J. Microbiol. Biotechnol. (2004), **14**(1), 162–166

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

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Evidence of Interaction of Phage P22 Tailspike Protein with DnaJ During Translational Folding

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Received: September 6, 2003
Accepted: September 25, 2003

Abstract Phage P22 tailspike is a thermostable homotrimeric protein, and temperature-sensitive folding (*tsf*) and global suppressor mutations affect its folding yields at elevated temperatures. We earlier suggested that the folding of the tailspike protein in *Escherichia coli* requires an unidentified molecular chaperone. Accordingly, in the present study, the interactions of purified DnaK, DnaJ, and GrpE heat-shock proteins with the tailspike protein were investigated during the translation and folding of the protein. The cotranslational addition of DnaJ to the tailspike protein resulted in the arrest of folding, when Dnak and GrpE were missing. However, the presence of DnaK, DnaJ, and GrpE had no effect on the folding yield of the tailspike protein, thus, providing evidence for the binding of the nascent tailspike protein with DnaJ protein, a member of DnaK chaperoning cycle.

Key words: Bacterophage P22, tailspike protein, protein folding, molecular chaperone, DnaJ

Molecular chaperones have been defined as proteins that bind to and stabilize an otherwise unstable conformer of another protein and by controlled binding and release, facilitating its correct fate *in vivo* [10, 15]. Heat shock proteins such as Hsp60 and Hsp70 are molecular chaperones that not only regulate the heat-shock response but also are required for folding a newly synthesized polypeptide under normal growth conditions [9]. The numerous structural and biochemical studies have revealed a detailed molecular mechanism for the two well-known chaperones, Hsp60 and Hsp70. Hsp60 recognizes collapsed folding intermediates and facilitates the global folding in the central cavities within each heptameric ring structure, while Hsp70 binds an extended conformation of unfolded polypeptides in the peptide-binding groove of the substrate-binding domain and facilitates the polypeptide folding locally. Evidence has accumulated that these chaperones work cooperatively in that Hsp70 acts on the nascent or importing polypeptide chain and Hsp60 subsequently performs chaperoning [6, 16]. It then has an option of either folding, rebinding to Hsp70, or transferring to another chaperone system such as Hsp60 for final folding [16, 29]. However, not all the proteins require all the cheperones for folding. It is not yet clear which proteins require chaperones, and if they do, which chaperons are required.

The tailspike protein of *Salmonella* phage P22 is a homotrimer, whose subunit has a molecular mass of 72 kDa [23]. Its X-ray crystallographic structure reveals dominance of a β-helix motif [27]. The folding pathway of the tailspike protein is consecutively comprised of single chain folding, subunit association, and a rate-limiting folding reaction to form the native trimer. Many temperature-sensitive folding (*tsf*) mutations of the tailspike protein have been characterized which block the folding and maturation at the high restrictive temperature [33]. The *tsf* mutations destabilize a folding intermediate and consequently reduce the folding yield of the tailspike at temperatures optimal for cell growth [4, 18]. Interestingly, the folding of the wild-type tailspike itself is temperature-sensitive [13]. One possible explanation for the folding defect of *tsf* mutants is the relatively limited availability of a molecular chaperone [22]. Van Dyk *et al.* [31] previously reported that the overexpression of GroEL/ES, an *E. coli* homolog of Hsp60, can rescue a subset of *tsf* mutations in *Salmonella* P22 phage, yet subsequent work showed that GroEL/ES does not mediate refolding only through binding to and sequestering substrates [2, 8]. It was shown that the yield of mature tailspike did not increase in the presence of excess groE gene products, that tailspike folded and matured in an *E. coli* strains that carries a mutation in the groEL gene, and that the folding of the

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tailspike protein *in vivo* requires an unidentified molecular chaperone under the regulation of a heat-shock transcriptional factor σ^{32} protein [14]. The folding polypeptides are presented in a vectorial manner during translation, and it is unclear whether their interaction with chaperones is governed by the same principles that function during refolding.

In the present study, it was examined whether the molecular chaperone DnaK, as Hsp70 homolog in *E. coli*, was involved in the folding of the tailspike protein. The folding of tailspike *in vivo* was analyzed in a DnaK-deficient mutant *E. coli* strain, and the chaperone action of the DnaK, DnaJ, and GrpE system was also analyzed in a synchronized translation reaction in a reticulocyte lysate. It was anticipated that the *E. coli* chaperones, added at levels comparable to their intracellular abundance, would not interact efficiently with the eukaryotic chaperones, which are present in the translation extract at a fraction of their cellular concentration. We provided the evidence for binding of the nascent tailspike protein with DanJ protein, a member of the DnaK chaperoning cycle.

MATERIALS AND METHODS

Materials

The molecular chaperones, DnaK, DnaJ, and GrpE proteins, were all bought from Epicentre Technologies. The mCAP™ mRNA capping kit was purchased from Stratagene, and the Rabbit Reticulocyte Lysate System from Promega. The [^{35}S]methionine was purchased from Amersham. All other chemicals were reagent-grade.

Strains and Plasmids

The bacterial strains, *E. coli* MC4100 [*araD139Δ(argF-lac)U169 rpsL150 relA1 deoC1 ptsF25 rpsR flbB301*] and BB1553 [MC4100 *ΔdnaK52::Cm^R sidB1*] were kindly provided by Schroder *et al.* (1993). The cells were grown in a Luria Broth supplemented with ampicillin (100 μg/ml) or chloramphenicol (100 μg/ml) as indicated. The expression plasmid encoding the wild-type P22 tailspike protein, PIN3, and plasmid carrying the *tsf* mutation have previously been described in [12] and [17], respectively. The plasmid pTG9 and construction of the plasmid carrying the tailspike protein and *tsf* mutant have also been previously described [1].

In Vitro Translation of Cloned Genes

The P22 tailspike mRNA was produced by the transcription of plasmid pTG9. The *in vitro* translation was performed using a rabbit reticulocyte lysate system at 37°C for 1 h according to the supplier's recommendation. A typical translation reaction mixture contained 55% of the reticulocyte lysate treated with micrococcal nuclease.

Folding and Maturation of Tailspike Protein

The formation of the native trimer was analyzed by SDS-polyacrylamide gel electrophoresis [28] of the cell lysate or *in vitro* translation mixture samples without boiling. At room temperature, the native tailspike trimers are not dissociated and all the incompletely folded polypeptide chains form an SDS-polypeptide complex [7, 13]. The folding yields of the native tailspike trimers were calculated based on a quantitation of the autoradiograms of the [^{35}S]-labeled protein separated by SDS-PAGE.

RESULTS AND DISCUSSION

The most abundant Hsp70 homologue of *E. coli*, DnaK, functions together with two heat-shock proteins, DnaJ and GrpE, cooperatively as a chaperone machine [3, 9]. In this system, DnaJ tags selected a nascent polypeptide or unfolded protein to prevent any aggregation. Subsequently, in the presence of ATP, a complex is formed including the unfolded substrate polypeptide, DnaJ, and DnaK. ATP hydrolysis by DnaK then shifts this complex to an ADP state, a substrate transfer to DnaK, and causes the release of DnaJ. Thereafter, the nucleotide exchange factor GrpE binds to the DnaK-ADP-substrate complex and promotes the release of ADP from DnaK. The substrate dissociates from DnaK upon the binding of a second molecule of ATP to DnaK [20, 24].

Whether mutation in the gene encoding the *E. coli* chaperone DnaK affects the folding and maturation of the P22 tailspike protein *in vivo* was investigated. For this purpose, the wild-type strain (MC4100) and *ΔdnaK52* mutant strain (BB1553) were used. Each strain was transformed with the PIN3 plasmid expressing the P22 tailspike gene under the control of a *tac* promoter. The formation of the tailspike trimers and unfolded monomer were analyzed using SDS-PAGE. The folding yield of the mature tailspike polypeptides decreased at a permissive temperature (39°C) in the BB1553 strain, and most of the unfolded polypeptide chains remained as a monomer, however, the tailspike trimer formation did not vary as in the wild-type strain (Fig. 1). In the MC4100 strain, the result was similar to that obtained previously in *E. coli* JM109 [17]. Therefore, the incomplete tailspike protein maturation in the *dnaK* mutant strain indicated that DnaK appeared to be involved in the folding process of the tailspike protein *in vivo*.

Next, the tailspike proteins were translated in a reticulocyte cell-free translation system, resulting in the specific labeling of the tailspike polypeptide chain with [^{35}S]methionine. The yield of wild-type tailspike trimers reached a maximal value after 60 min at 37°C. The amount of monomer tailspike polypeptides was also significantly higher than with the *E. coli* plasmid expression system.

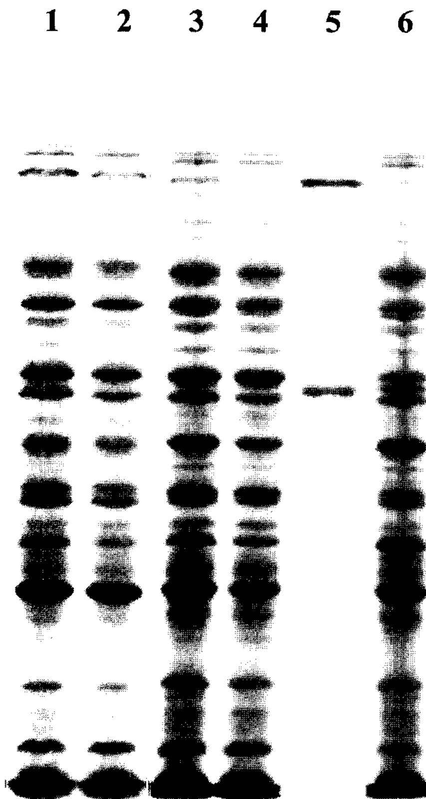

Fig. 1. Tailspike maturation in host lacking DnaK function.
E. coli MC4100 and BB1553 cells transformed with pIN3 were grown at 37°C, and the synthesis of the tailspike was induced at an early log phase by adding 1 mM IPTG. At the time of induction, aliquots of each culture were transferred to various temperatures. The cells were further grown for 3 h at 39°C and lysates were prepared using an SDS gel sample buffer. The samples were kept below room temperature, and the electrophoresis was performed on a 7.5% SDS-polyacryamide gel. Lane 1: 28°C in MC4100. Lane 2: 39°C in MC4100. Lane 3: 28°C in BB1553. Lane 4: no IPTG induction control of Lane 3. Lane 5: purified tailspike protein (trimer and monomer). Lane 6: 39°C in BB1553.

The apparent change in the folding yields of the translated trimer in this system might have been due to the different translation machinery. The level of the protein band was sufficient to obscure the estimated level of the native trimer protein produced in the system. The time course of the formation of the *tsf* mutants was similar to that of the wild-type.

The chaperone cycle of the DnaK system was also investigated to examine its interaction with the tailspike polypeptide folding. The addition of DnaJ alone to the translation reaction mixture prevented the folding of the newly synthesized tailspike polypeptides (Fig. 2A), whereas DnaK or GrpE had a minimal effect (data not shown). There was no change in the folding yield when DnaK, DnaJ, and GrpE were added simultaneously to the translation reaction mixture. Five mM DnaJ almost completely prevented the formation of tailspikes as a trimer without significantly inhibiting the translation. The complete course of protein folding in a cell-free translation system is still unknown. The inhibitory effect of DnaJ was strictly cotranslational. The translated trimer formation was reduced by the addition of DnaJ only early during the translation (Fig. 2B), since the addition of DnaJ late to the translation reaction mixture had no effect on the yield of folded trimers. As such, these results indicate that DnaJ is able to recognize unfolded nascent polypeptides early in translation.

In the suggested model, DnaJ functions cooperatively with DnaK and GrpE as a chaperone machine [3, 9]. The functional interaction of DnaJ with unfolded nascent polypeptides leads to the blockage of folded trimers

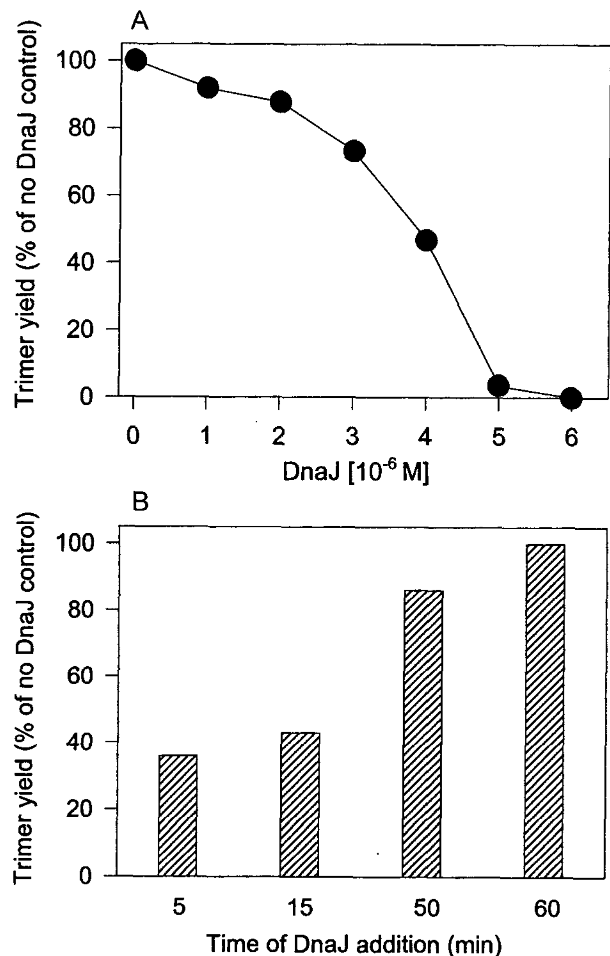

Fig. 2. Maturation of tailspike polypeptides into SDS-resistant trimer in the presence of DnaJ.
(A) Trimer synthesis of tailspike protein determined as a function of DnaJ added during translation reaction. The folding yields measured in the absence of any DnaJ were set at 100%. The reaction was initiated by the addition of mRNA to a translation mixture carrying each concentration of the DnaJ protein and carried out for 60 min at 37°C. (B) Prevention of protein folding by cotranslational addition of DnaJ. The trimer was synthesized in the presence of DnaJ (5 μM) added 5, 15, or 50 min after the initiation of the translation reaction. The amount of the folded trimer tailspike protein was analyzed 60 min after the initiation of the translation reaction.

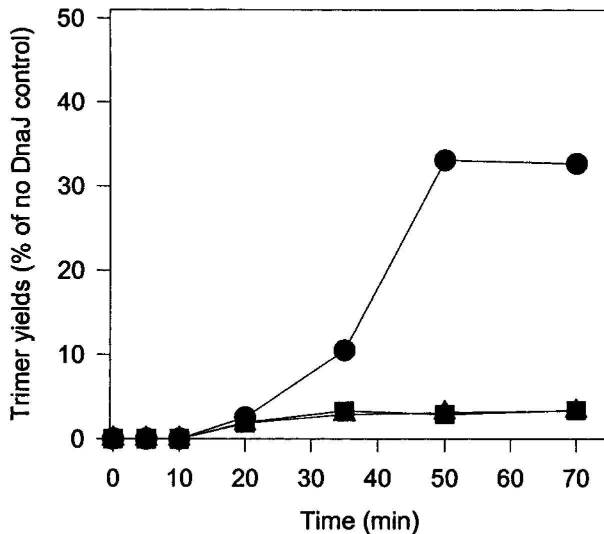

Fig. 3. Restoration of post-translational folding by addition of DnaK and GrpE.

The folded tailspike trimer was determined according to a time course after the post-translational addition of DnaK (■), GrpE (▲), or both (●) to the translation reaction sample in the presence of 5 μM DnaJ. The folding yield measured in the absence of DnaJ was set at 100%.

formation in the absence of DnaK and GrpE. If these interactions of DnaJ reflect the normal function during protein biosynthesis, the folding arrest by DnaJ must be reversible by the further addition of DnaK and GrpE: When DnaJ is added at a high concentration to eukaryotic *in vitro* translation reactions, the cotranslation binding of DnaJ blocks the folding of luciferase and chloramphenicol acetyltransferase until exogeneous DnaK and GrpE are added to allow the continuation of the chaperone cycle and polypeptide folding [11]. The antifolding effect of DnaJ was found to be reversed by DnaK and GrpE, yet unaffected by either protein alone (Fig. 3). The reversibility of the DnaJ inhibition was observed in the trimer formation of the tailspike protein. About 30% of the trimers were recovered from the translation reaction mixture arrested by DnaJ when both DnaK and GrpE were added post-translationally, suggesting that DnaJ binds to nascent chains very early in translation and that this interaction is responsible for folding in the native state upon the addition of DnaK and GrpE. The low recovery suggested the possibility that the sole DnaJ binding state results in an increased off-pathway aggregation or may cause proteolytic attack. The chaperone function is important for both the folding of newly synthesized polypeptide chains and the degradation of misfolded proteins [32]. Distribution between competent folding and misfolded polypeptide chains may occur at the level of the molecular chaperones by kinetic partitioning. The fraction that fails to achieve native conformation by remaining for a prolonged time period in association with chaperones may then undergo efficient degradation by protease. In contrast to monomeric proteins that are stably folded, the subunits of oligomeric proteins that are unable to fold may be cycled post-translationally between the chaperone system and the bulk solution. This iterative cycling would then allow incomplete polypeptides to be degraded [6]. Alternatively, or in addition, eukaryotic Hsp70 homologue proteins may recognize the DnaJ binding state or intervene in the DnaK cycle for folding. The Hsp70 system in the eukaryote has now become more complex with the discovery of novel binding Hsp70 proteins [20].

The binding specificity of DnaJ with polypeptides has not yet been elucidated. The DnaK/DnaJ chaperone machinery appears to play a significant role in the folding of growing polypeptide chains, and its binding capacity may depend on the structural properties of the polypeptides in a folding state. The apparent difference between the *tsf* mutant, suppressor mutant, and wild-type tailspike protein was not observed in regards to their interaction with the molecular chaperone (data not shown). Accordingly, it is proposed that the interaction of the tailspike polypeptide with the molecular chaperones may not act on the folding and maturation of the mutant polypeptide chain. Thus, the folding of the tailspike polypeptide may also occur spontaneously without the assistance of molecular chaperones. One further possibility is that the Hsp70 homologue [18, 26, 30] of unknown function in *E. coli* can substitute for the chaperone function of the DnaK system or GroEL chaperoning.

Acknowledgment

This work was supported by Grant E71840 from the Korean Ministry of Sciene and Technology.

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