fractions were pooled and stored in 2.8 M ammonium sulfate suspension at 4°C. Before use, *T.*GrpE was heat-treated for 15 min at 70°C to remove highly oligomerized forms of *T.*GrpE which were heat-labile.

### 2.2. Molecular mass estimation of T.GrpE

The purified *T.*GrpE (170 μg) was loaded on a gel filtration HPLC column (TSK G2000SWXL) equilibrated with 50 mM HEPES-NaOH buffer, pH 6.8, containing 100 mM $Na_2SO_4$ and eluted at a flow rate of 0.5 ml/min. Molecular size standards used are ferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa) and ribonuclease A (13.7 kDa). Analytical ultracentrifugation was performed with the analytical centrifuge Optima XL-A (Beckman). Sample was analyzed at 2.8 mg/ml (*T.*GrpE). Sedimentation equilibrium run was done at 14 000 rpm and 20°C with sample volumes of about 100 μl. Equilibrium profiles recorded at 280 nm were analyzed and partial specific volume 0.739, obtained from amino acid composition, was used for calculation. Mass spectrum (time of flight)

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*Abbreviations:* Hsp70, heat-shock protein of 70 kDa; *T.*DnaK·DnaJ complex, DnaK·DnaJ complex of *T. thermophilus*; *T.*GrpE, GrpE of *T. thermophilus*


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analysis was performed with TOF-MS (LASERMAT2000, Finning MAT).

*2.3. Assay of ATPase activity and other methods*

ATPase activities were assayed at 75°C in a 100 μl reaction mixture containing 25 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 150 mM KCl, 1 mM ATP, and 0.6 μM *T.*DnaK·DnaJ complex. In the case of measurement of salt-dependent ATPase activity, KCl and NaCl concentration in reaction mixture were changed as indicated. The reaction was initiated by addition of ATP, and terminated after a 15 min incubation by addition of 25 μl of 20% perchloric acid. As a control experiment (0% ATPase activity), *T.*DnaK·DnaJ complex was injected into the reaction mixture containing perchloric acid. Mixtures were centrifuged at 16000 rpm for 3 min at 4°C and released Pi in the supernatant was measured by malachite green assay [19,20]. Polyacrylamide gel electrophoresis (PAGE) was carried out on 15% polyacrylamide gel in the presence of 0.1% sodium dodecyl sulfate (SDS-PAGE) [21]. Gels were stained with Coomassie Brilliant Blue R-250. Protein concentrations were assayed by the method of Bradford with bovine serum albumin as a standard [22].

## 3. Results and discussion

*3.1. Thermus thermophilus GrpE*

The genes of DnaK chaperone system are located in the gene cluster in most bacteria and in the case of *T. thermophilus*, a *dnaK* gene cluster contains the genes in the order *dnaK–grpE–dnaJ–dafA* from upstream [17]. The *T.grpE* gene encodes 177 amino acid residues, 18 residues shorter (at N-terminus) than *E. coli* GrpE, and predicted molecular mass of *T.*GrpE was 20026 Da. Deduced amino acid sequences of *T.*GrpE are 26% identical to *E. coli* GrpE [23] (Fig. 1). N-terminal region of *E. coli* GrpE is susceptible to protease digestion, presumably a flexible loop, but some function of *E. coli* GrpE lacking N-terminal 33 residues is impaired [24]. Comparison of N-terminal region of GrpE from both bacteria suggests that first 18 residues out of 33 residues of *E. coli* GrpE are dispensable. *T.*GrpE was successfully expressed in a soluble fraction in *E. coli* and purified. The N-terminus of the expressed *T.*GrpE was analyzed by Edman degradation, and the sequence MEERNHEN was obtained. This sequence agreed with the predicted N-terminal amino acid sequence of the *T.grpE* gene (Fig. 1). Although molecular mass of *T.*GrpE monomer was estimated to be 23000 Da from SDS-PAGE, mass spectrography provided a value (20195 ± 31 Da) close to the molecular mass predicted from the gene (Table 1).

Table 1
Molecular mass of *T.*GrE

| Method | Molecular mass (Da) |
|---|---|
| TOF-MS | 20195 |
| SDS-PAGE | 23000 |
| Gel filtration | 180000 |
| Equilibrium centrifugation | 44700 |

To know oligomeric state of intact *T.*GrpE, purified *T.*GrpE was analyzed by gel filtration and sedimentation equilibrium centrifugation. *T.*GrpE was eluted from gel filtration HPLC as a single peak and an apparent molecular mass estimated from its retention time (15.2 min) was 180000 Da. The shape of the protein has serious effect on the retention time of gel filtration chromatography and molecular mass estimation by this method is valid only for globular proteins. We then analyzed *T.*GrpE by sedimentation equilibrium centrifugation by which determination of the molecular mass is possible without influence of shape of proteins and without reference of molecular mass standards. The value 44700 Da was obtained by this method (Table 1). From comparison of molecular masses of monomer and intact GrpE molecule, we conclude that intact *T.*GrpE is a homodimer with an elongated molecular shape. Behaviors of *E. coli* GrpE in analysis by gel filtration chromatography and sedimentation equilibrium [25] were very similar to those of *T.*GrpE and indeed the elongated homodimer structure of *E. coli* GrpE was recently proved by X-ray crystallography [24].

*3.2. Effects of T.GrpE for ATPase activity of T.DnaK·DnaJ complex*

*T.*DnaK·DnaJ complex exhibits a weak steady-state ATPase activity [16,17]. Similar to the *E. coli* DnaK system [8], this activity was stimulated by *T.*GrpE up to 4-fold (Fig. 2A). The extent of stimulation increased as the amount of added GrpE increased and was saturated when about 3 mol *T.*GrpE (counted as a dimer) per mol of *T.*DnaK·DnaJ complex was added. Because *T.*DnaK·DnaJ complex contains three mole-

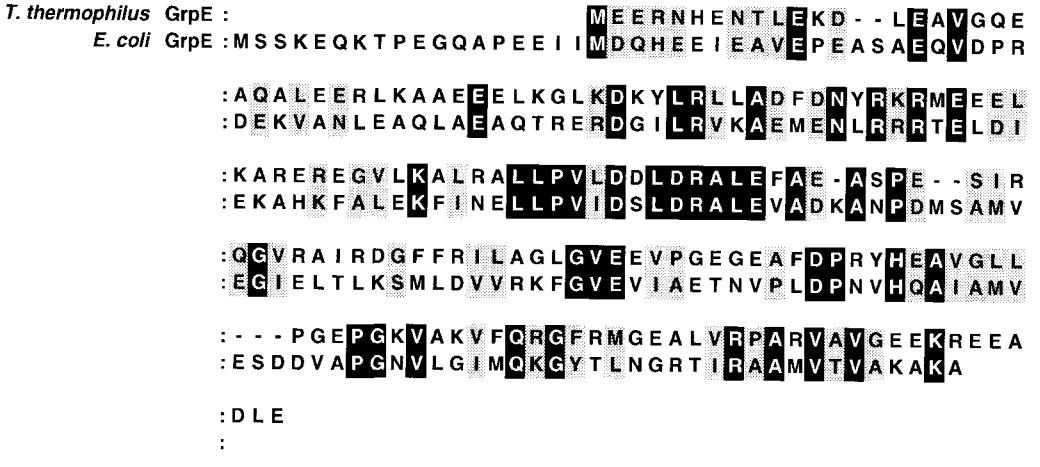


Fig. 1. Alignment of amino acid sequences of *T.*GrpE and *E. coli* GrpE. Identical amino acid residues are shown by *white letters* on *black background*, and similar ones are shown by *letters* on *shaded background*. Similar residues are grouped as follows: A, G, P, S, T; L, I, V, M; D, E, N, Q; K, R, H; F, Y, W; and C.

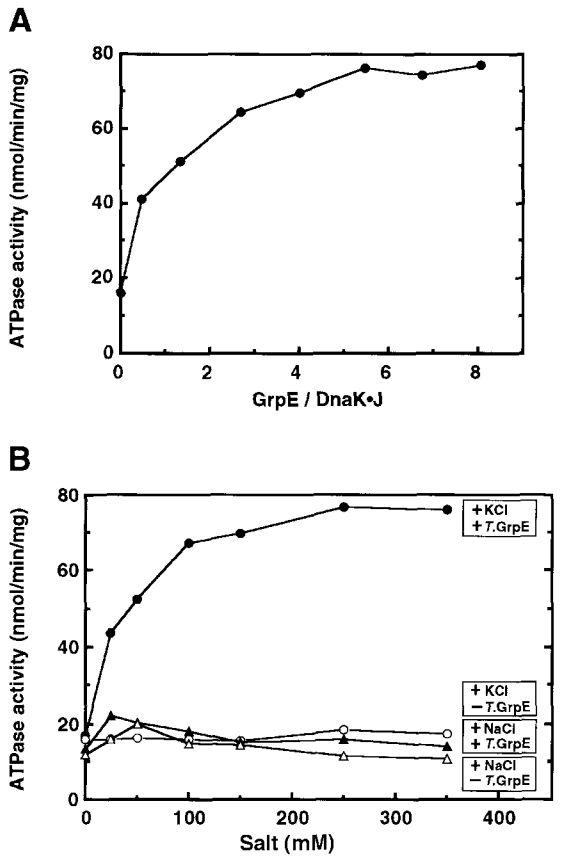


Fig. 2. Effects of *T*.GrpE on ATPase activity of *T*.DnaK·DnaJ complex. A: Titration of GrpE stimulation of ATPase activity of *T*.DnaK·DnaJ complex. The assay solution contained 150 mM KCl. GrpE/DnaK·DnaJ represents molar ratio of *T*.GrpE (as a monomer) to *T*.DnaK·DnaJ complex (as a complex). *B*, Effect of *T*.GrpE and $K^+$ on the ATPase activity of *T*.DnaK·DnaJ complex. ATPase activity of *T*.DnaK·DnaJ complex was measured in the presence of varying amount of KCl with (●) and without (○) *T*.GrpE, and in the presence of varying amount of NaCl with (▲) and without (△) *T*.GrpE. Other experimental details are described in Section 2.

cules of DnaK, the above result indicates that a homodimer GrpE interacts with a DnaK molecule in the complex. It is consistent with the result of X-ray crystallography of GrpE–DnaK complex in which a homodimer GrpE associates a single DnaK (ATPase domain) [24].

We carried out ATPase assay of the experiment of Fig. 2A in the presence of 150 mM KCl. Stimulation of ATPase activity of the DnaK chaperone system by GrpE has been always measured in the presence $K^+$ at considerable concentrations [8] and the study focused to the effect of $K^+$ on the function of GrpE has not been reported yet. Then we examined the effect of $K^+$ and found that stimulation of ATPase activity of *T*.DnaK·DnaJ complex by *T*.GrpE was absolutely dependent on the presence of $K^+$ in the assay solution (Fig. 2B). In other words, simultaneous presence of *T*.GrpE and $K^+$ is required and neither *T*.GrpE nor $K^+$ alone is effective. NaCl cannot substitute KCl at all, indicating specific interaction of $K^+$ ion(s) with the component(s) of *T*.DnaK·DnaJ complex. Half maximum concentration of $K^+$ for the stimulation was 40 mM and the effect was almost saturated at 150 mM KCl, which is a concentration in the physiological range [26].

Our observation is in contrast to the previous report by Feifel et al. that ATPase activity of *E. coli* DnaK was stimulated by $K^+$ alone [15]. This apparent discrepancy is attributable to the difference of the state of DnaK; they tested ATPase of DnaK alone in the absence of DnaJ and GrpE and we measured the DnaK chaperone system.

For cytosolic Hsp70 proteins in eukaryotes, $K^+$ has been known as an indispensable cofactor for optimal ATPase activity [12–14] and the X-ray crystallographic study of ATPase domain of Hsc70 provided its structural base, that is, there are two $K^+$ ions at the nucleotide binding cleft interacting with MgADP and Pi [14]. Although $K^+$ is an indispensable cofactor for optimum ATPases of both the DnaK system and eukaryotic cytosol Hsp70 members, the manner of action of $K^+$ on bacterial DnaK system may not be simply analogous, even if part of the mechanism could be common, to that of the eukaryotic cytosolic Hsp70 system because a GrpE homolog has not yet been found in the eukaryotic cytosol. The absence of a GrpE homolog in eukaryotic cytosol may imply that the cytosolic Hsp70 system does not require a nucleotide exchange factor because the rate-limiting step in its ATPase cycle is not the dissociation of ADP but rather the hydrolysis of ATP itself [13,27]. Recent investigations of Hsp70 chaperone system in various organisms show a diversity of components in this chaperone system [5]. For example, Höhfeld et al. found Hip, a stabilizer of Hsp70–nucleotide complex [28]. Effects of $K^+$ on the ATPase activity represents one of aspects of the diversity of Hsp70 systems of various organisms and further analysis of the step affected by $K^+$ will help clarify the conservation and diversion of the regulatory mechanism of Hsp70 system.

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