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Autostimulation of the DnaK (HSP 70) ATPase of *Escherichia coli*

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The ATPase activity of DnaK, the 70-kDa chaperone of *Escherichia coli*, is stimulated by an unfolded protein. However, the stimulation of the DnaK ATPase by unfolded bovine pancreatic trypsin inhibitor can only be observed at low DnaK protein concentrations. At higher DnaK concentrations, the ATPase activity of DnaK cannot be stimulated by the addition of unfolded bovine pancreatic trypsin inhibitor. This is a consequence of the autostimulation of the DnaK ATPase at higher DnaK concentrations. The autostimulation of DnaK is reflected by a non-linear dependence of ATP hydrolysis on DnaK concentration. Furthermore, DnaK exists as a mixture of monomers and dimers in equilibrium, and the dimers dissociate into monomers in the presence of ATP.

Heat shock protein; Chaperone; Peptide-dependent ATPase; Dimerisation

1. INTRODUCTION

Several proteins referred to as chaperones are involved in: (a) facilitating other polypeptides in maintaining the unfolded state, thus enabling their correct transmembrane targeting, intracellular folding or oligomeric assembly; (b) removal of denatured proteins after stress; and (c) disassembly of several protein complexes (reviewed in [1–3]). One class of these chaperones is the 70 kDa heat shock protein (hsp70), which comprises the DnaK protein of *Escherichia coli* [4]. Members of this family can distinguish native proteins from their non-native forms [4,5] due to the specificity of their peptide binding site. They bind reversibly unfolded proteins (and peptides) with concomitant ATP hydrolysis, and display a peptide-dependent ATPase activity [4–7]. We show in this study that the stimulation of the DnaK ATPase activity by unfolded bovine pancreatic trypsin inhibitor is apparent only if DnaK is assayed at a low DnaK protein concentration (less than 0.1 μM). At higher DnaK concentrations, unfolded bovine pancreatic trypsin inhibitor does not appear to stimulate significantly the ATPase activity of DnaK. This results from an autostimulation of the DnaK ATPase. This autostimulation is reflected by a non-linear dependence of ATP hydrolysis on DnaK concentration. Furthermore, DnaK exists as a mixture of monomers and dimers in equilibrium, and the dimers dissociate into monomers in the presence of ATP.

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2. MATERIALS AND METHODS

2.1. *ATPase activity*

1 μl of purified DnaK in 50 mM Tris-hydrochloride, pH 7.4, 50 mM KCl, 0.06 M sodium phosphate, 5 mM 2-mercaptoethanol, was incubated for 1 h at 20°C with 1 μl of 100 μM [^{3}H]ATP (1.5 Ci/mmol) containing 300 μM $MgCl_2$, and 1 μl of unfolded BPTI or buffer (Tris-hydrochloride, pH 7.4) as indicated. The reaction (linear as a function of time) was terminated by applying 2 μl of sample to polyethyleneimine cellulose thin-layer chromatography plates that had been spotted with carrier nucleotide as described in [6].

2.2. *Purification of DnaK*

DnaK was purified as described in [8], and further chromatographed on a hydroxylapatite column (Bio-Gel HTP from Bio-Rad Laboratories) equilibrated in 50 mM Tris-hydrochloride, pH 7.4, 50 mM KCl, 5 mM 2-mercaptoethanol. It was eluted (at 0.06 M sodium phosphate) with a linear gradient of 0–0.2 M sodium phosphate, pH 7.4, in the same buffer. DnaK was more than 99% pure, as judged by electrophoresis in sodium dodecyl sulfate polyacrylamide gel. ^{125}I-labelled DnaK was prepared by the chloramine-T method as described in [9]. The ^{125}I-labelled DnaK was separated from [^{125}I]iodide by chromatography on a P-2 gel permeation column equilibrated in buffer A (50 mM Tris-hydrochloride, pH 7.4, 50 mM KCl, 5 mM 2-mercaptoethanol, 0.06 M sodium phosphate) (Bio-Rad Laboratories).

2.3. *Preparation of unfolded BPTI*

Unfolded BPTI was prepared as described in [10]. It was separated from iodoacetamide and urea by chromatography on a Bio-Gel P-2 gel permeation column (from Bio-Rad Laboratories) equilibrated in 50 mM Tris-hydrochloride, pH 7.4.

2.4. *Protein determination*

Protein was determined by the method of Bradford [11].

2.5. *Gel filtration*

Gel chromatography of purified DnaK was carried out on a Bio-Gel P-200 column (from Bio-Rad Laboratories, 5 ml bed volume) equilibrated with buffer A. 50 μl of [^{125}I]DnaK (0.9 μM) in buffer A was loaded onto the gel. Catalase (molecular weight 240,000), serum albumin (molecular weight 67,000), and maltose binding protein (molecular weight 42,000) were used as molecular weight standards.

2.6. *Materials*

ATP disodium salt was from Sigma. [^{3}H]ATP and [^{125}I]iodine were obtained from Amersham. All other products were from Sigma and were reagent grade.

3. RESULTS

3.1. *Stimulation of DnaK by unfolded bovine pancreatic trypsin inhibitor at various DnaK concentrations*

It has been shown previously that unfolded proteins bind specifically to chaperones and stimulate their ATPase activity [4,6,7]. Unfolded BPTI binds to DnaK, in contrast to native BPTI [4]. However, the stimulation of the DnaK ATPase by unfolded BPTI has not been reported. As shown in Fig. 1, the stimulation of the DnaK ATPase by unfolded BPTI is apparent only if the assay is made at a low DnaK concentration. Unfolded BPTI stimulates nearly four-fold the ATPase activity of DnaK (from 4 to 14 nmol/min per mg of DnaK), at a DnaK concentration of 0.1 μM. However, if the assay is made at a higher DnaK concentration (0.7 μM), the DnaK ATPase activity is close to 14 nmol/min per mg of DnaK, in the absence or in the presence of unfolded BPTI, and it does not appear to be stimulated by unfolded BPTI.

3.2. *Dependence of the DnaK ATPase activity on DnaK concentration*

DnaK has been shown to possess a low ATPase activity (around 10 nmol of ATP hydrolyzed per min per mg of protein) which is stimulated several-fold by DnaJ and GrpE [12]. The results presented in Fig. 2 suggest that the DnaK ATPase is stimulated by itself as DnaK concentration increases: the rate of ATP hydrolysis by DnaK does not show a linear dependence with respect to DnaK concentration, but displays a curvilinear dependence on DnaK concentration, with concavity upwards. This behaviour is characteristic of associating enzyme systems, with a higher specific activity of multimers over monomers: the increase in multimers with increasing enzyme concentration leads to an increase of the enzyme specific activity.

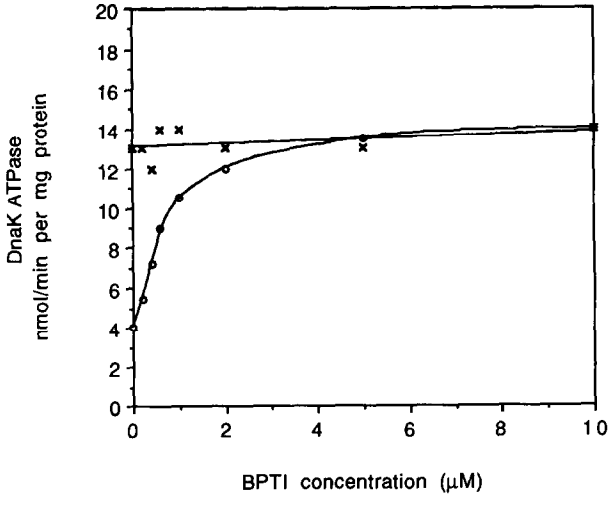

Fig. 1. Stimulation of the DnaK ATPase by unfolded BPTI. The ATPase activity of DnaK was measured in the presence of unfolded BPTI at the concentrations indicated in abscissa. The concentration of DnaK was 0.1 μM (○), or 0.7 μM (×).

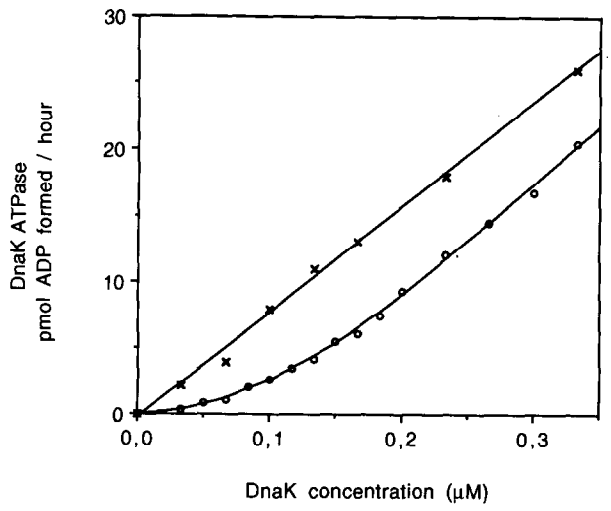

Fig. 2. Dependence of the DnaK ATPase on DnaK concentration. The ATP hydrolysis by DnaK was measured at the DnaK concentrations indicated in abscissa, in the absence (○) or in the presence of 1.5 μM unfolded BPTI (×).

In the presence of a concentration of unfolded bovine pancreatic trypsin inhibitor sufficient to stimulate DnaK, the rate of ATP hydrolysis by DnaK varies linearly with respect to DnaK concentration. Thus when DnaK is stimulated by an unfolded protein its specific activity is not affected by DnaK concentration. These results suggest that DnaK interacts with unfolded BPTI in a similar way as DnaK interacts with itself, and that both interactions would involve the peptide binding site of DnaK.

3.3. *Dimeric forms of DnaK*

It has been reported previously that DnaK exists mainly as a monomer with a very low amount of dimers [13]. The autostimulation of DnaK described here reflects protein–protein interactions and indicates the importance of multimeric forms of DnaK. A sample of pure DnaK protein (at 0.9 μM) was passed through a gel permeation column in the absence and in the presence of ATP. In the absence of ATP, the protein elutes at a position corresponding to an apparent molecular weight of approximately 100,000 Da, suggesting that it migrates as a mixture of monomers and multimers in equilibrium. A shoulder is seen at a position corresponding to a molecular weight of 140,000 Da which would correspond to a more stable dimeric form of DnaK. In the presence of ATP, most of the protein elutes (as bovine serum albumin) at a position corresponding to a molecular weight of 70,000 Da, and a low

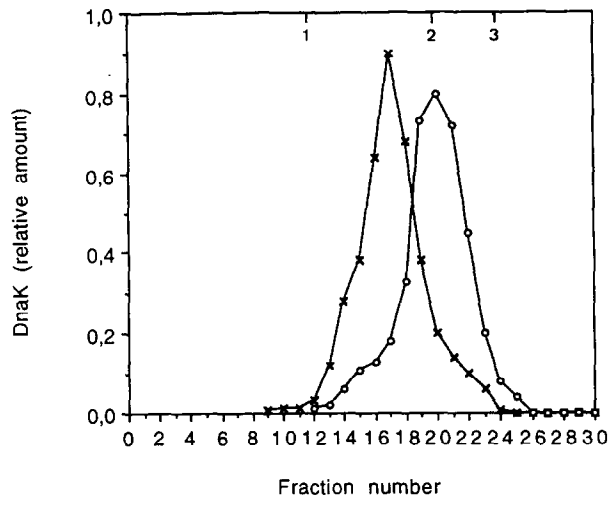

Fig. 3. Filtration of DnaK on a P-200 gel permeation column. A 50 μl sample of purified [^{125}I]DnaK (0.9 μM) was loaded on a P-200 column (5 ml bed volume). Fractions (100 μl/5 min) were collected and counted for radioactivity. Column buffer and DnaK sample contained no ATP (×) or 100 μM ATP (○). In a similar experiment, the DnaK protein sample was mixed with protein standards: (1) catalase; (2) serum albumin; (3) maltose binding protein.

amount elutes earlier at positions corresponding to dimeric forms (similar results were obtained with unlabeled DnaK). The occurrence of dimeric forms of DnaK correlates with the autostimulation of the DnaK ATPase. ATP triggers dissociation of dimers into monomers. This is in accordance with a proposed role of ATP in the dissociation of chaperone–protein complexes (reviewed in [14]).

4. DISCUSSION

The present results show that the stimulation of the DnaK ATPase by exogenous peptides can only be observed at low DnaK concentrations where the autostimulation of DnaK is negligible. The basal DnaK ATPase activity displays autostimulation at concentrations as low as 0.1 μM, and the autostimulation masks the effect of unfolded bovine pancreatic trypsin inhibitor on the DnaK ATPase. It can be suspected that other peptide-dependent ATPases would display a similar autostimulation. For this reason, such peptide-dependent ATPases should be assayed at a low ATPase concentration. In fact, the stimulation of the chaperone BiP by peptides has been observed at a 0.5 μM BiP concentration [6], and the stimulation of the protease La by casein, at a protease concentration of 0.8 μM [15]. The autostimulation of DnaK and the stimulation of DnaK by unfolded bovine pancreatic trypsin inhibitor are mutually exclusive. This result suggests that the autoassociation of DnaK implicates its peptide binding site, thereby hindering its interaction with unfolded BPTI. It would be interesting to determine whether the autostimulation of DnaK has a physiological function or if it is a consequence of its peptide binding activity. It will be interesting (and the peptide-dependent ATPase activity of DnaK should help for this purpose) to determine the motifs recognized by DnaK in unfolded proteins (amino acid side chains, polypeptide backbone, secondary structures), and the properties of native proteins (DnaJ, GrpE, sigma 32, lambda P, p53) (discussed in [16]) which allow their interaction with DnaK.

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