

DnaK ATPase activity revisited

Daniel R. Palleros, Katherine L. Reid, Li Shi, Anthony L. Fink*

Department of Chemistry and Biochemistry, University of California, Santa Cruz, CA 95064, USA

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It has recently been reported that the ATPase activity of DnaK, a 70 kDa heat shock protein from *E. coli*, is autostimulated by increasing protein concentration [(1993) FEBS Lett. 322, 277–279], suggesting that the DnaK dimer may be the enzymatically active species. In this paper we investigated the ATPase activity of different DnaK preparations; we found that the turnover number was very dependent on protein purification. With HPLC-purified DnaK we found a turnover number 20- to 50-fold lower than typical values previously published and no evidence of autostimulation, indicating that the monomer is the active species.

DnaK; hsp70; Molecular chaperone; ATPase; Oligomer

1. INTRODUCTION

Members of the 70 kDa family of heat-shock proteins, hsp70, play a complex housekeeping role in stressed as well as normal cells (reviewed in [1–3]). They have been shown to interact with unfolded proteins [4–9], nascent polypeptide chains [10,11], and short peptides [12,13]. The release of substrate proteins from hsp70 is triggered by Mg-ATP [4,5,10,12,14]. We have recently shown that ATP binding *in vitro*, in the presence of potassium ions, causes a conformational change in DnaK probably linked to substrate–protein dissociation; ATP is hydrolyzed subsequent to substrate–protein dissociation [15].

The hsp70 family has been conserved throughout evolution. Its members possess two functional domains: a C-terminal domain responsible for binding to substrate proteins [16] and a highly conserved N-terminal domain which contains the nucleotide binding site. The three-dimensional structure of the N-terminal domain of bovine brain hsp73 (also known as hsc70, a constitutive form of hsp70) is known at 2.2 Å resolution [17]. All members of the hsp70 family bind ATP very tightly [18, 19] and have a very low intrinsic ATPase activity [20–22]. It has been shown that the ATPase activity of hsp70 family members is increased 3- to 5-fold by the

presence of peptides [12] and unfolded proteins [15,20,22].

Most hsp70s have been shown to exist as monomers, dimers and trimers in a concentration-dependent equilibrium, a process which is strongly dependent on the presence of nucleotides. Mg-ATP, but not Mg-ADP, dissociates dimers and trimers to monomers [4,7,22–25]. Recent studies of the intrinsic ATPase of DnaK showed a curvilinear dependence with DnaK concentration; this behavior was interpreted as autostimulation of ATPase activity by self-association [26]. As reported here, we have carefully investigated the ATPase activity of DnaK and find no evidence of autostimulation. However, the intrinsic ATPase activity is strongly dependent on protein purification; the turnover number reported here is at least 20- to 50-times lower than the values reported by other laboratories [26,27] due to removal of contaminants which possess higher intrinsic ATPase activity or stimulate DnaK ATPase activity.

2. MATERIALS AND METHODS

2.1. Materials

[2,8-³H]ATP, tetrasodium salt, 1 μCi/μl, 34 μM, in ethanol/water 1:1 was from NEN. Liquid scintillation cocktail was Bio-Safe II from Research Products International Corp. PEI-cellulose F TLC plates were from Merck. Bovine serum albumin (BSA, crystalline 99%) was from Calbiochem. Heparin–Sephacrose was from Pharmacia; ATP-agarose was from Sigma. All other chemicals were from the same sources as reported elsewhere [4,7].

2.2. DnaK preparation

Escherichia coli DnaK was isolated and purified by a modification of procedures previously described [28,29]. We added an ammonium sulfate precipitation step and used a Phenomenex Bio-Sep 3000 size exclusion column (SEC) as a final purification step before ATPase activity experiments. JM1174 bacteria containing the pJM6 plasmid [28] (in which the DnaJ gene was deleted) were grown in two 250 ml flasks containing 50 ml of media at 30°C overnight. The flasks were

*Corresponding author. Fax: (1) (408) 459-2935.

Abbreviations: BSA, bovine serum albumin; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HMWS, high molecular weight species; HPLC, high performance liquid chromatography; hsp70, 70 kDa heat-shock proteins; PAGE, polyacrylamide gel electrophoresis; RCMLA, reduced carboxymethylated α-lactalbumin; SDS, sodium dodecyl sulfate; SEC, size exclusion chromatography; TLC, thin-layer chromatography.

used to inoculate 2 l of media containing 100 µg/ml of carbenicillin. The culture was allowed to incubate for 5 h (late log phase O.D. ≈ 2) at 30°C. The cells were then induced for 3 h by shifting the temperature to 37°C and by adding 1 mM iso-propyl-β-D-thiogalactopyranoside. The cells were then centrifuged at 8,000 rpm for 20 min at 4°C. The resulting pellet was stored at -40°C overnight. The frozen pellet was allowed to thaw slowly on ice with the addition of 80 ml of 10% (w/v) sucrose in 50 mM Tris-HCl (pH 8); 20 ml of buffer K (180 mM spermidine-HCl, 50 mM DTT, 50 mM EDTA, and 0.9 M ammonium sulfate) containing 2 mg/ml of lysozyme (freshly prepared solution) were added. After the pellet thawed, the mixture was incubated at 37°C for 4 min, returned on ice for 10 min, and centrifuged at 30,000 rpm for 30 min at 2°C in a 50.2 Ti Beckman rotor. The supernatant was collected and slowly precipitated at 4°C with ammonium sulfate at 65% saturation. The mixture was centrifuged in a 50.2 Ti Beckman rotor at 20,000 rpm for 30 min at 2°C. The pellet was resuspended in 10 ml of buffer A (25 mM imidazole, pH 7.0, 10% sucrose, 25 mM NaCl, 5 mM MgCl₂, 5 mM β-mercaptoethanol). After resuspension, the protein solution was dialyzed (12–14,000 MW cut-off membrane) overnight at 4°C against 1 l of the same buffer A. The protein solution was passed through a heparin-Sepharose column (2 ml) previously washed with buffer A. The flow-through was then passed over an ATP-agarose column (≈ 2.5 ml) that had been equilibrated with 10 vols. of buffer A. The column was washed with 10 vols. of buffer A containing 500 mM NaCl. The protein was eluted with 5 column vols. of buffer Q (25 mM HEPES-KOH, pH 7.6, 50 mM KCl, 1 mM EDTA, 5 mM β-mercaptoethanol, 10% glycerol) containing 5 mM ATP. The eluted protein was collected in 1 ml fractions and the purity of each fraction was determined by SDS-PAGE. The flow-through of the first application was saved and loaded again after the column was regenerated with 10 vols. of buffer A. To the collected fractions from the ATP-agarose column, 0.1 vols. of 150 mM EDTA in 100 mM Tris-HCl, pH 7, was added and the solution incubated at 4°C for 0.5–1 h. DnaK was precipitated by slowly adding solid ammonium sulfate (70% saturation), spun down at 17,000 rpm (Sorvall SS-34 rotor) for 1 h, at 4°C. The pellet was resuspended in about 7 ml of buffer T (100 mM Tris-HCl, 5 mM EDTA, 5 mM β-mercaptoethanol, pH 7.2) and extensively dialyzed against the same buffer (4 × 1 l) and then against the same buffer without EDTA (3 × 1 l). Protein concentration was about 20 µM (molar extinction coefficient 27,000 M⁻¹ · cm⁻¹ [30]). The ratio of the absorbance at 280 and 260 nm (A_{280}/A_{260}) was 1.3–1.4; total amount of protein was about 30 mg. Protein solution was stored at -70°C. For ATPase activity experiments DnaK was further purified (see section 3.1.).

2.3. DnaK analysis

Size-exclusion HPLC was carried out as previously described [7,30] using a Beckman instrument. Columns were Bio-Sil SEC-250 (600 × 7.8 mm; from Bio-Rad) or Bio-Sep 3000 (600 × 7.8 mm; from Phenomenex) as indicated. Flow rate was 1 ml/min; detection was by absorbance at 215 nm. Unless otherwise indicated the HPLC mobile phase was 20 mM sodium phosphate, 200 mM potassium chloride, pH 6.5. The Bio-Sil SEC-250 column (molecular weight cut-off 300 kDa) was calibrated using the following standards: blue dextran (void volume) 10.15 ml; BSA dimer, 13.14 ml; BSA monomer, 15.44 ml; ovalbumin, 16.8 ml; and sodium azide (total solvent-accessible volume), 24.12 ml. SDS and native-PAGE were run on a Pharmacia Phast system using 8–25% polyacrylamide-gradient gels and Coomassie blue staining. Densitometry was performed using an ISCO gel scanner (ISCO model 1312) coupled to an absorbance monitor (ISCO model UA5) and integrator (Spectraphysics, model 4270).

2.4. ATPase activity

Aliquots of DnaK (6.5 µl; 0.38–3.8 µM) in 20 mM Tris-HCl, 200 mM NaCl, pH 6.5, were mixed with buffer M (12.0 µl; 20 mM Tris-HCl, 19.0 mM NaCl, 0.58 M KCl, 9.3 mM MgCl₂, pH 7.2), a solution of BSA (8 µl; 16 mg/ml in 20 mM Tris-HCl, pH 7.2), a stock solution of Mg-ATP (16.0 µl; 62.5 µM Na₂-ATP in 20 mM Tris-HCl, 20 mM NaCl, 10 mM MgCl₂, pH 7.2) and [2,8-³H]ATP (1 µl; 34 µM in

ethanol/water 1:1, 1 µCi/µl). The reaction mixture (50 µl) was incubated at 37.0 ± 0.5°C or 22.0 ± 0.5°C, aliquots (2 µl) were taken at different times and applied on cellulose TLC plates spotted with ATP, ADP and AMP markers. Plates were developed with an aqueous solution containing 0.7 M LiCl and 1 M formic acid. The spots for ATP, ADP and AMP, respectively, were scraped into 10 ml of scintillation cocktail and the radioactivity counted using a Beckman LS-230 instrument. For each time-point the concentration of ADP was calculated by multiplying the initial ATP concentration (20.7 µM) by the ratio of the radioactivity associated with the ADP spot with respect to the total radioactivity in the lane (ADP + ATP). No AMP was formed over the course of the reaction. The V_{max} reported in Fig. 4A are the slopes of the linear correlations of [ADP] vs. time. Plots were linear in the length of time investigated (10–30% reaction); at least 12 points were taken.

3. RESULTS

3.1. DnaK purification

SDS-PAGE analysis followed by densitometry showed that DnaK purity before SEC-HPLC purification was higher than 97% (Fig. 1A). This level of purity is enough for most practical applications, however it was found unsatisfactory for the determination of ATPase activity (see below). DnaK was further purified by SEC-HPLC using a Bio-Sep 3000 column and 20 mM Tris-HCl, 200 mM NaCl, pH 6.5, as the mobile phase. When a 200 µl sample (48 µM) was injected into the HPLC, peaks for monomeric and oligomeric DnaK were detected with the monomer accounting for 50% of the area. The peak for the monomer was collected and concentrated to a final volume of 500 µl and a concentration of about 10 µM and re-injected; it afforded peaks for monomer (70%) and oligomers indicating re-association of DnaK. The monomer peak was collected and concentrated to a final concentration of 3.8 µM. Re-injection into the HPLC (20 µl) indicated the presence of a single peak (Fig. 1B, trace b). Under the same conditions (concentration and volume of injection) DnaK before HPLC purification showed peaks at higher molecular weight accounting for about 10% of the total area (Fig. 1B, trace a). The area under these peaks diminished to less than 3% when DnaK (3.8 µM) was co-injected with an excess of Mg-ATP (20–1,000 µM; data not shown).

3.2. Oligomeric forms of DnaK

The oligomeric state of DnaK was analyzed by non-denaturing gel electrophoresis as a function of the protein concentration. The results of the native gels are shown in Fig. 2. As can be observed, the amount of oligomeric forms (dimer, trimer, tetramer) present is highly dependent on the total DnaK concentration. At a concentration of 10 µM the oligomers account for 30–40% of the area (Fig. 2a). Below 3 µM (Fig. 2b), DnaK is mainly present as monomers. DnaK (10 µM) was also analyzed by SEC-HPLC with and without nucleotides in the running buffer; Fig. 3a, shows peaks for oligomeric species which correspond to about 20% of

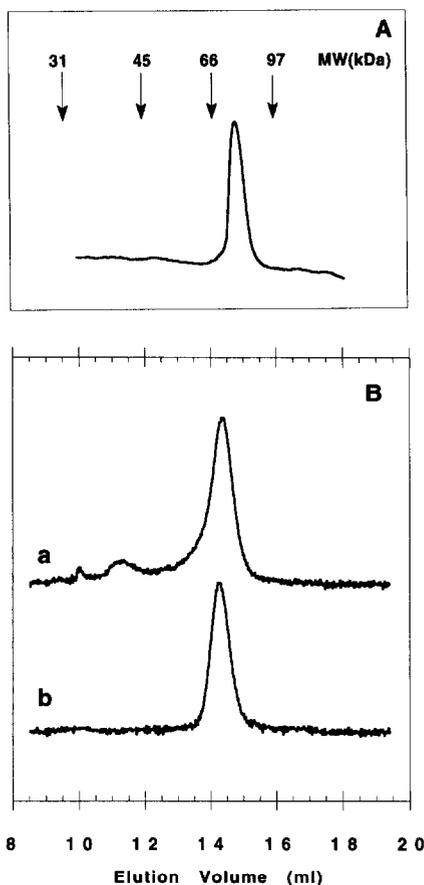


Fig. 1. Study of DnaK purity. (A) Densitometry trace of SDS-PAGE; protein concentration, $10 \mu\text{M}$; volume loaded, $1 \mu\text{l}$. Arrows indicate the position of molecular weight standards. (B) SEC-HPLC traces; protein concentration, $3.8 \mu\text{M}$; HPLC column, Bio-Sil SEC-250; volume injected, $20 \mu\text{l}$. Trace: a, DnaK before HPLC purification; b, HPLC-purified DnaK.

the area. Oligomers were dissociated in the presence of Mg-ATP (Fig. 3b) but not in the presence of Mg-ADP (Fig. 3c) which, in fact, seemed to favor their formation.

3.3. ATPase activity

DnaK ATPase activity was studied at 37°C as a function of protein concentration in the range $[\text{DnaK}] = 0.05\text{--}1.00 \mu\text{M}$, and $[\text{ATP}] = 20.7 \mu\text{M}$. To avoid possible losses of protein (especially at very low protein concentrations) due to adsorption to the plastic reaction vessel walls, a carrier protein, BSA, at a concentration of about 2.5 mg/ml was used in the experiments. In separate controls, determined at $[\text{DnaK}] = 0.5 \mu\text{M}$ (data not shown), it was found that BSA has no effect on DnaK ATPase activity. The results of DnaK ATPase activity ($[\text{ADP}]$ produced ($\mu\text{M}/\text{min}$) as a function of DnaK concentration, using HPLC-purified DnaK, are shown in Fig. 4A. As can be observed, ATPase activity (V_{max}) increases linearly with $[\text{DnaK}]$, as expected for a monomeric active enzyme; that these values truly represent V_{max} was tested at $[\text{DnaK}] = 0.5 \mu\text{M}$ and different ATP concentrations.

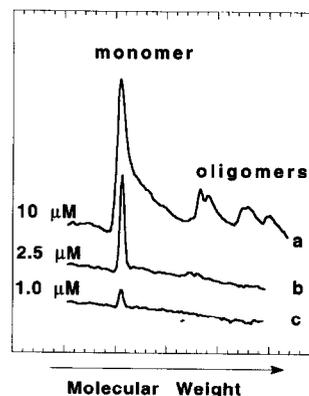


Fig. 2. Association state of DnaK. Densitometry traces of native-PAGE at $[\text{DnaK}]$ indicated in the figure; $1 \mu\text{l}$ samples at the concentrations indicated in the figure (in 100 mM Tris-HCl, 5 mM β -mercaptoethanol, $\text{pH } 7.2$) were applied to 8–25% gradient gels.

The initial velocity was independent of the nucleotide concentration in the range $[\text{ATP}] = 10\text{--}100 \mu\text{M}$, indicating enzyme saturation (data not shown; K_d for DnaK and Mg-ATP is estimated as $1.5 \mu\text{M}$; Shi and Fink, unpublished results). The turnover number is $0.087 \pm 0.007 \text{ min}^{-1}$, at 37°C and $\text{pH } 7.2$. On the other hand, the turnover number for DnaK before HPLC purification was $0.42 \pm 0.02 \text{ min}^{-1}$ at 37°C as shown in Fig. 4B, which also includes the kinetics, under the same conditions, using HPLC-purified DnaK. The ATPase activity of HPLC-purified DnaK was also studied at 22°C (at $[\text{DnaK}] = 0.5$ and $3.25 \mu\text{M}$; and $[\text{ATP}] = 20.7 \mu\text{M}$), the turnover number was $0.016 \pm 0.04 \text{ min}^{-1}$, and independent of protein concentration.

4. DISCUSSION

The results shown in Fig. 2 indicate that the associa-

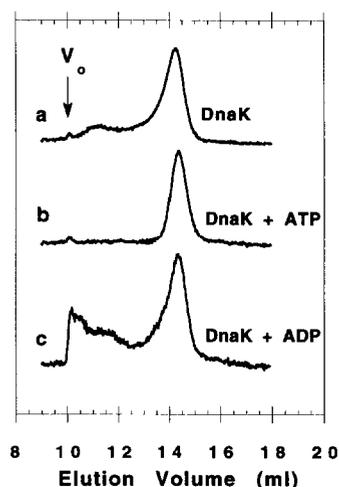


Fig. 3. SEC-HPLC analysis of DnaK. In all cases protein concentration was $10 \mu\text{M}$; $20 \mu\text{l}$ were injected into a Bio-Sil SEC-250 column; V_0 indicates void volume (blue dextran; see text). Trace: a, mobile phase was 20 mM sodium phosphate, 200 mM KCl, $\text{pH } 6.5$; b, mobile phase as in a + $100 \mu\text{M}$ Mg-ATP; c, mobile phase as in a + $100 \mu\text{M}$ Mg-ADP.

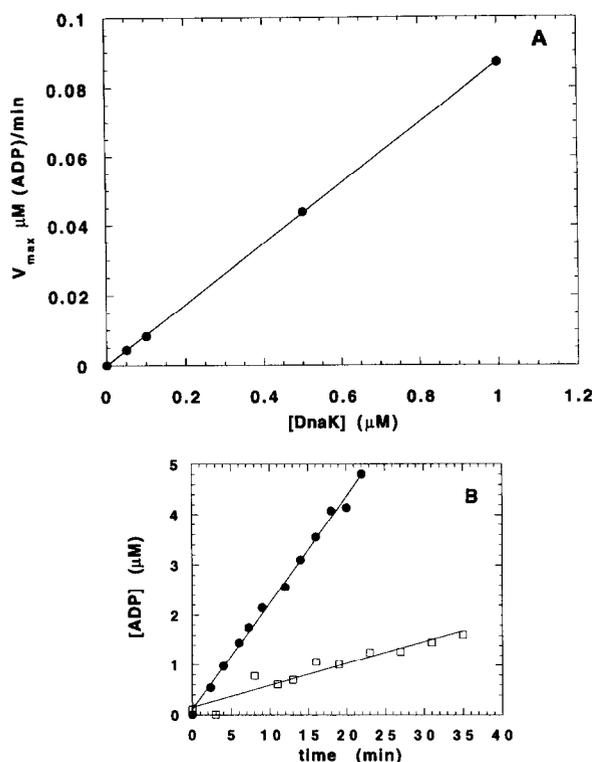


Fig. 4. Intrinsic ATPase activity of DnaK at 37°C. (A) ADP (μM) produced per min as a function of DnaK concentration; HPLC-purified DnaK was used; $[\text{ATP}] = 20.7 \mu\text{M}$, pH 7.2. (B) ADP (μM) produced as a function of time using: (●) DnaK before HPLC purification (see text) and (□) HPLC-purified DnaK; protein concentration was $0.5 \mu\text{M}$; $[\text{ATP}] = 20.7 \mu\text{M}$; pH 7.2.

tion state of DnaK is highly dependent on protein concentration, as expected for an equilibrium process. When samples of DnaK of similar concentrations were analyzed by native gels and SEC-HPLC, smaller amounts of oligomers were detected by SEC-HPLC due to a larger dilution factor involved in this technique as compared to native gels. This explains why only about 20% of oligomers are observed on the HPLC analysis (Fig. 3a) as compared to 30–40% observed for the same nominal concentration ($10 \mu\text{M}$) by native gels (Fig. 2a). As explained below, these oligomers are not exclusively dimers and trimers of native DnaK. According to Fig. 2, below $1 \mu\text{M}$, the concentration range used in the ATPase activity experiments, DnaK is mainly present as monomers. The HPLC analysis (Fig. 3b and c) indicates that Mg-ATP, but not Mg-ADP, dissociates the oligomers; $20 \mu\text{M}$ Mg-ATP, the concentration used in the ATPase experiments, was enough to cause complete dissociation (not shown). Taken together, these results clearly indicate that DnaK at the submicromolar level and in the presence of Mg-ATP exists exclusively as monomers.

The results presented in Fig. 1B, trace a, show that before HPLC purification, the DnaK sample contained high molecular weight species, HMWS, (about 10% of total area); on the other hand, SDS-PAGE analysis

showed that more than 97% of the preparation migrated as a 70 kDa polypeptide (Fig. 1A). This implies that no more than 3% of these HMWS could be due to foreign proteins and the remainder must be DnaK; this rules out the possibility that the HMWS are mainly complexes of DnaK with other proteins. Most of these HMWS are likely to be complexes of native DnaK with denatured DnaK rather than just oligomers of native DnaK, as the following fact indicates: HPLC-purified DnaK does not show HMWS. Under the same conditions (concentration, temperature and injected volume) HPLC analysis of highly pure DnaK showed a single peak (Fig. 1B, trace b). This indicates that at this level of concentration the HMWS are not just oligomers of native DnaK. If this were the case HPLC-purified DnaK would reassociate and show oligomer peaks. This was confirmed by native-PAGE analysis; HPLC-purified DnaK at a concentration of $6 \mu\text{M}$ showed oligomer bands (data not shown). Fig. 3b shows that, like most hsp70-substrate protein complexes studied [4,5,10,12,14], the HMWS dissociate in the presence of Mg-ATP (except for a small fraction, see below). This eliminates the possibility that the HMWS are aggregates of denatured DnaK. We have previously observed that the soluble aggregates of thermally denatured bovine brain hsp73 are not dissociated by Mg-ATP [7].

Some of the HMWS are resistant to the addition of Mg-ATP (Fig. 3b), i.e. about 3% remain after addition of Mg-ATP as very high molecular weight aggregates, therefore at least part of them must be due to species other than DnaK-substrate protein complexes since, as indicated above, these complexes are expected to dissociate completely after addition of Mg-ATP. Probable candidates are aggregates of denatured DnaK and/or GroEL, a bacterial heat-shock protein of MW 800 kDa which binds tightly to ATP-agarose columns, a crucial step in the purification of DnaK.

The turnover numbers for HPLC-purified DnaK reported in this paper, 0.087 ± 0.007 (37°C , pH 7.2) and 0.016 ± 0.004 (22°C , pH 7.2), are lower than previously published values. Liberek et al. reported 0.25 min^{-1} at 30°C [27], a 3-fold larger figure without considering the temperature effect. Richarme and Kohiyama [26] found that the turnover number for the intrinsic DnaK ATPase activity was dependent on the protein concentration; their reported intrinsic activity (at 20°C) was in the range $0.28\text{--}0.90 \text{ min}^{-1}$ for $[\text{DnaK}] = 0.1\text{--}0.7 \mu\text{M}$. As Fig. 4A indicates, our results do not support such findings; our intrinsic ATPase activity was 20- to 50-times lower and independent of DnaK concentration in the range $0\text{--}1.00 \mu\text{M}$ (a range similar to that studied by Richarme and Kohiyama [26]), as expected for a monomeric active protein. However, the intrinsic activity of DnaK prior to HPLC purification at 37°C was $0.43 \pm 0.02 \text{ min}^{-1}$, Fig. 4B, a value very close to those reported by other laboratories [26,27]. Our turnover numbers are comparable with those reported by Mc-

Carty and Walker [28] (0.06 min^{-1} (30°C , pH 8.1) and 0.15 min^{-1} (37°C , pH 8.1)), who used high resolution SEC and ion-exchange chromatography as final purification steps. This indicates that the intrinsic ATPase activity of DnaK is very sensitive to the method of protein preparation and its final purity. Most standard techniques to assess purity, such as SDS-PAGE and HPLC, may show small amounts of contaminating proteins (less than 3%), however, this amount could be enough to give an erroneous measurement of DnaK ATPase activity. The 5-fold decrease in our intrinsic ATPase activity after purification by HPLC indicates that DnaK was contaminated with other ATPases of much higher specific activity (a likely candidate is GroEL [31]), and/or polypeptides that stimulated its own ATPase activity, including denatured DnaK, which were detected as HMWS. The possibility that the lower turnover number reported here is due to denaturation of DnaK can be ruled out based on the HPLC of Fig. 1B, trace b, which shows no evidence of denatured DnaK. Our previous investigations on the stability of hsp70 indicate that denatured forms of DnaK elute earlier from the HPLC column [7,30]; also, no evidence of denaturation was observed by circular dichroism and fluorescence analyses (not shown). We previously studied the protein-stimulated ATPase of DnaK in the presence of RCMLA (using HPLC-purified DnaK at 0.5 and $2.4 \mu\text{M}$ [15]); the turnover number, $0.24 \pm 0.02 \text{ min}^{-1}$ at 37°C , was independent of DnaK concentration. Thus, RCMLA, a permanently unfolded form of α -lactalbumin, caused a 3-fold stimulation in the ATPase activity of DnaK.

The results presented here indicate that DnaK under the conditions of the ATPase activity experiments ($[\text{DnaK}] \leq 1 \mu\text{M}$; $[\text{ATP}] = 20.7 \mu\text{M}$) exists mainly as a monomer. The lack of autostimulation of its ATPase activity support the idea that the monomer is the enzymatically active species. The observation by Richarme and Kohiyama of autostimulation of the intrinsic ATPase activity coupled with the detection of dimeric forms of DnaK suggested that DnaK dimers were the enzymatically active species [26]. It was also observed that the addition of unfolded bovine pancreatic trypsin inhibitor resulted in lack of DnaK autostimulation, suggesting that DnaK self-association involves its peptide binding site [26]. On the basis of the results presented in this paper, it is likely that the autostimulation observed by Richarme and Kohiyama was due to contamination with denatured DnaK rather than a true autostimulation; their gel permeation experiment (Fig. 3 in [26]) indicates that their DnaK preparation was not homogeneous.

The intrinsic ATPase activity reported here is comparable with values found for other hsp70 family members. Bovine brain hsc70 has a turnover number of 0.075 min^{-1} (based on monomers) at 37°C [20]; recombinant rat hsc70 showed an ATPase activity of 0.12

min^{-1} (37°C) [21]. BiP or Grp78, an hsp70 present in the endoplasmic reticulum, displays intrinsic ATPase activity in the range 0.031 – 0.10 min^{-1} (37°C), depending on the protein source [22].

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