Trigger Factor from the Psychrophilic Bacterium *Psychrobacter frigidicola* Is a Monomeric Chaperone[▽]

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In eubacteria, trigger factor (TF) is the first chaperone to interact with newly synthesized polypeptides and assist their folding as they emerge from the ribosome. We report the first characterization of a TF from a psychrophilic organism. TF from *Psychrobacter frigidicola* (TF_{pf}) was cloned, produced in *Escherichia coli*, and purified. Strikingly, cross-linking and fluorescence anisotropy analyses revealed it to exist in solution as a monomer, unlike the well-characterized, dimeric *E. coli* TF (TF_{ec}). Moreover, TF_{pf} did not exhibit the downturn in reactivation of unfolded GAPDH (glyceraldehyde-3-phosphate dehydrogenase) that is observed with its *E. coli* counterpart, even at high TF/GAPDH molar ratios and revealed dramatically reduced retardation of membrane translocation by a model recombinant protein compared to the *E. coli* chaperone. TF_{pf} was also significantly more effective than TF_{ec} at increasing the yield of soluble and functional recombinant protein in a cell-free protein synthesis system, indicating that it is not dependent on downstream systems for its chaperoning activity. We propose that TF_{pf} differs from TF_{ec} in its quaternary structure and chaperone activity, and we discuss the potential significance of these differences in its native environment.

The folding of cytosolic proteins is coordinated by three chaperone systems in eubacteria: trigger factor (TF), DnaK and its cofactors DnaJ and GrpE, and GroEL, together with its cofactor GroES. As TF alone interacts with the ribosomes, it is the first chaperone to bind newly synthesized polypeptides and assist their folding, whereupon it passes them to downstream chaperone systems (12). It has recently been shown that, for multidomain proteins, TF and the DnaK system cooperate to slow down folding and cause a shift to a posttranslational folding mode (24). TF also plays an important role in the regulation of protein translocation due to its position at the ribosome exit tunnel (1, 24).

TF is highly abundant in *E. coli* (up to 40 μ M), with most TF molecules existing in an equilibrium between its monomeric and dimeric states in the cytosol (28). The high cytosolic concentration of TF has recently been linked to a distinct functional role of the chaperone in maintaining newly translated polypeptides in a folding-competent state in the *E. coli* cytoplasm (35). A distinct antichaperone activity has also been described for TF, however, whereby substoichiometric concentrations of TF lead to increased polypeptide aggregation, whereas high TF/polypeptide ratios can also delay folding as the chaperone maintains polypeptides in a non-native state without promoting their complete refolding (9).

E. coli TF (TF_{ec}) is composed of an N-terminal ribosome-binding tail (domain I), an internal domain with peptidyl-prolyl *cis/trans* isomerase (PPIase) activity (domain II), and a C-terminal domain III that is involved in its chaperone activity

(26). The importance of the PPIase domain remains unclear as TF binds non-native nascent polypeptides lacking proline residues (29), while its PPIase activity is also not required for its chaperoning function (15).

Cold-adapted microorganisms, such as psychrophiles and psychrotrophs, are capable of growth at temperatures as low as 0°C. Few molecular chaperones from such bacteria have been investigated in detail, and although TF homologues have been identified in the genomes of psychrophiles such as *Pseudoalteromonas haloplanktis*, *Psychrobacter arcticus*, or *Shewanella frigidimarina*, neither their biochemistry nor their importance in cold adaptation has been characterized to the same degree as for GroEL (36). Here, therefore, we present the first detailed investigation of a cold-adapted TF homologue, from *Psychrobacter frigidicola* (TF_{pf}), and reveal its unexpected behavior: TF_{pf} displays no dimerization, and, importantly, does not exhibit the in vitro chaperonelike holding activity characteristic of TF.

MATERIALS AND METHODS

Bacterial strains and plasmids. *P. frigidicola* (DSM 12411) was obtained from DSMZ, Braunschweig, Germany and grown in Marine broth. *E. coli* TOP10 (Invitrogen) and *E. coli* BL21(DE3) (Novagen) strains were used for cloning and recombinant protein production, respectively, while *E. coli* W3110 and *E. coli* W3110 Δ tig (6) were used for protein translocation studies. The pLG6scFv2H12 vector was used for expression of the 2H12 scFv antibody fragment (7). pACYC184 and pBADHisB were obtained from NEB Biolabs and Invitrogen, respectively.

Cloning of *P. frigidicola* tig gene. DNA manipulations were carried out according to the method of Sambrook and Russell (30a). A 3' partial sequence of the *tig* gene was initially amplified from *P. frigidicola* genomic DNA by PCR using degenerate primers. The 5' region was then amplified with the primers TFS1 (CTTGCTACGCGCAGCATTTTGTATTCG) and TFS2 (GCCGCTTTAC CAGCCAATTCTTCAGCTTGG) using an LA Taq PCR in vitro cloning kit (Takara Corp.). The complete arabinose promoter cassette from pBADHisB was amplified and cloned into HindIII-digested pACYC184 to yield the expression

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vector p15ara. Finally, complete *tig* genes from *P. frigidicola* (*tig_{PF}*) and *E. coli* (*tig_{EC}*) were amplified by PCR with or without a C-terminal His₆ tag and 5'-NdeI and 3'-PstI flanking restriction sites and cloned using these sites into p15ara to yield p15aratighisPF and p15aratighisEC vectors. The *tig_{PF}* and *tig_{EC}* genes were subcloned without His₆ tags as BamHI/PstI and BamHI/XhoI fragments, respectively, into pBADHisB.

Expression and purification of TFs from *E. coli* and *P. frigidicola*. TF_{PF} and TF_{EC} were expressed from p15aratighisPF and p15aratighisEC, respectively, in *E. coli* BL21(DE3). Expression was induced at 25°C in Luria-Bertani medium by the addition of 1 mg of arabinose/ml. The cells were harvested, and proteins were extracted in buffer A (100 mM NaCl, 10 mM imidazole, 2 mM β-mercaptoethanol, 20 mM Tris-HCl [pH 8.0]) with the addition of 5% CellLytic reagent (Sigma-Aldrich), 0.3 kU of rLysozyme (Invitrogen)/ml, and 50 μg of DNase I (Sigma-Aldrich)/ml. After centrifugation at 14,000 rpm for 30 min at 4°C, the samples were loaded onto a Ni²⁺-NTA HiTrap cartridge (GE Healthcare), followed by stepwise washing of the resin with buffer A containing NaCl from 100 to 500 mM. The resin was then washed with buffer A containing imidazole from 20 to 250 mM, and fractions containing eluted TF were pooled, dialyzed against 20 mM Tris-HCl (pH 8.0) and 1 mM dithiothreitol at 4°C overnight, concentrated, and snap-frozen in liquid nitrogen. Fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting (16).

GAPDH refolding assay. Refolding of denatured GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was performed based on Huang et al. (10). Denatured enzyme was diluted 50-fold in GAPDH buffer (0.1 M potassium phosphate [pH 7.5], 1 mM EDTA, 5 mM dithiothreitol) containing 0.625, 1.25, 2.5, 3.75, 5, 7.5, 10, or 20 μM TF. The reaction was maintained at 4°C for 30 min, followed by 180 min at 25°C or 15°C. The GAPDH activity was measured by mixing 1-μl reaction aliquots with 99 μl of GAPDH buffer in the presence of 700 μM β-NAD (Sigma-Aldrich) and 700 μM D-L-glyceraldehyde-3-phosphate (Sigma-Aldrich). This was followed by measurement of the synthesized NADH at 340 nm (Tecan microplate spectrophotometer; Genios). The data were fitted to a single exponential equation (Prism program; GraphPad Software), and the relative activity recovered was calculated by dividing the rate constant for refolded GAPDH by the rate constant determined with nondenatured enzyme.

RCM-RNase T₁ refolding assay. RNase T₁ from *Aspergillus oryzae* (Sigma-Aldrich) was denatured, reduced, and carboxymethylated using dithiothreitol as the reducing agent, according to the published protocol (27). Refolding was induced by dilution of reduced and carboxymethylated RNase T₁ (RCM-RNase T₁) to a final concentration of 0.5 μM in 1.6 M NaCl–0.1 M Tris-HCl (pH 8.0) in a spectrofluorometer (Cary Eclipse; Varian). TF_{PF} or TF_{EC} was included in the reaction mixture at 0.1 or 0.2 μM. The data were fitted to a single-exponential equation (Prism program).

Glutaraldehyde cross-linking. TF_{PF} or TF_{EC} (5 μg) was incubated at concentrations of 0.5, 2.5, 10, 20, or 30 μM for assays at 25°C and at 0.15, 0.625, 2.5, 10, or 20 μM for assays at 15°C in 20 mM HEPES (pH 7.5)–100 mM NaCl–1 mM EDTA for 25 min. Cross-linking was initiated by the addition of 0.1% glutaraldehyde, and the reaction was quenched after 15 min by incubation in 100 mM Tris-HCl (pH 8) for 10 min at the relevant assay temperature. Samples were precipitated for 10 min on ice using 10% trichloroacetic acid and centrifuged at 14,000 rpm and 4°C for 30 min. Pellets were solubilized in 8 M urea in phosphate-buffered saline (PBS), and samples were analyzed by SDS-PAGE (10% gel) and Coomassie brilliant blue staining.

Fluorescence anisotropy. Both TF_{PF} and TF_{EC} were labeled by using the Alexa Fluor 532 monoclonal antibody kit (Molecular Probes) according to the manufacturers' protocol. The procedure was optimized to achieve a labeling ratio of approximately one fluorophore per protein. The concentration of protein (*M*) was calculated by using UV-visible absorption spectroscopy and the following formula: $M = (A_{280} - (A_{530} \times 0.09)) / \epsilon_{280}$, where A_{280} is the absorbance at 280 nm and A_{530} is the absorbance at 530 nm, with ϵ_{280} TF_{PF} = 10,240 cm⁻¹ M⁻¹ and ϵ_{280} TF_{EC} = 15,930 cm⁻¹ M⁻¹. Labeling ratios (LR) of 1.1 for TF_{PF} and 1.3 for TF_{EC} were calculated by using the following formula: $LR = A_{530} / (81,000 \times M)$.

Alexa Fluor 532-labeled TF_{PF} or TF_{EC} (50 nM) was titrated with increasing concentrations of the corresponding unlabeled TF, followed by detection of TF homodimerization by observation of the change in fluorescence anisotropy (*r*). Fluorescence was measured in a Cary Eclipse spectrofluorometer (Varian) equipped with a variable temperature, Peltier-controlled cell holder (TLC-50F Quantum Northwest) and manual excitation and emission polarizers. The excitation wavelength was 532 nm, and the emission wavelength was 554 nm. The excitation and emission slits were set at 5 and 20 nm, respectively. Anisotropy was measured by using an L-format detection configuration. All titration measurements were performed in 20 mM Tris-HCl–150 mM NaCl–1 mM EDTA buffer (pH 7.5), and samples were incubated at 25°C. Experiments were per-

formed in triplicate, and data were fitted (Origin Software, version 7.5; Origin-Lab) by using the following equation:

$$r_{\text{obs}} = \frac{r_{\text{min}} \times 2 \times [TF_{\text{lab}}] + (F \times r_{\text{max}} - r_{\text{min}}) \times (a - \sqrt{a^2 - 4 \times [TF_{\text{lab}}] \times [TF]})}{2 \times [TF_{\text{lab}}] + (F - 1) \times (a - \sqrt{a^2 - 4 \times [TF_{\text{lab}}] \times [TF]})}$$

where r_{obs} is the observed change in anisotropy, TF_{lab} is the fluorescently labeled TF, $a = K_d + [TF_{\text{lab}}] + [TF]$, and K_d is the dissociation constant. Since increased concentrations of unlabeled TFs led to an increase in fluorescence intensity, a correction factor (*F*), where $F = \text{intensity}_{\text{max}} / \text{intensity}_{\text{min}}$, was applied. The $\text{intensity}_{\text{max}}$ is understood to be the emission intensity from the bound form, while $\text{intensity}_{\text{min}}$ is the intensity of the unbound form (17).

TF-assisted cell-free protein synthesis. The gene encoding the 2H12 anti-domoic acid scFv antibody fragment was amplified from pIG6scFv2H12 (7), and a T7 promoter was added by overlap PCR. A recombinant M2 FLAG tag was also added at the C terminus of the encoded scFv to detect the synthesized protein. Cell-free translation of the scFv was carried out using 2 pmol/μl of linear template in the PURE System (Post Genome Institute Co., Ltd.), according to the manufacturer's instructions. Where indicated, TF_{PF} or TF_{EC} was added at 2.5, 5, 10, or 20 μM. After incubation for 1 h at 37°C, an equal volume of water was added, and samples were centrifuged at 14,000 rpm and 4°C for 20 min. Pellets were solubilized in 8 M urea in PBS, and soluble and insoluble fractions were analyzed by SDS-PAGE and Western blotting with an anti-M2 FLAG antibody. The binding of scFv molecules was tested by enzyme-linked immunosorbent assay (ELISA) according to published procedures (7). Briefly, wells of a 96-well microtiter plate were coated with 10 μg of domoic acid (Calbiochem, United Kingdom)/ml conjugated to ovalbumin, followed by blocking with 5% milk powder in PBS. Detection of bound scFv was carried out with an anti-M2 FLAG antibody, followed by the addition of an anti-mouse peroxidase-conjugated antibody and TMB substrate (Sigma-Aldrich).

scFv2H12 export monitoring. *E. coli* BL21(DE3) cells containing pIG6scFv2H12 and either p15aratigPF or p15aratigEC plasmids were grown in LB medium containing 1 mg of arabinose/ml at 37°C. Alternatively, *E. coli* W3110 or *E. coli* W3110 Δ*tig* cells containing pIG6scFv2H12 and pBADHisB, pBADtigPF, or pBADtigEC constructs were grown at 37°C in LB medium containing 0.05 to 0.125 mg of arabinose/ml. When the optical density at 600 nm reached ~0.5, scFv expression was induced by the addition of 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside), followed by growth at 25°C for 3 h. The cells were harvested, and protein was extracted with 5% CellLytic reagent, 0.3 kU of rLysozyme/ml, and 50 μg of DNase I/ml in PBS. Insoluble protein fractions were pelleted by centrifugation at 14,000 rpm and 4°C for 20 min and solubilized in 8 M urea in PBS. Soluble and insoluble fractions were analyzed by SDS-PAGE and Western blotting with an anti-His₆ tag peroxidase-conjugated antibody.

RESULTS AND DISCUSSION

Isolation of the *tig* gene from *P. frigidicola*. The *tig* gene from *P. frigidicola* was cloned and sequenced, and its sequence was confirmed in a further, independent cloning before deposition in GenBank under accession no. EU221522. The 1,341-bp open reading frame encodes a predicted protein of 447 amino acids with an expected mass of 50,629 Da and 58.3% sequence homology with TF_{EC}. Despite this relatively low primary amino acid sequence identity, however, the predicted TF_{PF} protein reveals a secondary structure organization very similar to that of TF_{EC} (Fig. 1). Conserved, putative domains corresponding to the three known domains found in TF's distinctive "crouching dragon" conformation (22) were identified: the TF_{PF} N-terminal domain exhibits a characteristic TF signature sequence (⁴⁷GFRKGNVP; underlined residues are conserved) essential for ribosome binding (14), while the conserved residues Phe171, Phe180, Met197, Ile198, Phe201, Val218, Phe220, Pro221, Tyr224, and Phe236 (Fig. 1, diamonds) in the PPIase catalytic site, of which Ile198, Tyr224, and Phe236 are strictly conserved in the FK506-binding protein (FKBP) PPIase family, were also identified in the predicted *P. frigidicola* protein sequence (Fig. 1). In addition to the overall organization of TF domains I, II, and III, the *P. frigidicola* chaperone

FIG. 1. Sequence alignment of trigger factors from *P. frigidicola* and *E. coli*. The predicted amino acid sequences of *E. coli* and *P. frigidicola* TFs were aligned using CLUSTAL W (34). Invariant residues are indicated on a black background, and similar residues are indicated on a gray background. Predicted secondary structures, determined with PORTER (30), are shown above the TF_{PF} and below the TF_{EC} sequences: α -helices are drawn as cylinders, β -strands are drawn as arrows, and other elements are drawn as solid lines. The conserved ribosome-binding motif is boxed in dashed lines, and residues conserved in the PPIase catalytic site are indicated by diamonds.

exhibits an extended cluster of glutamine residues at its C-terminal end. Similar clusters of amino acids have been noted previously in TF proteins from psychrobacteria, but their role is unclear.

PPIase activity of TF_{PF}. TF_{PF} and TF_{EC} were purified to homogeneity using Ni²⁺-IMAC (immobilized metal affinity chromatography). Although the PPIase activity of TF is not essential for its in vivo function and appears to be unnecessary also for its chaperone activity (13), the FKBP-like substrate-binding pocket of TF is nevertheless known to play a role in the recognition of and interaction with hydrophobic patches in nascent or unfolded proteins (29). The PPIase activity of TF_{PF} was investigated, therefore, by following refolding of RCM-T1, which is limited by the rate of *trans* to *cis* isomerization of its peptidyl-prolyl bonds at Pro39 and Pro55 (31). After denaturation at 15°C (pH 8.0) in the absence of salt, RCM-T1 was

found to refold slowly into its active form in 1.6 M salt in the presence of TF_{PF} (Fig. 2). This refolding confirmed the presence of a PPIase activity in the *P. frigidicola* chaperone, as predicted from analysis of the primary sequence.

TF_{PF} does not present the holding-chaperone activity characteristic of TF_{EC}. The chaperone activity of TF can be monitored, independently of its peptidyl-prolyl isomerization activity, by measuring its ability to refold GAPDH in vitro (10, 13, 15). In the present study, GAPDH exhibited a low level of spontaneous refolding (ca. 10%) (Fig. 3A), while the yield of reactivated enzyme increased with increasing TF_{EC} concentration, up to a maximum of 40% at 3.75 μ M TF_{EC}. At higher chaperone concentrations, however, the reactivation yield decreased dramatically, to as low as 3% in the presence of 20 μ M TF_{EC}. This well-known phenomenon, whereby TF_{EC} protects non-native substrates from aggregating but without promoting

FIG. 2. PPIase activity of *P. frigidicola* and *E. coli* TFs. PPIase activity of TFs against the RCM-RNase T₁ model substrate. RCM-RNase T₁ refolding was monitored by an increase in intrinsic tryptophan fluorescence at 320 nm after excitation at 268 nm. Symbols: ●, TF_{Pf}; ■, TF_{Ec}; ×, buffer.

their complete folding, has been characterized extensively with substrates such as lysozyme (9), carbonic anhydrase II, and creatine kinase (21), as well as GAPDH (10), and is proposed to result from the formation of a stable complex between partially folded substrate and dimeric TF (10, 20).

In the case of TF_{Pf}-assisted refolding, the GAPDH reactivation yield again increased with increasing TF concentration, until a plateau at almost 40% of GAPDH activity was reached at 5 μ M TF_{Pf}. Surprisingly, however, the slowdown in reactivation apparent at higher concentrations of TF_{Ec} was not observed with TF_{Pf}, which suggests that TF_{Pf} cannot stably bind GAPDH folding intermediates in the same manner as TF_{Ec}. In this regard, the behavior of TF_{Pf} is more similar to that of “true” chaperones such as GroEL (38), DsbC (4), and PDI (2) than to TF_{Ec}. In order to further investigate this phenomenon, GAPDH refolding was also analyzed at 15°C, the optimal growth temperature of *P. frigidicola*. Although the spontaneous refolding of GAPDH was slightly increased at the lower temperature (Fig. 3B) and the holding effect of TF_{Ec} was now clearly evident at chaperone concentrations as low as 2.5 μ M, TF_{Pf} exhibited a pattern of activity very similar to that observed at 25°C (Fig. 3A), but with a slightly increased (ca. 44%) maximal recovery of GAPDH activity.

Since the suppression of GAPDH reactivation by TF_{Ec} has been attributed to the ability of TF_{Ec} to dimerize and complex with partially folded GAPDH, we compared the dimerization ability of both TFs in order to determine the basis for the difference between the polypeptide-holding properties of the two chaperones.

TF_{Pf} does not form dimers in vitro. Previously published studies indicate that TF_{Ec} exists in a monomer-dimer equilibrium in solution (28). In order to investigate TF_{Pf} dimerization, therefore, we initially utilized a glutaraldehyde cross-linking approach to determine the quaternary structure of the purified chaperone at different concentrations.

As expected, TF_{Ec} exhibited extensive, concentration-dependent dimerization, leading to the appearance of a dominant product with abnormal migration that corresponds to ~175 kDa (Fig. 4A, star). Higher-molecular-weight products visible in the gel at higher TF_{Ec} concentrations are most likely higher oligomeric or aggregated species. Strikingly, TF_{Pf} did not undergo a monomer-to-dimer conversion, even at concentrations as high as 30 μ M. While this observation is important

FIG. 3. Effect of TF concentration on reactivation of GAPDH at 25°C and 15°C. Refolding of denatured GAPDH was monitored by measuring its enzymatic activity 3 h after 50-fold dilution (final concentration 2.5 μ M) in the presence of increasing concentrations of TF_{Pf} (●), TF_{Ec} (■), or BSA (×) at 25°C (A) or at 15°C (B).

in demonstrating that dimerization of TF is not a prerequisite for its chaperone function reported in Fig. 3, it also supports the hypothesis that dimer formation may be important in its polypeptide holding ability. In order to investigate dimerization under conditions more physiologically relevant to TF_{Pf}, cross-linking was repeated at 15°C, at which temperature both TFs presented behavior similar to that observed at 25°C (Fig. 4B).

While glutaraldehyde cross-linking has been widely used to characterize TF dimers (26), cross-linking experiments can be limited by the low specificity of the cross-linking agent and the positioning of the target amino acids at the protein surface. In order to confirm these initial observations, therefore, the quaternary structure of TF_{Pf} was further analyzed by using steady-state fluorescence anisotropy to characterize changes in protein size (3).

Titration of fluorescently labeled TFs with the corresponding unlabeled TF allowed the resultant changes in anisotropy to be plotted against the concentration of unlabeled TF, with data fitted to a model specific for dimerization, according to the equation for calculating r_{obs} provided above. Equilibration of 50 nM labeled TF_{Ec} with the unlabeled chaperone at concentrations of up to 100 μ M yielded a significant increase in anisotropy (Fig. 5), indicating multimerization of TF_{Ec} with a

FIG. 4. Analysis of quaternary structures of TFs at 25°C and 15°C by cross-linking. Increasing concentrations (indicated by triangles above the lanes) of TF_{Pf} and TF_{Ec} were cross-linked using 0.1% glutaraldehyde, precipitated using trichloroacetic acid, and separated by SDS-10% PAGE. (A) Cross-linking at 25°C, with TF concentrations of 0.5, 2.5, 10, 20; and 30 μ M; (B) cross-linking at 15°C, with TF concentrations of 0.15, 0.625, 2.5, 10, and 20 μ M. Equal amounts of protein were loaded in each lane. The first lane of each TF gradient was not treated with glutaraldehyde. Cross-linked dimers are indicated by stars.

dimer-monomer dissociation constant of 2.8 ± 0.3 μ M. This measurement is consistent with the range of K_d values previously determined for TF_{Ec} by using high-pressure liquid chromatography (1 μ M) (28) and analytical ultracentrifugation (23, 28), which validated the approach prior to characterization of the *P. frigidicola* chaperone.

As shown in Fig. 5, and in accordance with results from the cross-linking analysis, no significant change in anisotropy was detected for TF_{Pf}, even at up to 100 μ M unlabeled protein, whereas dimerization was clearly evident with the *E. coli* chaperone at concentrations as low as 0.5 μ M. Similar results were observed with TF_{Pf} at 15°C (data not shown). Although both the N-terminal and C-terminal domains of TF molecules have been demonstrated to be involved in dimerization (22, 26, 35, 39), and shortening of the TF_{Ec} C terminus by only 13 amino acids almost completely inhibits dimerization while leaving the chaperone activity of the protein unaffected (39), the specific residues that mediate TF dimerization have yet to be identified. It remains difficult to explain the structural basis for the lack of dimerization of TF_{Pf}, therefore, but these results indicate nonetheless that, unlike the previously characterized TF from *E. coli*, TF_{Pf} may exist in exclusively monomeric form in the *P. frigidicola* cytoplasm.

In order to further delineate functional differences between the *E. coli* and *P. frigidicola* TFs that may arise from their different quaternary structures, we investigated the ability of TF_{Pf} to complement a Δ tig mutation in the *E. coli* GP179 (MC4100 Δ tig Δ dnaKJ) host strain (6). Despite successful complementation by TF_{Ec}, however, it was not possible to assess complementation by TF_{Pf} due to its low-level expression under the same conditions in the *E. coli* host (data not shown).

TF_{Pf} exhibits reduced retardation of protein export in *E. coli*. Previous studies indicate that TF_{Ec}, upon binding nascent polypeptides under physiological conditions, can impede their

FIG. 5. Analysis of quaternary structure of TFs at 25°C by fluorescence anisotropy. Alexa Fluor 532-TF (50 nM) was titrated against increasing concentrations of unlabeled TF. The change in anisotropy is plotted against the unlabeled protein concentration, as indicated. Symbols: $\bullet$, TF_{Pf}; $\blacksquare$, TF_{Ec}.

export from the cytoplasm. Since this effect is inversely proportional to the concentration of TF in the cytoplasm (19), it raises the possibility that it might be mediated by the dimeric form of TF (20). Previous work by our own group also demonstrated that periplasmic export of a recombinant scFv antibody fragment, with an OmpA leader peptide, was severely impaired upon coproduction of TF_{Ec} (8). In order to further investigate the potential significance of the lack of dimerization of TF_{Pf} observed in vitro, therefore, we used the same scFv expression model to examine the effect of TF_{Pf} on retarding polypeptide translocation of the cytoplasmic membrane in *E. coli*.

Upon coproduction of TF_{Ec}, the 2H12 scFv occurred almost exclusively in its unprocessed form in *E. coli* BL21, i.e., with the OmpA leader peptide still attached at its N terminus (Fig. 6A). This indicates that translocation of the recombinant polypeptide across the cytoplasmic membrane is almost completely blocked by elevated intracellular concentrations of TF_{Ec}. Coproduction of TF_{Pf} at a similar level and under the same conditions had no such effect on scFv secretion, however, since only the processed (and, therefore, translocated) form of the scFv was detectable in cellular preparations (Fig. 6A). In order to investigate whether this lack of retardation could be due to competition for substrate between TF_{Pf} and endogenous TF_{Ec} in the cell, the export analysis was repeated in parallel in *E. coli* W3110 and *E. coli* W3110 Δ tig strains. While an unprocessed scFv product could be detected in TF_{Pf}-expressing cultures in this analysis, albeit at a dramatically lower concentration than with the *E. coli* chaperone, no difference in protein export was observed between the parental and Δ tig strains (Fig. 6B). Therefore, the lack of retardation observed with TF_{Pf} is likely to be due to its inability to form stable complexes with newly translated polypeptides during folding, unlike TF_{Ec} which remains stably bound to nascent polypeptide chains for extended periods (19) and so impedes their secretion.

TF_{Pf} can assist folding of newly translated proteins independently of other chaperones. In order to better evaluate the ability of TF_{Pf} to fold newly translated polypeptides, we ex-

FIG. 6. Effect of TFs on the periplasmic export of 2H12 scFv in *E. coli*. (A) 2H12 scFv was expressed in *E. coli* BL21(DE3) cells, with TF_{Pf} and TF_{Ec} coexpressed from p15aratigPF and p15aratigEC, respectively. Soluble (upper) and insoluble (lower) fractions were analyzed by Western blotting with an anti-His₆ tag antibody. (B) pBADHisB + *tig*_{Pf} and + *tig*_{Ec} represent *E. coli* W3110 or *E. coli* W3110 Δ*tig* cells expressing 2H12 scFv in the presence of pBADHisB control, pBADtigPF, and pBADtigEC plasmids, respectively. Whole-cell extracts were analyzed as described above. Arrows indicate bands of the expected sizes of the processed and unprocessed scFv polypeptides.

pressed the 2H12 scFv, in the absence of a leader peptide, in a cell-free protein synthesis system. Systems such as the PURE model used in the present study (33), which is based on purified cellular (*E. coli*) components but lacks endogenous chaperones, allow delineation of the role of individual chaperones in cotranslational protein folding, as recently demonstrated with *E. coli* TF (11, 18).

We added TF_{Ec} or TF_{Pf} during translation of the scFv to create a coupled translation-folding scheme in vitro. Analysis of the reactions clearly indicated that TF_{Pf} does not simply suppress the aggregation of the scFv, as indicated by Western blot analysis, but also assists its correct folding, as judged by ELISA (Fig. 7). This effect was independent of TF_{Pf} concentration in the range 2.5 to 20 μM, whereas the addition of up to 20 μM TF_{Ec} led to no improvement in either the solubility or the yield of functional scFv. Conversely, the two TFs led to similar increases in the solubility and functionality of the same 2H12 scFv in *E. coli* cell expression experiments, however, resulting in up to sixfold increases in the volumetric yield of functional scFv (data not shown). This apparent discrepancy between the effects of *E. coli* TF on scFv folding in cell-free and cell-based environments can be explained by its requirement to transfer many aggregation-sensitive proteins to downstream chaperone systems such as DnaK/J/GrpE or GroES/EL in the cell to ensure their successful folding (11). Of considerably more interest to the present study, however, is the fact that TF_{Pf} could assist the folding of the target protein independently of downstream chaperone machineries, providing another indication of the significantly different folding properties of the *P. frigidicola* and *E. coli* chaperones.

Conclusion. In the present study, we have demonstrated that TF from *P. frigidicola* does not dimerize or exhibit the characteristic holding-chaperone activity of its *E. coli* homologue. In addition, it refolds denatured RCM-T1, a process that requires peptidyl-prolyl isomerization, and it can promote the correct

folding of a model scFv antibody fragment in the absence of additional chaperones in a cell-free protein synthesis system. The results presented indicate, therefore, that TF_{Pf} behaves as a “true” monomeric chaperone in its ability to independently promote the correct folding of polypeptide substrates. Since chaperones from psychrophiles have previously been found to have new adaptive features to facilitate protein folding and

FIG. 7. Effect of increasing concentrations of TFs on cell-free protein synthesis. Various concentrations of TF_{Pf} and TF_{Ec} were added to a cell-free protein synthesis system prior to production of the 2H12 scFv recombinant antibody fragment. Activity of the soluble scFv was monitored by ELISA. Soluble and insoluble fractions were separated by centrifugation and analyzed by Western blotting with an anti-M2 FLAG antibody. The results are representative of two independent experiments.

stability at low temperatures (5), it will be instructive to determine whether these novel characteristics of TF_{PF} reflect its cold adaptation or distinctive, as-yet-undiscovered, mechanisms in *P. frigidicola*.

This first characterization of a TF from a psychrophilic species has revealed striking properties of TF_{PF}. In combination with other recent studies in the field (18, 25, 32, 37), it significantly advances our molecular understanding of the functioning of TF. Given in particular that a specific mechanistic role has been proposed for dimeric TF_{EC} in posttranslational protein folding (20), the present results highlight that dimerization may not be a universal feature among bacteria and that the physiological relevance of dimer formation remains to be fully understood.

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