FEBS Letters 506 (2001) 108–112 FEBS 25293

The chaperone activity of trigger factor is distinct from its isomerase activity during co-expression with adenylate kinase in *Escherichia coli*

Zhen-Yu Li, Chuan-Peng Liu, Liu-Qin Zhu, Guo-Zhong Jing, Jun-Mei Zhou*

National Laboratory of Biomacromolecules, Institute of Biophysics, Academia Sinica, 15 Datun Road, Beijing 100101, PR China

Received 30 July 2001; revised 5 August 2001; accepted 6 August 2001

First published online 18 September 2001

Edited by Judit Ovádi

Abstract To investigate the molecular chaperone function of trigger factor (TF) and its relationship with isomerase activity in vivo, the assisted folding of adenylate kinase (AK) by TF in *Escherichia coli* was examined by measuring the amounts of soluble AK produced during co-expression. When the mutant of chicken AK, P17G, is expressed in plasmid pBVAK, 95% of the protein is found in inclusion bodies. Co-expression of AK with TF was achieved using a plasmid pBVAT that allowed expression of TF and AK in the same plasmid under separate control. Co-expression with TF resulted in an increase in the amount of soluble AK, with a higher increase when TF was expressed at higher levels in the cell. Co-expression of AK with the two TF mutants, Y221G and F233Y, in which peptidyl-prolyl *cis/trans* isomerase activity was 1% of wild-type, gave the same results as wild-type TF. This provides in vivo evidence that the molecular chaperone activity of TF is distinct from its isomerase activity. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Trigger factor; Molecular chaperone; Peptidyl-prolyl *cis/trans* isomerase; Inclusion body; Adenylate kinase; Co-expression

1. Introduction

The *Escherichia coli* trigger factor (TF) was originally identified as being involved in the maintenance of a translocation-competent conformation of the precursor protein proOmpA (outer membrane protein A) in a cell-free translation system [1,2], although subsequent research failed to verify this [3]. Later it was identified as a peptidyl-prolyl *cis/trans* isomerase (PPIase) [4,5] and was detected in the 50S subunit of the functional ribosome, which covers the exit domain of the nascent polypeptide chain [5,6]. In further studies, presecretory and non-secretory proteins have been crosslinked to TF while still associated with the ribosome [5,7]. Further, TF is active in cooperating with chaperones such as GroEL and DnaK and is itself an important chaperone in nascent peptide folding [7–12]. We found in previous in vitro studies on guanidine denatured GAPDH that an excess of TF assists reactivation and prevents aggregation, by repeated binding and release cycles between TF and folding intermediates during refolding [13]. TF recognizes random coil or loosely formed secondary structures, suggesting that TF interacts with the nascent chain and is involved in the earliest stages of peptide folding [14]. A recent study showed that co-expression of TF in combination with GroEL/GroES is helpful in preventing aggregation of a number of recombinant proteins [15]. These results suggest that TF might be useful in preventing aggregation of recombinant proteins in *E. coli* and thus improve the yield of soluble protein.

TF is a protein that shows both molecular chaperone and PPIase activities. In order to understand the relationship between the chaperone activity and PPIase activity, we used two TF mutants that greatly reduced PPIase activity. The mutants are Y221G (C.P. Liu, G.C. Huang, Z.Y. Li and J.M. Zhou, unpublished results) and F233Y [21]. In order to study the relationship between the chaperone and PPIase activities in vivo, we constructed an expression system to allow co-expression of adenylate kinase (AK) and TF in stoichiometric concentrations but induced by different promotors. The results show clearly that co-expression with TF resulted in a higher yield of soluble AK and more soluble AK was obtained when the amount of TF produced was higher. Mutants, Y221G and F233Y, show the same ability as wild-type TF in assisting folding of AK but possess only 1% of wild-type PPIase activity. This provides in vivo evidence that the molecular chaperone activity of TF is distinct from its isomerase activity.

2. Materials and methods

2.1. Bacterial strain and plasmids

E. coli strain JM109 {*endA1, recA1, gyrA96, thi, hsdR17* (r_k^-, mk^+), *relA1, supE44,* Δ(*lac-proAB*), [*F′, traD36, proAB, lacI*^{q}*ZΔM15*], *pREP4*} was used throughout this study. An expression vector (pBVAK, derived from pBV220) for chicken AK mutant P17G (Fig. 1A) was donated by Dr. X.-R. Sheng. Expression of AK was induced by heat shock at 42°C. Plasmid pQE60 contains the wild-type *tig* gene that encodes TF and was donated by Professor G. Fischer. TF mutants Y221G (C.P. Liu, G.C. Huang, Z.Y. Li and J.M. Zhou, unpublished) and F233Y [21] were obtained via site-directed mutagenesis of plasmid pQE60.

The vector for co-expression of AK and TF under control of different promotors was constructed as follows: a pair of PCR primers was designed to amplify the *tig* gene in pQE60 along with the T5 promotor and SD sequence and to introduce a *Bam*H1 restriction site at both 5′ and 3′ ends. The resulting PCR product was inserted into pBVAK at the *Bam*H1 site, downstream of the AK gene. The desired clones were selected and sequenced. This plasmid was named pBVAT (Fig. 1B), and allows AK and TF to be induced separately. When induced by heat shock at 42°C, AK and TF are expressed together from the same mRNA. Activation of the T5 promotor by addition of isopropyl β-D-thiogalactoside (IPTG) results in expression of only TF.

*Corresponding author. Fax: (86)-10-64872026.
E-mail address: zhoujm@sun5.ibp.ac.cn (J.-M. Zhou).

Abbreviations: AK, adenylate kinase; TF, trigger factor; PPIase, peptidyl-prolyl *cis/trans* isomerase; IPTG, isopropyl β-D-thiogalactoside

PII: S0014-5793(01)02896-4

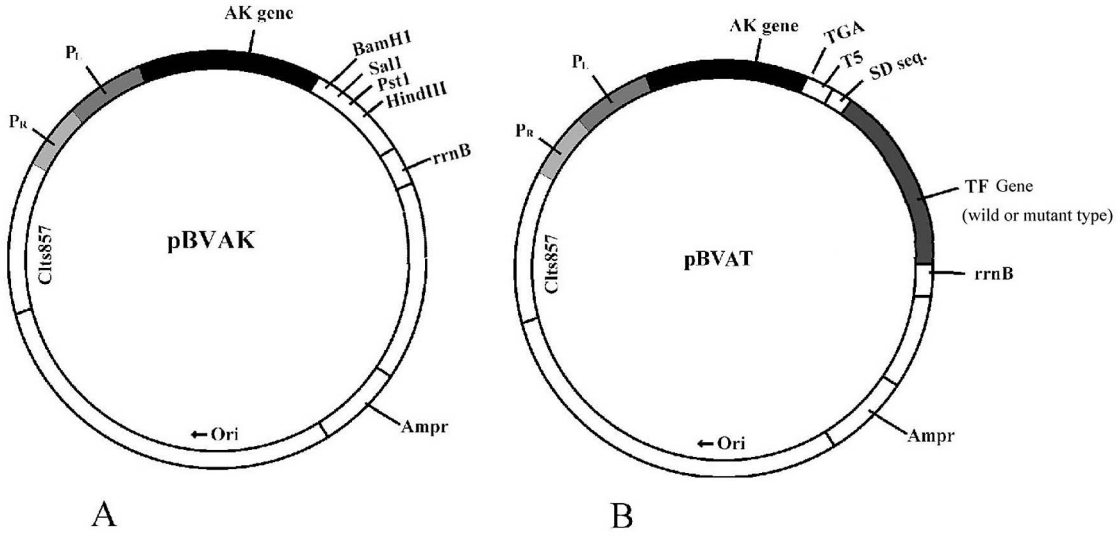

Fig. 1. Plasmids for expression of AK (A) and for controlled co-expression of TF with AK (B). Abbreviations: *ori*, replication origin of pMB1; *Ampr*, ampicillin resistant gene; P_R/P_L, promotor from λ phage; Clts857, P_R/P_L repressor gene.

2.2. Media, chemicals, and culture conditions

2TY broth was used throughout. Tryptone and yeast extract were from Oxoid, IPTG was from Promega and other chemicals were local products of analytical purity.

Strains harboring the respective plasmids were cultured overnight at 30°C. A 2 ml culture was added into 40 ml of media and grown with shaking at 250 rpm at 30°C until $A_{600\,nm}$ reached about 0.6∼0.8. IPTG was then added to a final concentration of 0.1 mM for the strain harboring pBVAT to induce TF expression (under the T5 promotor) and the temperature was immediately increased to 42°C for all cultures to induce AK expression. After 4 h growth at 42°C, cultures were centrifuged at 8000 rpm at 4°C for 10 min to collect the cell pellets.

2.3. Sample processing

All the procedures of cell lyses and separation were performed at 4°C. The pellets were washed with 0.9% NaCl then resuspended in 400 μl lysis buffer (50 mM Tris, 150 mM NaCl, pH 8.0) plus 50 μg lysozyme and 100 μM phenylmethylsulfonyl fluoride (PMSF). The cells were lysed by sonication for 2 min using a Cole-Parmer ultrasonic homogenizer CPX 600. Lysis products were centrifuged at 12 000 rpm for 10 min and the supernatant was set aside for analysis. The pellets containing the inclusion bodies were washed with 2 M urea, 0.1% Triton X-100, and 0.9% NaCl in turn with centrifugation between each washing step and finally dissolved in 400 μl 6 M urea and allowed to stand for 2 h. After centrifugation at 12 000 rpm for 10 min, the supernatants were collected for further analysis.

For each sample, 1 μl of supernatant and 1 μl of 6 M urea-solubilized inclusion bodies were analyzed by 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) in duplicate. After electrophoresis, one gel was subjected to Coomassie brilliant blue staining and the other gel was used for the Western blot. The AK bands on the nitrocellulose membrane were detected with anti-AK McAb3D3 [16] and AP-conjugated goat anti-mouse IgG.

AK activities of supernatants or 6 M urea-solubilized inclusion bodies were measured by the method following the reduction of NADP in a coupled enzyme solution with hexokinase and glucose-6-phosphate dehydrogenase in 50 mM Tris–HCl buffer (pH 8.1) [17]. 10 μl aliquots were taken from each reaction mixture for activity measurements. The amount of AK was then calculated from the activity (the specific activity was 0.23 mmol mg^{-1} min^{-1}).

PPIase activity of TF was assayed at 10°C by the chymotrypsin-

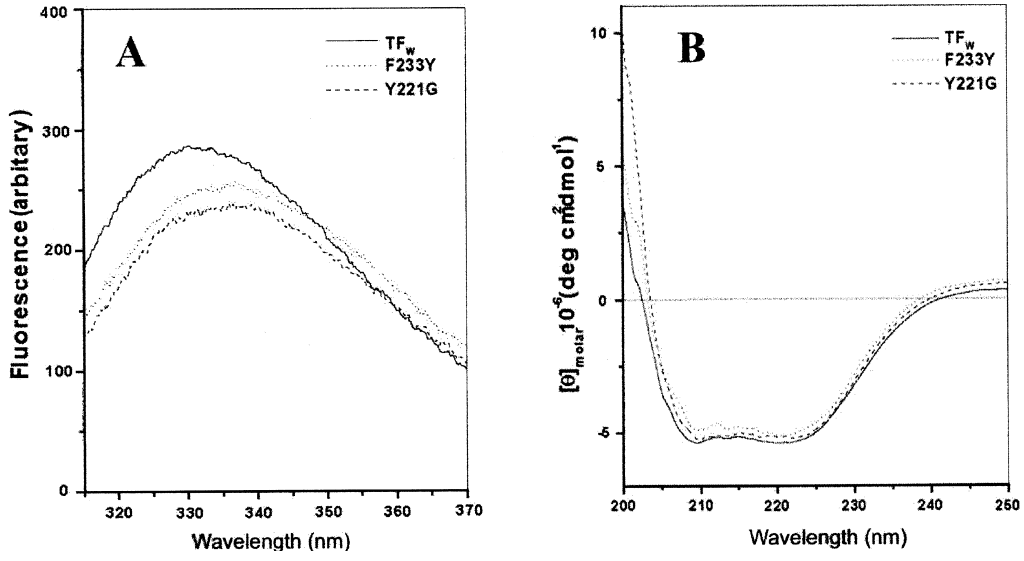

Fig. 2. Spectral properties of the two TF mutants. A: Trp fluorescence spectra of wild-type TF and the mutants. B: Far-UV CD spectra of wild-type TF and the mutants.

coupled method [18] using a tetrapeptide (succinyl-Ala-Ala-Pro-Phe-4-nitroanilide, Peptide Institute Inc. Japan) as a substrate. An apparent specificity constant 1.01 μM^{-1} s^{-1} [19] was used to calculate the amount of TF. Thus the TF/AK ratio of each sample could be calculated.

Since the PPIase activity of the mutants, Y221G and F233Y, is too low to be detected under the experimental conditions, the TF/AK ratio of TF mutants was calculated by analyzing the images of Coomassie blue stained gels (dry gels scanned with a flatbed scanner) using the gel analyzing software Totallab™ V1.01 (Nonlinear Dynamics Ltd), using the average result of five gels.

Wild-type and the two TF mutants, Y221G and F233Y, were purified according to the method of Stoller [4] and were quantified as described [20]. Fluorescence measurements were carried out on a Hitachi F4500 fluorescence spectrophotometer. Fluorescence emission spectra were recorded between 300 and 400 nm after excitation at 295 nm in a 1 cm cell. Both excitation and emission slits were set at 5 nm. Far-UV circular dichroism (CD) spectra were measured with a Jasco J720 spectrophotometer in a 0.1 cm cell. The enzyme concentration used was 0.2 mg/ml.

3. Results and discussion

3.1. Activity and conformation properties of TF mutants, Y221G and F233Y

The mutants Y221G and F233Y used in the present study show very low PPIase activity, so that the residual activities of the mutants could only be measured with purified enzyme at 100 times higher amounts than the wild-type enzyme. The substrate specificity of wild-type TF for the substrate succinyl-Ala-Ala-Pro-Phe-4-nitroanilide was 1540 mM^{-1} s^{-1} and substrate specificity of F233Y was 15 mM^{-1} s^{-1}, i.e. 1% of that for wild-type TF. For the mutant Y221G, the measured substrate specificity was 20 mM^{-1} s^{-1}, about 1.3% of wild-type. These results support the suggestions that residues Y221 and F233 of TF are involved in enzyme catalysis [21].

As shown previously, changes in the Trp fluorescence mainly reveal conformational changes in the middle domain of TF while changes in the far-UV CD spectra principally reflect the changes in the structure of terminal domains of TF [19]. The Trp fluorescence of the two mutants showed a red shift compared with wild-type TF (Fig. 2A). This indicates loosening of the structure of the middle domain. The CD spectra of the mutants show no significant difference to wild-type TF (Fig. 2B), indicating that the structures of the two terminal domains (N and C) were not affected by the mutations. The isomerase activity of TF is dependent on the middle domain and the observed minor conformational change may account for the loss in isomerase activity.

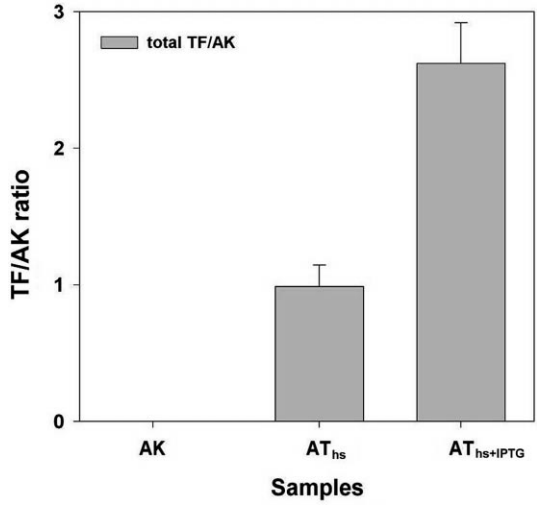

Fig. 3. The relative expression levels of total TF (wild-type) to AK under different conditions. Samples '$AT_{hs+IPTG}$', 'AT_{hs}' and 'AK' represent proteins expressed by plasmids pBVAT induced by heat shock and IPTG, plasmid pBVAT induced by heat shock only and plasmid pBVAK induced by heat shock, respectively. The ratios were measured by activity assay as described in Section 2.

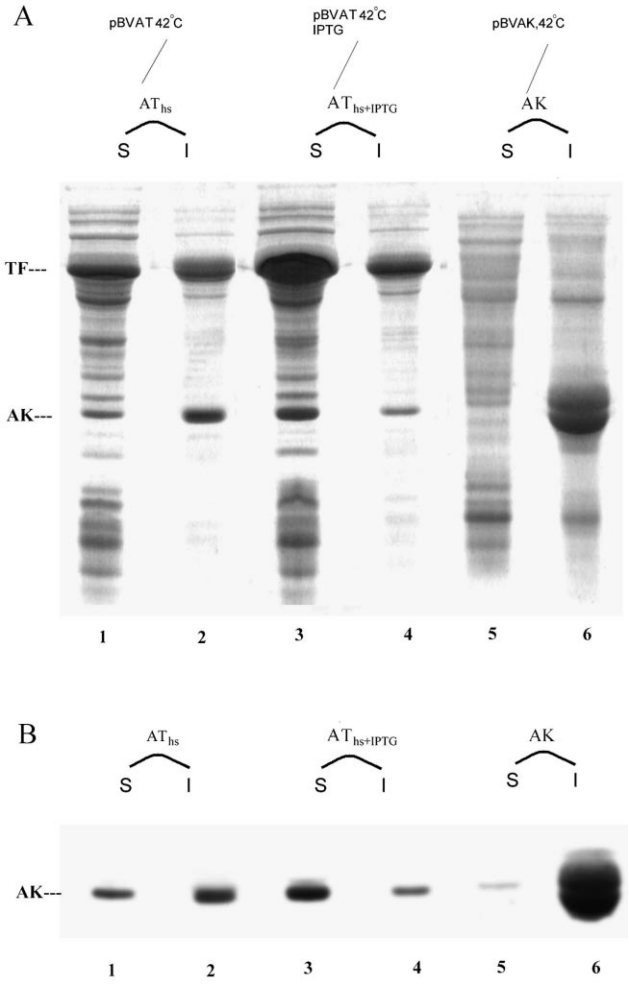

Fig. 4. The co-expressed wild-type TF and AK in supernatants and inclusion body fractions. A: Proteins analyzed by SDS–PAGE (15% polyacrylamide gel) stained by Coomassie blue R250. B: The bands of AK detected by Western blot. Columns 1, 3, 5: supernatants (S) of samples 'AT_{hs}', '$AT_{hs+IPTG}$' and 'AK', respectively. Columns 2, 4, 6: insoluble (I) components of samples 'AT_{hs}', '$AT_{hs+IPTG}$' and 'AK', respectively. Samples were treated as described in Section 2.

3.2. Controlled expression of TF and AK

In the present study, the plasmid pBVAK was used to express AK alone and pBVAT was constructed to express AK and TF together under the control of separate promotors. When AK was induced by heat shock at 42°C of the strain harboring pBVAK, 95% of the expressed AK was found in inclusion bodies. The proteins expressed from pBVAK or pBVAT under heat shock alone were nominated as samples 'AK' or 'AT_{hs}'. The proteins expressed by pBVAT induced using heat shock and 0.1 mM IPTG were nominated as sample '$AT_{hs+IPTG}$'. For the strain harboring pBVAT, TF and AK genes were proceeded by T5 and P_R/P_L promotors respec-

Table 1
The relationship between soluble AK and the amount of co-expressed TF

Expression conditions		Soluble AK	TF/AK
pBVAK	heat shock	$< 5\%$	0
pBVAT	heat shock (sample 'AT_{hs}')	$25 \pm 4\%$	1.1 ± 0.2
	heat shock, 0.1 mM IPTG (sample '$AT_{hs+IPTG}$')	$65 \pm 7\%$	2.8 ± 0.3
$pBVAT_{Y221G}$	heat shock (sample '$AT_{Y221G,hs}$')	$15 \pm 3\%$	1.2 ± 0.4[a]
	heat shock, 0.1 mM IPTG (sample '$AT_{Y221G,hs+IPTG}$')	$52 \pm 7\%$	2.9 ± 0.5[a]
$pBVAT_{F233Y}$	heat shock (sample '$AT_{F233Y,hs}$')	$20 \pm 3\%$	1.1 ± 0.3[a]
	heat shock, 0.1 mM IPTG (sample '$AT_{F233Y,hs+IPTG}$')	$60 \pm 8\%$	2.7 ± 0.5[a]

[a]The TF/AK ratio for TF mutants was calculated by analyzing images of Coomassie blue stained gels using Totallab™ V1.01 (Nonlinear Dynamics Ltd), using the average result of five gels.

tively, so TF was under the control of both the T5 and the P_R/P_L promotors while AK was under the control of only the P_R/P_L promotor. When cultured at 30°C without IPTG, there was no recombinant TF expressed. Thus, under the experimental conditions, strains harboring pBVAK or pBVAT under heat shock or both heat shock and IPTG could express AK only, AK and certain amount of TF, or AK and large amount of TF, respectively. The expression levels of wild-type TF and AK in different strains were presented as a ratio of total TF/AK and are compared in Fig. 3. The total amount of TF expressed in pBVAT induced by heat shock and IPTG was 2.8 times the amount of AK. In pBVAT induced by only heat shock the amounts of TF and AK were approximately equal.

3.3. Co-expression of TF with AK increases the yield of soluble AK

The effects of co-expressed TF on the solubility of AK were further analyzed and the results are shown in Fig. 4A and B. The SDS–PAGE and the western blot of the supernatant and inclusion bodies for the proteins expressed by pBVAK and pBVAT strains under different culture conditions show dramatic differences. In sample 'AK', about 95% of the recombinant AK was found in the insoluble fraction (lanes 5, 6 in Fig.

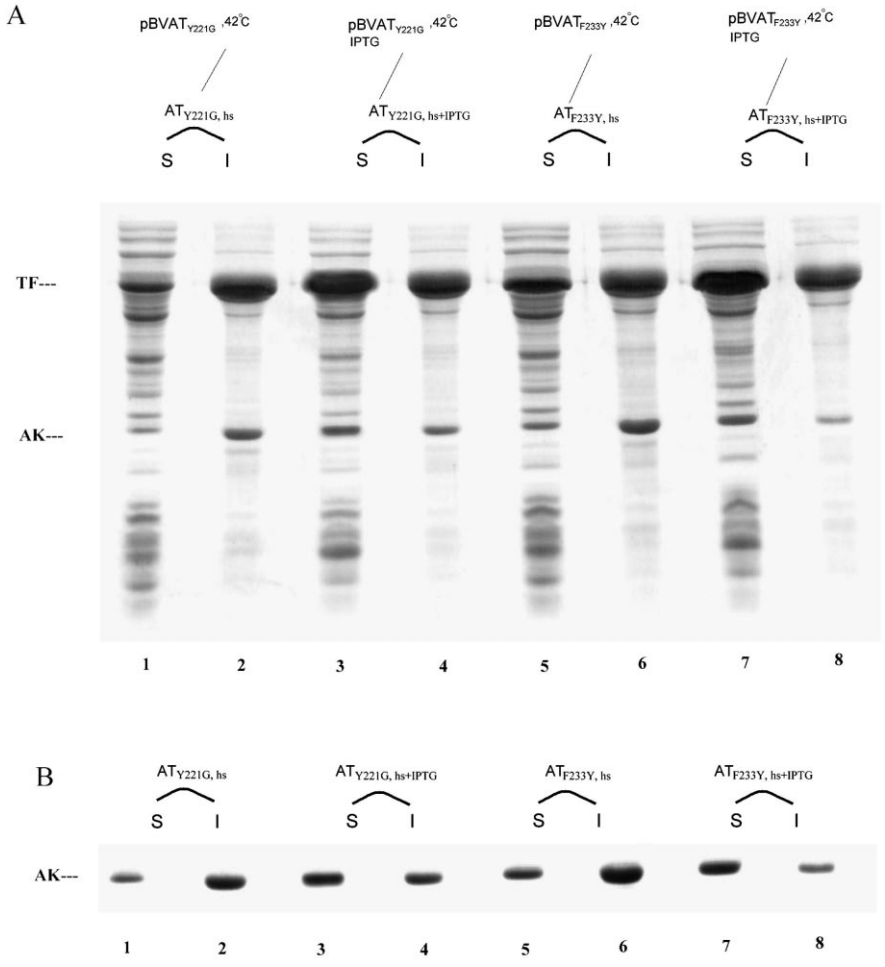

Fig. 5. The co-expressed TF mutants and AK in supernatants and inclusion body fractions. A: Proteins analyzed by SDS–PAGE (15% polyacrylamide gel) stained by Coomassie blue R250. B: The bands of AK detected by Western blot. Columns 1, 3, 5, 7: supernatants (S) of samples '$AT_{Y221G,hs}$', '$AT_{Y221G,hs+IPTG}$', '$AT_{F233Y,hs}$', '$AT_{F233Y,hs+IPTG}$', respectively. Columns 2, 4, 6, 8: insoluble (I) components of samples '$AT_{Y221G,hs}$', '$AT_{Y221G,hs+IPTG}$', '$AT_{F233Y,hs}$', '$AT_{F233Y,hs+IPTG}$', respectively. Samples were treated as described in Section 2.

4A and B). When TF was introduced into the expression system (samples 'AT_{hs}' and '$AT_{hs+IPTG}$'), the total amount of recombinant AK decreased to a certain degree. However, the amount of soluble AK increased significantly (lanes 1–4 in Fig. 4A and B). The quantities of TF and AK in each sample were determined by measuring enzyme activities of AK and TF in both supernatant and refolded inclusion body fractions. As shown in Table 1, soluble AK accounted for more than 60% of the total synthesized AK in sample '$AT_{hs+IPTG}$', in which the molar ratio of expressed TF to AK was about 2.8:1 (Fig. 2). There was 25% soluble AK in sample 'AT_{hs}', in which the molar ratio of expressed TF to AK was about 1:1. For sample 'AK', in which no TF was expressed, soluble AK was only 5%. This shows clearly that co-expression of TF with AK increases the amount of soluble AK and it is clearly related to the quantity of co-expressed TF.

When TF mutants Y221G or F233Y were co-expressed with AK, the amount of soluble AK also increased with increasing levels of mutant TF co-expression (Fig. 5). In this case the amount of mutant TF was quantified by SDS–PAGE since the mutants had no detectable activity in the co-expressed systems. The soluble AK was determined using the activity assay as before. As shown in Table 1, the results are essentially the same as for wild-type TF. This provides direct evidence that in vivo chaperone activity of TF is distinct from its PPIase activity.

From the current study we cannot rule out the possibility that a low level of PPIase activity is required for AK folding in addition to the chaperone activity. However, even if this is the case, it is clear that the PPIase activity alone is not sufficient for AK folding. Further, it is the chaperone activity that is limiting for AK folding and at least stoichiometric binding is required. The level of chaperone activity in the mutant TF proteins is essentially undiminished and is clearly distinct from the PPIase activity.

It has been suggested that TF assists GAPDH refolding in vitro by repeated binding–releasing cycles [16]. A number of TF molecules may bind to a single substrate molecule where there are two or more hydrophobic binding sites available on the target protein. The higher the concentration of TF, the larger the number of binding–release cycles and the higher the degree of protection of target proteins from aggregation. Hence more folding intermediates have the chance to reach the native state. The results presented here show that different levels of TF co-expression resulted in different chaperone efficiency in *E. coli*. When the TF level was low (as in the sample 'AT_{hs}'), AK is still prone to aggregation. This suggests either that each nascent AK molecule needs help from more than one TF molecule or that the binding affinity requires higher levels of TF to achieve effective binding. The similarity between these and in vitro results [16] suggests that TF assisted folding in the two systems may occur by a similar mechanism. Early in folding of the nascent polypeptide, interaction of exposed hydrophobic surfaces may be the main route to aggregation. High concentrations of TF may block this interaction and decrease the rate of aggregation, thus allowing more nascent polypeptides to follow the pathway to correct folding.

Maturation of nascent AK requires a certain microenvironment. It is important that nascent polypeptides be insulated from each other. For recombinant proteins, large quantities of nascent polypeptides are synthesized simultaneously. This crowding then results in aggregation. When a sufficient number of TF molecules are available in the surrounding environment, these TF molecules can bind to nascent polypeptides so as to form a relatively closed microenvironment and prevent interaction between nascent polypeptides. Thus, the productivity of nascent polypeptide folding can be improved. This indicates the potential of TF to improve the yield of recombinant heterologous proteins for medicinal and other biotechnological purposes.

Earlier studies have shown hints that TF binds to nascent polypeptides and cooperates with chaperones such as GroEL and DnaK [7–12,15]. Here we provide experimental evidence that TF is effective as a molecular chaperone in the folding of a heterologous protein in vivo.

Acknowledgements: This work was supported in part by the 973 Project of the Chinese Ministry of Science and Technology (G1999075608) and the Chinese National Natural Science Foundation (30070163). The authors would like to thank Dr. S. Perrett of this laboratory for a critical reading of this paper and helpful suggestions.

References

[1] Crooke, E. and Wickner, W. (1987) Proc. Natl. Acad. Sci. USA 84, 5216–5220.
[2] Crooke, E., Guthrie, B., Lecker, S., Lill, R. and Wickner, W. (1988) Cell 54, 1003–1011.
[3] Guthrie, B. and Wickner, W. (1990) J. Bacteriol. 172, 5555–5562.
[4] Stoller, G., Rücknagel, K.P., Nierhaus, K.H., Schmid, F.X., Fischer, G. and Rahfeld, J.U. (1995) EMBO J. 14, 4939–4948.
[5] Hesterkamp, T., Hauser, S., Lütcke, H. and Bukau, B. (1996) Proc. Natl. Acad. Sci. USA 93, 4437–4441.
[6] Scholz, C., Stoller, G., Zarnt, T., Fischer, G. and Schmid, F.X. (1997) EMBO J. 16, 54–58.
[7] Valent, Q.A., Kendall, D.A., High, S., Kusters, R., Oudega, B. and Luirink, J. (1995) EMBO J. 14, 5494–5505.
[8] Kandror, O., Sherman, M., Rhode, M. and Goldberg, A.L. (1995) EMBO J. 14, 6021–6027.
[9] Kandror, O., Sherman, M.Y., Moerschell, R. and Goldberg, A.L. (1997) J. Biol. Chem. 272, 1730–1734.
[10] Göthel, S.F., Scholz, C., Schmid, F.X. and Marahiel, M.A. (1998) Biochemistry 37, 13392–13399.
[11] Lyon, W.R., Gibson, C.M. and Caparon, M.G. (1998) EMBO J. 17, 6263–6275.
[12] Deuerling, E., Schulze-Specking, A., Tomoyasu, T., Mogk, A. and Bukau, B. (1999) Nature 400, 693–696.
[13] Huang, G.C., Li, Z.Y., Zhou, J.M. and Fischer, G. (2000) Protein Sci. 9, 1254–1261.
[14] Huang, G.C., Li, Z.Y. and Zhou, J.M. (2000) Biochim. Biophys. Acta 1480, 77–82.
[15] Nishihara, K., Masaaki, K., Hideki, Y. and Takashi, Y. (2000) Appl. Environ. Microbiol. 66, 884–889.
[16] Cheng, P., Zhou, J.M. and Guo, Z.Q. (1998) Biochem. Biophys. Res. Commun. 244, 556–560.
[17] Hill, A., Muller, G., Noda, L., Pinder, T., Schirmer, H., Schirmer, I. and Zabern, I.V. (1974) Eur. J. Biochem. 43, 131–144.
[18] Fischer, G., Bang, H. and Mech, C. (1984) Biomed. Biochim. Acta 43, 1101–1111.
[19] Zarnt, T., Tradler, T., Stoller, G., Scholz, C., Schmid, F.X. and Fischer, G. (1997) J. Mol. Biol. 271, 827–837.
[20] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[21] Tradler, T., Stoller, G., Rucknagel, K.P., Schierhorn, A., Rahfeld, J.U. and Fischer, G. (1997) FEBS Lett. 407, 184–190.