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Original Communication

Comparative chaperone activities of trigger factors from mesophilic and psychrophilic bacteria

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ABSTRACT

The refolding level of thermally inactivated bacterial luciferases by trigger factor (TF) of the mesophilic bacterium *Escherichia coli* (TF_{Ec}) and the psychrophilic bacterium *Psychrobacter frigidicola* (TF_{Pf}) was measured. Refolding of heat inactivated luciferases reached a maximum of 25-30% in *E. coli ΔdnaKdnaJ* cells containing plasmids with a *tig* gene (encoding TF) and *luxAB* genes (encoding heterodimeric (αβ) luciferase from *Photobacterium leiognathi*). However, while the activity of TF_{Ec} was characterized by a significant reduction in refolding with an increase in TF concentration, the chaperone activity of the psychrophilic TF remained at a plateau at higher concentrations. TF_{Pf} also did not affect the growth kinetics of the host bacterial cells at high TF concentrations, unlike TF_{Ec}, which exerted a lethal effect on bacterial cells with increased concentration. Moreover, TF_{Ec} and TF_{Pf} effectively assisted in refolding dimeric forms of luciferase but were unable to refold an enzyme variant in monomeric form. Finally, luciferase refolding by TF_{Pf} was found to be more efficient in *E. coli* strains lacking the ClpB chaperone than in *clpB*$^+$ strains.

KEYWORDS: chaperone, trigger factor, *Psychrobacter*, refolding, luciferase

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INTRODUCTION

The folding of newly synthesized polypeptide chains is the main function of the molecular chaperone trigger factor from *Escherichia coli* (TF_{Ec}). This role is closely associated with the work of the 50S ribosomal subunit with which it forms a stable complex during protein synthesis [1, 2, 3]. TF localizes at the ribosomal exit tunnel from which nascent polypeptide chains are released into the cytoplasm and is involved in the primary steps of protein assembly and folding, a process that is subsequently completed in the cytoplasm with input from the ATP-dependent DnaKJE and GroEL/ES chaperones [4, 5, 6]. In addition, the co-purification and stable association of TF with many full-length cytoplasmic polypeptides [7] indicate that TF is involved not only in co-translational folding of polypeptide chains at the ribosome, but also in post-translational stabilization of their native protein structures. TF_{Ec} has been demonstrated to possess chaperone activity and can partially renature substrates such as urea denatured glyceraldehyde-3-phosphate dehydrogenase (GAPDH) enzyme *in vitro* [8, 9]. We have reported that TF_{Ec} also assists the refolding of heat inactivated luciferase, both *in vitro* and *in vivo*, and, unlike the DnaK-ClpB bi-chaperone system, is able to recover the activity of heterodimeric but not monomeric luciferases [10, 11]. Furthermore, we demonstrated a rapid decrease, to negligible levels, in the ability

of TF_{Ec} to refold luciferases with increasing TF_{Ec} intracellular concentration. Since TF_{Ec} forms a homodimer at higher protein concentrations [12], it is thought that denatured proteins which associate with dimeric TF_{Ec} are unable to be released from the complex, resulting in reduced efficiency of the refolding process [10]. Trigger factor from *Psychrobacter frigidicola* TF_{Pf}, alternatively, exists in solution exclusively in monomeric form, unlike its dimeric TF_{Ec} counterpart [13]. Therefore, in the current report we present a comparative analysis of the chaperone activities of TF_{Ec} and TF_{Pf} *in vivo* in the refolding of heat-inactivated heterodimeric and monomeric bacterial luciferases and thus, shed light on the mechanism of action of this molecular chaperone.

MATERIALS AND METHODS

Bacterial strains and plasmids

E. coli strains K12 MG1655 (prototroph) and its deletion mutant PK202 *ΔdnaK14 ΔdnaJ14 dksA::kan* were kindly provided by Dr. E. A. Craig (USA) [14]. SG20250 Δ*lac*U169 *araD flbB relA* and SG22100 *clp*B*::kan* (other markers are the same as SG20250) *E. coli* K12 strains were kindly provided by S. Gottesman and Y. Zhou (USA) [15]. Cells were grown in Luria-Bertani (LB) broth and LB agar containing ampicillin (100 μg/ml), kanamycin (20 μg/ml), and chloramphenicol (15 μg/ml) at 28 °C. The pLeo1 (Amp^r) plasmid contains the *Photobacterium leiognathi luxCDABE* genes inserted into the pUC18 vector under the *lac* promoter [16]. The pKlux (Amp^r) hybrid plasmid contained the BglII-SalGI fragment with *Vibrio harveyi* 392 *luxAB* genes inserted into the pBlueScript KS II vector under the *lac* promoter [17]. The pXen4 plasmid that contains *luxAB* genes from *Photorhabdus luminescens* Zm1 was prepared via subcloning of the XhoI-PstI digested DNA fragment from the pXen7, containing the complete *P. luminescens lux* operon in pUC19 vector under the control of the *lac* promoter [18]. The pPho1 plasmid contains *Photobacterium phosphoreum luxAB* genes under the control of the *lac* promoter [19]. The p15aratighisPF plasmid (pACYC184 vector containing *P. frigidicola tig* gene under the control of the arabinose (*araB*) promoter) was provided by Dr. S. Robin (NUI Galway, Ireland) [13]. Plasmid pT7-mut3 (Amp^r) was kindly provided by Prof. E. A. Meighen (USA). It contains the *V. harveyi* monocistronic *luxAB* gene that encodes a luciferase enzyme that is monomeric due to mutagenesis of the intercistronic region between the *luxA* and *luxB* genes and the operon sequence immediately upstream to the *luxB* transcription start position. Thus, the ATG initiation codon of *luxB* is changed to a CAG codon for glutamine in the *mut3* gene, which was cloned in the expression vector pT7-5 [20]. Plasmid pTf16 (Cm^r) contains the *tig* gene of *E. coli* inserted into the pACYC184 vector under the control of the *araB* promoter and was obtained from the TAKARA BIO INC collection, Japan (Chaperone Plasmid Set).

Measurement of bioluminescence intensity

Bacterial luciferases catalyze the oxidation of an aliphatic aldehyde (RCHO) by oxygen (O_2) in the presence of the reduced form of flavinmononucleotide ($FMNH_2$) as follows:

$$FMNH_2 + RCHO + O_2 = FMN + RCOOH + H_2O + \text{light quantum } (\lambda_{max} = 490 \text{ nm})$$

The bioluminescence of cell suspensions (200 μl) was measured using a LM-01T (Immunotech) or a Biotox 7 (“Ekon” LTD) luminometer with the addition of 2 μl of 0.001% *n*-decanal alcohol solution (Sigma) as a substrate to the sample. In experiments with *E. coli* cells (pLeo1), the addition of *n*-decanal was not necessary. The intensity of bioluminescence (μV) was measured at room temperature.

Thermo-inactivation and refolding of luciferases *in vivo*

Thermal inactivation of luciferases was performed in a water bath at a fixed temperature. To suppress protein synthesis chloramphenicol (167 μg/ml) was added to the cell suspension. Refolding of luciferases was carried out at 22 °C. Bioluminescence was measured in time series aliquots. An aliquot of 200 μl of suspension was mixed with 2 μl of 0.001% *n*-decanal in ethanol for 30 s before measurement of bioluminescence. Preliminary heat shock was performed by incubating *E. coli* cells in LB medium in a thermostat at 42 °C for 30 min without the addition of chloramphenicol.

RESULTS

TF-dependent refolding of thermally inactivated luciferases

TF-dependent refolding of thermally inactivated luciferases was measured *in vivo* in *E. coli* PK202 *ΔdnaKJ14 dksA::kan* cells that contained hybrid plasmids expressing *luxAB* (pLeo1) genes and a *tig* gene from either the mesophilic bacterium *E. coli* (pTf16) or the psychrophilic bacterium *P. frigidicola* (p15aratighisPF). Prior to measurement, protein synthesis was inhibited via the addition of chloramphenicol (167 μg/ml) to cell suspensions, and to inactivate luciferase, cells were incubated in a water bath at 46 °C for 5 min. The dependence of TF-refolding (expressed as a percentage of the initial activity level) of thermally inactivated *P. leiognathi* luciferase on the concentration of L-arabinose is presented in Fig. 1a. The level of *P. leiognathi* luciferase refolding is extremely low (less than 1%) in the presence of lysates of *E. coli* PK202 Δ*dnaKJ14 dksA::kan* (pLeo1) cells, in the absence of TF-expressing plasmids. The presence of the pTf16 or p15aratighisPF plasmids, encoding the *E. coli* and *P. frigidicola* TFs, respectively, led to an increase in refolding up to a maximum of approximately 20-25% of the initial level. Significant differences are apparent between the luciferase refolding abilities of the TF_{Ec} and TF_{Pf} chaperones depending on their intracellular concentration. In the case of *E. coli* cells expressing TF_{Pf} from the p15aratighisPF plasmid, the refolding ability of cell lysates increased and reached an activity plateau at approximately 18% of the initial luciferase activity with increasing concentration of the L-arabinose (Fig. 1a). In the case of lysates of cells expressing TF_{Ec} from the pTf16 plasmid, the efficiency of luciferase refolding dropped rapidly with increasing concentration of the inducer in the medium, from a peak of 24% in the absence of arabinose to nearly zero in the presence of 20 mM L-arabinose (Fig. 1a). High refolding level in the absence of added arabinose is achieved due to leak-through transcription of the arabinose promoter [21]. It should be noted that the refolding activity of the psychrophile-derived TF was at its maximal level in the presence of 20 mM L-arabinose.

Fig. 1b shows the dependence of refolding of *P. leiognathi* luciferase on the duration of incubation with TF-expressing *E. coli* cell lysates at 22 °C. In order to achieve maximal refolding levels for the two chaperones, TF_{Ec} was expressed in the absence of additional arabinose whereas TF_{Pf} was expressed in the presence of 50 mM arabinose, based on the results shown in Fig. 1a. In the case of TF_{Ec}-expressing cells, luciferase refolding proceeded during the 120 min incubation period and reached up to 30% in the *E. coli* PK202 (pTf16, pF2) cells that had been grown at 28 °C to mid-exponential phase in the absence of L-arabinose. In the case of TF_{Pf}-expressing *E. coli* PK202 (p15aratighisPF, pF2) grown at 28 °C to mid-exponential phase in the presence of 50 mM L-arabinose, Fig. 1b reveals a very similar luciferase refolding, albeit with a slightly reduced reaction rate and slightly lower maximum level of refolding in the case of the TF_{Pf} chaperone.

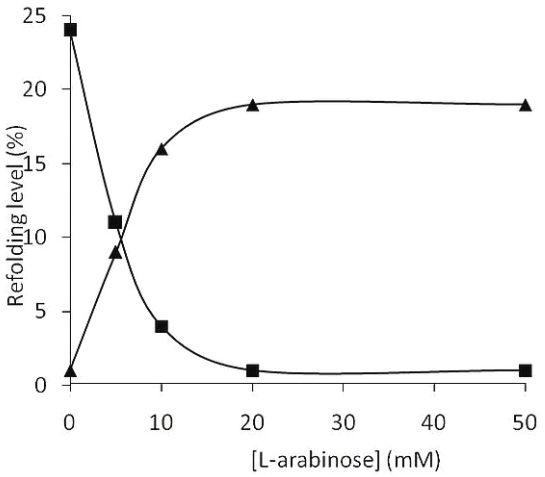

Fig. 1a. Maximum level of TF-mediated refolding of heat-inactivated *P. leiognathi* luciferase with increasing concentration of L-arabinose used as inducer: ▲ - TF_{Pf}; ■ - TF_{Ec}. *E. coli* PK202 *dnaKJ14 dksA*::*kan* cells containing pLeo1 (*P. leiognathi* luciferase) and either pTf16 (TF_{Ec}) or p15aratighisPF (TF_{Pf}) plasmids were incubated for 14 h at 22 °C after the addition of L-arabinose at concentrations from 0-50 mM to induce TF expression. Protein synthesis was stopped by the addition of chloramphenicol (167 μg/ml) and cells were incubated at 46 °C for 5 min to inactivate the luciferase. Refolding of thermally inactivated luciferase was carried out at 22 °C. The measured luciferase activity is presented as a percentage of the activity prior to inactivation.

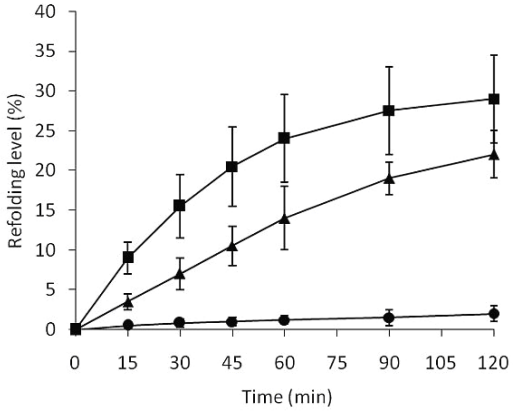

Fig. 1b. Refolding of thermo-inactivated *P. leiognathi* luciferase using lysates of *E. coli* PK202 *dnaKJ14dksA*::*kan* cells expressing ■ - TF_{Ec} (pLeo1, pTf16); ▲ - TF_{Pf} (pLeo1, p15aratighisPF); ● - no additional TF (pLeo1). *E. coli* PK202 *dna*KJ14 *dks*A::*kan* (pLeo1, pTf16) cells were grown without L-arabinose addition, while *E. coli* PK202 *dnaKJ14 dksA*::*kan* (pLeo1, p15aratighisPF) cells were grown with addition of the L-arabinose to the final concentration of 50 mM. After inhibition of protein synthesis via the addition of chloramphenicol (167 μg/ml) and inactivation of luciferase by incubation at 46 °C for 5 min, luciferase refolding was carried out at 22 °C. The enzymatic activity of luciferase is expressed as a percentage of the initial activity level.

It has previously been described that overproduction of TF_{Ec} in recombinant expression experiments exerts a lethal effect on the host bacterial cells [21, 22]. Fig. 2 shows the growth curves of *E. coli* PK202 cells, containing TF_{Pf}-encoding p15aratighisPF or TF_{Ec}-encoding pTf16 plasmids, in the presence of varying arabinose concentrations in the medium. As can be seen, very significant inhibition of the growth is observed with plasmid pTf16 encoding the dimeric *E. coli* TF in the presence of 50 mM L-arabinose, while the growth of bacterial cells containing the p15aratighisPF plasmid, encoding monomeric TF_{Pf}, is unchanged in the presence of the same L-arabinose concentration. Therefore, it can be proposed that accumulation of the dimeric *E. coli* chaperone to higher concentrations is responsible for the reduction in chaperone activity and the toxic effect on the cell physiology, whereas the monomeric TF_{Pf} molecule is tolerated better by the expressing cells at comparable protein levels.

Previously we reported that the efficiency of DnaKJE- and TF_{Ec}-dependent refolding of different luciferase enzymes decreased with increasing thermostability of the enzymes [10, 23]. Fig. 3 shows the renaturation of luciferases of differing

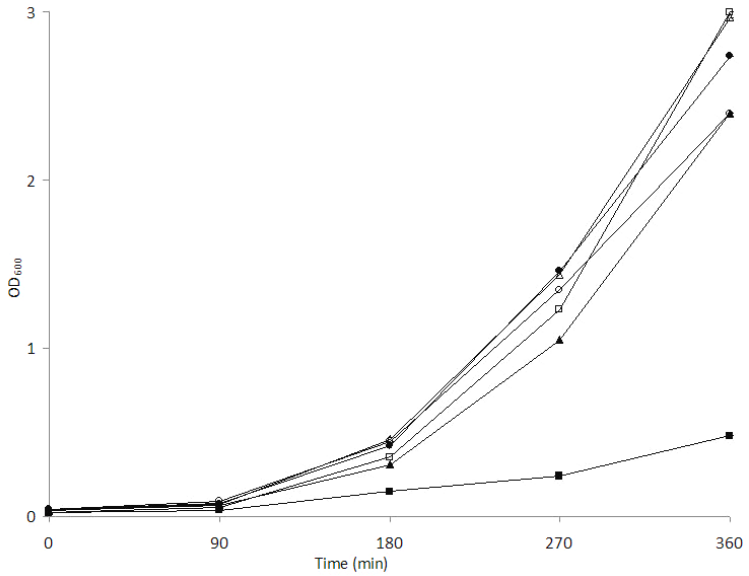

Fig. 2. Growth curves of *E. coli* PK202 cultures containing TF_{Ec}- or TF_{Pf}-encoding plasmids in the presence or absence of L-arabinose inducer in the medium. ○ – *E. coli* PK202 cells (no additional TF genes); ● - *E. coli* PK202 cells with 50 mM L-arabinose added; *E. coli* cells encoding: □ - TF_{Ec} , ■ - TF_{Ec} with 50 mM L-arabinose added; *E. coli* cells encoding: Δ - TF_{Pf}; ▲ - TF_{Pf} with 50 mM L-arabinose added.

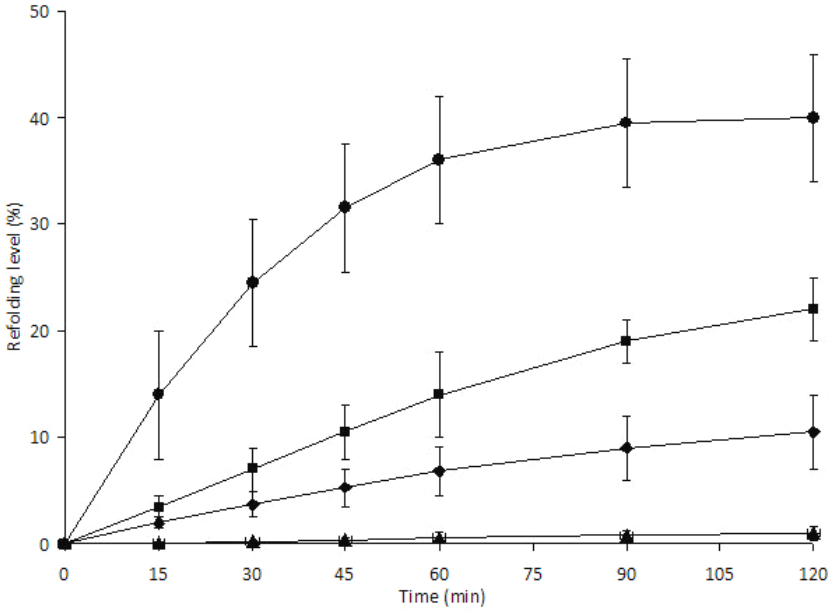

Fig. 3. TF_{Pf} - dependent refolding of thermo-inactivated luciferases by lysates of *E. coli* PK202 cells expressing TF_{Pf} and the following luciferases of differing thermal sensitivities: ● - *P. phosphoreum* (pPho1); ■ - *P. leiognathi* (pLeo1); ♦ - *V. harveyi* (pKlux); ▲ - *P. luminescence* (pXen7).
Cell growth and refolding conditions were as for Fig. 1b while cell suspensions were heated as follows for thermal inactivation of luciferases prior to refolding: *P. phosphoreum*, *P. leiognathi* - 46 °C, 5 min; *V. harveyi* - 47 °C, 15 min; *P. luminescence* - 48 °C, 15 min. Luciferase activity is plotted as a percentage of the initial activity level.

thermal stabilities in the presence of TF_{Pf}. Experiments were carried out by co-expressing TF_{Pf} from p15aratighisPF with *luxAB* luciferase genes on the following plasmids: pPho1 (from *P. phosphoreum*), pLeo1 (*P. leiognathi*), pKlux (*V. harveyi*) and pXen4 (*P. luminescens*) in *E. coli* PK202. According to previous measurements of thermal inactivation rate constants, thermolability of the luciferase enzymes investigated in this work decreases in the order *P. phosphoreum* > *P. leiognathi* > *V. harveyi* > *P. luminescens* luciferases [19]. In the case of TF_{Ec}, refolding of thermolabile luciferase from *P. leiognathi* reached significantly higher level (up to 30-40% of the initial enzyme activity) than in the case of the more thermostable *V. harveyi* (15%) and particularly *P. luminescens* (less than 1%) luciferases [10]. As can be seen from the data presented in Fig. 3, the TF_{Pf} chaperone exhibited a similar pattern of activity to that previously associated with TF_{Ec}, with a reduced refolding yield upon increasing luciferase thermostability. In the case of the most thermolabile luciferase from *P. phosphoreum*, TF_{Pf}-mediated refolding reached 40-50% of the original activity, while the yield for the most thermostable *P. luminescens* luciferase achieved a maximal activity level of only approximately 1%.

To investigate the influence of the quaternary structure of the substrate protein on the ability of TF_{Pf} to assist its refolding, we used the heterodimeric *V. harveyi* bacterial luciferase (encoded on the pKlux plasmid containing *luxAB* genes) and an engineered monomeric version thereof (on plasmid pT7-mut3). These two forms of the luciferase exhibit almost identical temperature sensitivities (Fig. 4) and activities [20], even though the former has been shown to exist in a largely dimeric form and the latter exclusively as a monomer. DnaKJE- dependent refolding of the heat-inactivated luciferase revealed almost identical refolding kinetics and maximal refolded level of both the luciferase isoforms, at approximately 40% of the initial level of activity (Fig. 5a). In Fig. 5b, however, TF-dependent refolding of the same heat-inactivated *V. harveyi* luciferase isoforms

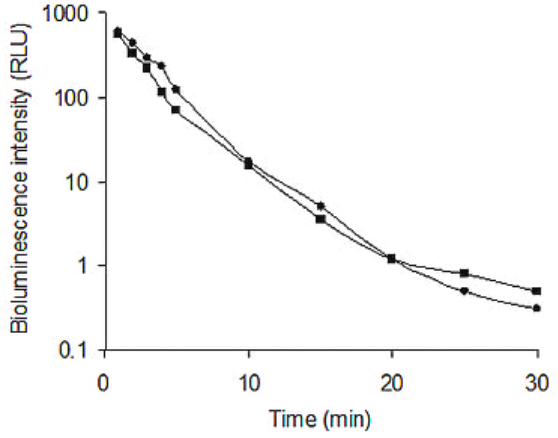

Fig 4. Thermo-inactivation of *Vibrio harveyi* luciferase and an engineered monomeric version thereof at 46 °C. *E. coli* PK202 Δ*dna*KJ14 *dks*A::*kan* (pKlux) cells were grown at 28 °C until the mid-exponential growth phase. Protein synthesis was stopped by the addition of chloramphenicol (167 μg/ml). ■ - monomeric form of *V. harveyi* luciferase (pT7-mut3); ● - heterodimeric form of *V. harveyi* luciferase (pKlux).

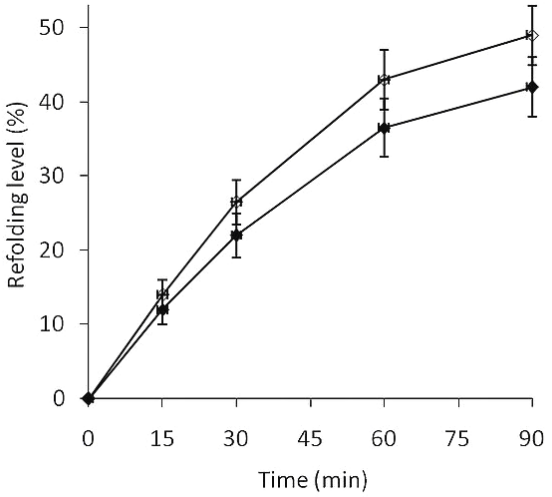

Fig. 5a. DnaKJE-dependent refolding of thermo-inactivated bacterial heterodimeric (αβ; ♦) and monomeric (◊) forms of *V. harveyi* luciferase expressed in *E. coli* MG1655 *tig::kan* cells.

demonstrates that TF_{Ec} (which forms dimers in solution) and TF_{Pf} (which exists in solution only in the form of a monomer) both promote refolding only of the heterodimeric luciferase variant. While refolding yields of up to 10% (TF_{Pf}) and 20% (TF_{Ec}) were achieved with the heterodimer under the assay conditions, both chaperones were almost completely ineffective in restoring the activity of the monomeric form of the same protein.

Disaggregation and refolding of proteins in *E. coli* are associated with the activity of the ATP-dependent DnaKJE-ClpB bichaperone system. ClpB is not directly involved in refolding of substrates, but acts to disaggregate proteins. This role of ClpB is especially important in the disaggregation of large aggregates, which the DnaKJE system alone is not able to destroy [24]. Recently we have reported that in the absence of ClpB, the chaperone activity of TF_{Ec} increases in *E. coli* cells [10]. Data presented in Fig. 6 indicate that this phenomenon is conserved in TF_{Pf}. Measurement of the level of refolding of heat inactivated *P. leiognathi* luciferase using TF_{Pf}-expressing *E. coli* SG22100 *clpB::kan* cell extracts revealed that the maximum level of the TF_{Pf}-dependent refolding reached approximately 40%, whereas in the corresponding *E. coli* $clpB^+$ strain, the maximal

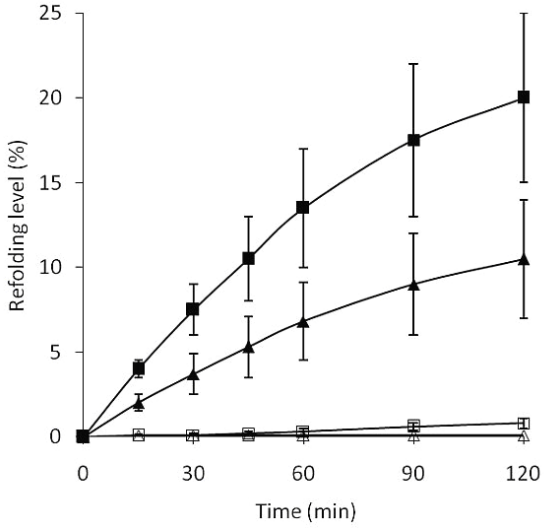

Fig. 5b. TF-dependent refolding of thermo-inactivated heterodimeric (αβ) and monomeric forms of *V. harveyi* luciferase expressed in *E. coli* PK202 cells: ■ - TF_{Ec} - dependent refolding of heterodimeric *V. harveyi* luciferase; □ - TF_{Ec} - dependent refolding of monomeric *V. harveyi* luciferase; ▲ - TF_{Pf} - dependent refolding of heterodimeric form of *V. harveyi* luciferase; Δ - TF_{Pf} - dependent refolding of monomeric *V. harveyi* luciferase. *E. coli* PK202 *dna*KJ14 *dks*A::*kan* (pKlux, pTf16) cells were grown without L-arabinose addition, while *E. coli* PK202 *dnaKJ14 dksA*::*kan* (pKlux, p15aratighisPF) cells were grown with addition of the L-arabinose to the final concentration of 50 mM.

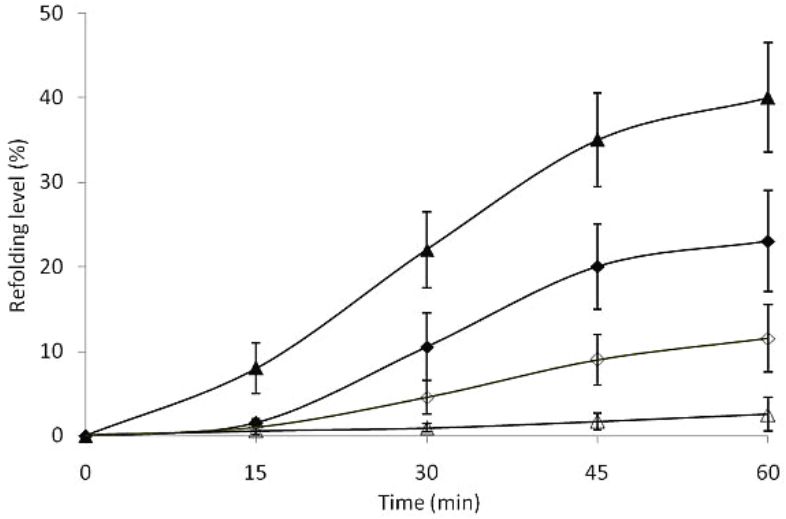

Fig. 6. The influence of ClpB chaperone on TF_{Pf}-dependent refolding of heat-inactivated *P. leiognathi* luciferase. The heat-inactivated luciferase was incubated with cell extracts of: ◊ - *E. coli* SG20250 *dnaKJ*$^+$*clpB*$^+$ (pLeo1) cells; ♦ - *E. coli* SG20250 *dnaKJ*$^+$*clpB*$^+$ (pLeo1, p15aratighisPF) cells; Δ - *E. coli* SG22100 *dnaKJ*$^+$*clpB*::*kan* (pLeo1); ▲ - *E. coli* SG22100 *dnaKJ*$^+$*clpB*::*kan* (pLeo1, p15aratighisPF) cells.

level of refolded luciferase reached was only 20%. We propose that this indicates that the ClpB chaperone competes with trigger factor for substrate binding and thus reduces the refolding activity of the latter, in contrast to the DnaKJE chaperone family that operates in a complex with ClpB in the cell [24].

DISCUSSION

Thermal inactivation of bacterial luciferases leads to exposure of hydrophobic epitopes with which DnaKJE family chaperones interact to refold the proteins [25]. Inability to refold denatured luciferases or refolding with a very low efficiency, is typical in the case of TF chaperones when monomeric luciferases are used as a substrate. This indicates a requirement of TF for a particular spatial, or rather quaternary, structure of the protein substrate for successful contact with the chaperone and folding. This assumption is supported by the data presented in [7], in which TF_{Ec} has been demonstrated to form complexes with various oligomeric proteins and to stabilize dimeric and oligomeric protein forms in the bacterial cytoplasm.

Chromosomally-encoded TF_{Ec} achieved refolding of only approximately 0.5-1.0% of the heat-inactivated *P. leiognathi* luciferase that was expressed in the same *E. coli* PK202 *ΔdnaKJ14 dksA::kan* cells, indicating the amounts of TF molecules expressed from the chromosomal gene were insufficient to promote efficient refolding. Introduction of an additional plasmid-encoded *tig* gene under the control of an inducible arabinose promoter increased the number of TF molecules in the cell and in addition, the efficiency of luciferase refolding, even in the absence of active induction of the plasmid-encoded P_{BAD} promoter. A significant reduction in the refolding efficiency occurred with a further increase in the TF_{Ec} intracellular concentration upon addition of arabinose, however, which is a previously reported peculiarity of TF from mesophilic bacteria and fundamentally different from the DnaKJE chaperone system, with which refolding efficiency reaches a plateau at increased DnaKJE concentrations rather than decreasing at increased chaperone levels [26]. It should be noted that an approximate four-fold increase in the intracellular TF concentration in *E. coli* cells leads to the reduced viability and even cell death [22]. As apparent from the present study, the increase of TF_{Ec} concentration in *E. coli* PK202 *ΔdnaKJ14 dksA::kan* cells is accompanied by a loss of cell viability and a concomitant reduction in the efficiency of the refolding process. The concentration of TF_{Ec} has also been demonstrated

to be crucial to the efficient refolding *in vitro* of GAPDH protein denatured using urea, with a significant reduction in renaturation of the enzyme at TF_{Ec} concentrations above optimal [9, 13].

It is hypothesized that these effects are determined by the peculiarities of the TF_{Ec} quaternary structure. TF_{Ec} exists in the form of a monomer and exhibits chaperone activity at low concentrations in the *E. coli* cytoplasm. This ability to form a complex with denatured proteins and release them in their native form disappears upon formation of dimers of TF_{Ec}, which occurs with increased concentration of the chaperone. As a result, proteins associated with TF_{Ec} are not able to escape from the complex, leading to reduced refolding efficiency. If proteins that are involved in cell division, such as FtsZ, a key cell division protein with which TF is known to form a complex [22], are bound by TF_{Ec} under the same conditions then inhibition of cell growth results. In this study, we have demonstrated that the chaperone activity of TF_{Pf} from the psychrophilic bacteria *P. frigidicola*, which exists exclusively in the form of a monomer irrespective of concentration, showed no reduction in refolding efficiency upon increasing its intracellular concentration. Furthermore, an increase in the intracellular concentration of TF_{Pf} to levels characterized by a dramatic growth inhibitory effect in the case of the mesophilic *E. coli* chaperone did not result in a lethal effect on the expressing cells. Thus, it is proposed that the dimeric form of TF determines its toxic effect on its host cells at high intracellular concentrations. Despite these structural and functional differences, it should be noted that TF_{Ec} from the mesophilic bacteria *E. coli* and TF_{Pf} from the psychrophilic bacteria *P. frigidicola*, while differing in their quaternary structures at higher concentrations [13], exhibited the same: 1) inability to refold a monomeric luciferase variant, demonstrating the importance of quaternary structure of protein substrates also for the TF folding process; 2) reduced refolding efficiency with increased thermostability of the substrate molecule, and 3) increased refolding efficiency in *E. coli clp*B^- compared with wild-type *E. coli* strains, suggesting competition of ClpB with the TFs for substrate binding and folding in *E. coli*.

CONFLICT OF INTEREST STATEMENT

There are no conflicts of interest.

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