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Functional adaptations of the bacterial chaperone trigger factor to extreme environmental temperatures

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Summary

Trigger factor (TF) is the first molecular chaperone interacting cotranslationally with virtually all nascent polypeptides synthesized by the ribosome in bacteria. Thermal adaptation of chaperone function was investigated in TFs from the Antarctic psychrophile *Pseudoalteromonas haloplanktis*, the mesophile *Escherichia coli* and the hyperthermophile *Thermotoga maritima*. This series covers nearly all temperatures encountered by bacteria. Although structurally homologous, these TFs display strikingly distinct properties that are related to the bacterial environmental temperature. The hyperthermophilic TF strongly binds model proteins during their folding and protects them from heat-induced misfolding and aggregation. It decreases the folding rate and counteracts the fast folding rate imposed by high temperature. It also functions as a carrier of partially folded proteins for delivery to downstream chaperones ensuring final maturation. By contrast, the psychrophilic TF displays weak chaperone activities, showing that these functions are less important in cold conditions because protein folding, misfolding and aggregation are slowed down at low temperature. It efficiently catalyses prolyl isomerization at low temperature as a result of its increased cellular concentration rather than from an improved activity. Some chaperone properties of the mesophilic TF possibly reflect its function as a cold shock protein in *E. coli*.

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Introduction

Living organisms have colonized nearly all environments on earth, from the permanently frozen polar regions or the arctic permafrost, to the extremely hot deep-sea hydrothermal vents, solfatares or hot springs. The range of temperatures compatible with life is wide and is currently estimated from –20°C, as exemplified by metabolically active bacteria between sea ice crystals (Deming, 2002), to 122°C in hydrothermal vents as exemplified by the archaeon *Methanopyrus kandleri* (Takai *et al*., 2008). Microbial life under these extreme environmental temperatures obviously requires a vast array of adaptations at all cellular levels (Gerday and Glansdorff, 2007; Horikoshi *et al*., 2011). Previous studies on temperature adaptations of proteins focused mainly on enzyme activity, protein stability and folding (Vieille and Zeikus, 2001; Feller, 2010; Struvay *et al*., 2013). However, a key determinant of these adaptations is the acquisition of the final, biologically active conformation of proteins aided by chaperones and folding catalysts, which remains almost unexplored. This prompted us to analyse the functions of homologous (orthologues) trigger factors (TFs) from the Antarctic psychrophile *Pseudoalteromonas haloplanktis* (*Ph*TF), the mesophile *Escherichia coli* (*Ec*TF) and the hyperthermophile *Thermotoga maritima* (*Tm*TF). This series covers nearly all temperatures encountered by bacteria and by most living organisms. *Pseudoalteromonas haloplanktis* thrive permanently at sub-zero temperatures in the Antarctic sea water and resist long-term frozen conditions when entrapped in the winter ice pack. On the other hand, *T. maritima* has been isolated from various geothermically heated sea sediments around 90°C.

TF (~ 50 kDa) is the first molecular chaperone interacting cotranslationally with virtually all newly synthesized polypeptides by the bacterial ribosome. It delays premature chain compaction and maintains the nascent polypeptide in a non-aggregated state until sufficient structural information (encoded in the amino acid sequence) is available for productive folding and subsequently promotes protein folding (Merz *et al*., 2008; Hartl and Hayer-Hartl, 2009; Hoffmann *et al*., 2010). Recently, an additional unfoldase activity of TF that reverses premature folding has been reported (Hoffmann *et al*., 2012). TF

transiently associates with ribosomes in a 1:1 stoichiometry via the ribosomal protein L23 located at the polypeptide exit channel of the large subunit (Kramer *et al*., 2002; 2004b). The intracellular molar concentration of TF exceeds that of ribosomes by a twofold to threefold factor, resulting in a large, unbound cytosolic pool. TF displays an unusual extended conformation (Merz *et al*., 2008; Martinez-Hackert and Hendrickson, 2009) containing three distinct domains: the N-terminal ribosome-binding domain, the C-terminal chaperone domain and a peptidyl-prolyl *cis-trans* isomerase (PPIase) domain of the FKBP family (Scholz *et al*., 1997; Kramer *et al*., 2004a; Merz *et al*., 2006). Although ribosome attachment of TF is crucial for its *in vivo* interaction with nascent chains (Kramer *et al*., 2002), its ability to prevent aggregation, to promote protein folding and to catalyse prolyl isomerization can be monitored *in vitro* using model substrates.

Because TF is conserved among bacteria and is the first chaperone interacting with proteins, using an ATP-independent mechanism, without co-chaperone, it represents an appropriate model for studying the effects of extreme environmental temperature on the chaperone function in a single polypeptide. In bacteria, *Ec*TF is by far the best characterized ribosome-associated chaperone (Merz *et al*., 2008; Hoffmann *et al*., 2010; Lakshmipathy *et al*., 2010), but is also a cold shock protein (Kandror and Goldberg, 1997) in sharp contrast to most folding helpers. In the psychrophile *P. haloplanktis*, *Ph*TF is a cold acclimation protein continuously overexpressed at low temperature (4°C versus 18°C), while most heat shock chaperones (classified as such in *E. coli*) are strongly downregulated, suggesting a crucial function of *Ph*TF for protein folding in the cold (Piette *et al*., 2010; 2011b). In the hyperthermophile *T. maritima*, the proteomic context of *Tm*TF with regard to temperature remains unclear (Pysz *et al*., 2004; Wang *et al*., 2012). We show here that these structurally homologous TFs display distinctive chaperone and prolyl isomerase properties that are correlated with the bacterial environmental temperature.

Results

Production of TFs

The genes coding for the three TFs were cloned from the genomic DNA of *P. haloplanktis*, *E. coli* and *T. maritima* on the basis of the known genome sequences. The native genes (devoid of purification tag) were expressed in *E. coli* grown at 18°C (to promote recombinant protein solubility), and the corresponding TF variants were purified to homogeneity. N-terminal amino acid sequencing by Edman degradation confirmed the identity of the proteins and indicated the persistence of an N-terminal de-formylated translation initiation methionine. Electrospray ionization-quadrupole-time of flight mass spectrometry also confirmed the expected mass of the proteins, as well as the absence of post-translational modifications such as proteolytic cleavages. Additional information on the investigated TFs is provided in Table S1.

Prevention of D-glyceraldehyde-3-phosphate dehydrogenase aggregation by TFs

In this standard assay of chaperone holdase activity (binding to non-native refolding intermediates and prevention of their aggregation), GAPDH (D-glyceraldehyde-3-phosphate dehydrogenase) unfolded in GdmCl (guanidine hydrochloride) is diluted 50-fold with aqueous buffer. This leads to slow aggregation of GAPDH, accompanied by a strong increase in light scattering. *Ec*TF has been reported to prevent this aggregation (Huang *et al*., 2000). At 20°C, *Tm*TF from the hyperthermophilic bacterium resembles *Ec*TF in its efficiency to prevent GAPDH aggregation (Fig. 1A) and displays a similar dependence on TF concentration (Fig. 1B). By contrast, at the same temperature *Ph*TF from the psychrophilic bacterium is virtually unable to prevent aggregation of GAPDH (Fig. 1A). This assay cannot be performed below 15°C because GAPDH aggregation becomes poorly reproducible. In order to examine the chaperone activity of *Ph*TF and of its homologues at low temperature, the dilution was performed under ice-cold conditions (no GAPDH aggregation) then slowly brought to 15°C (Piette *et al*., 2010). Figure S1 shows that *Ec*TF and *Tm*TF maintain their holdase activity while *Ph*TF delays aggregation during 10–20 min. Under these conditions, the concentration dependence of *Ph*TF holdase activity is qualitatively similar to that of its mesophilic and thermophilic homologues (Fig. 1B). This indicates that *Ph*TF requires low temperature incubation in order to perform its holdase activity.

GAPDH refolding assisted by TFs

The above-mentioned assay monitors the holdase activity of a chaperone but does not provide data for the foldase activity that leads to an increase in productive folding of the native state. This foldase activity is quantified by the recovery of the activity of the tetrameric GAPDH after dilution from the GdmCl-unfolded state under similar assay conditions as for aggregation prevention. Figure 2 illustrates the known *Ec*TF behaviour: the foldase activity increases with its concentration and a maximal reactivation of about 30% is reached at a ratio of three *Ec*TF molecules per GAPDH tetramer. At higher *Ec*TF concentrations, the yield of reactivation decreases possibly because the strong binding to non-native states of

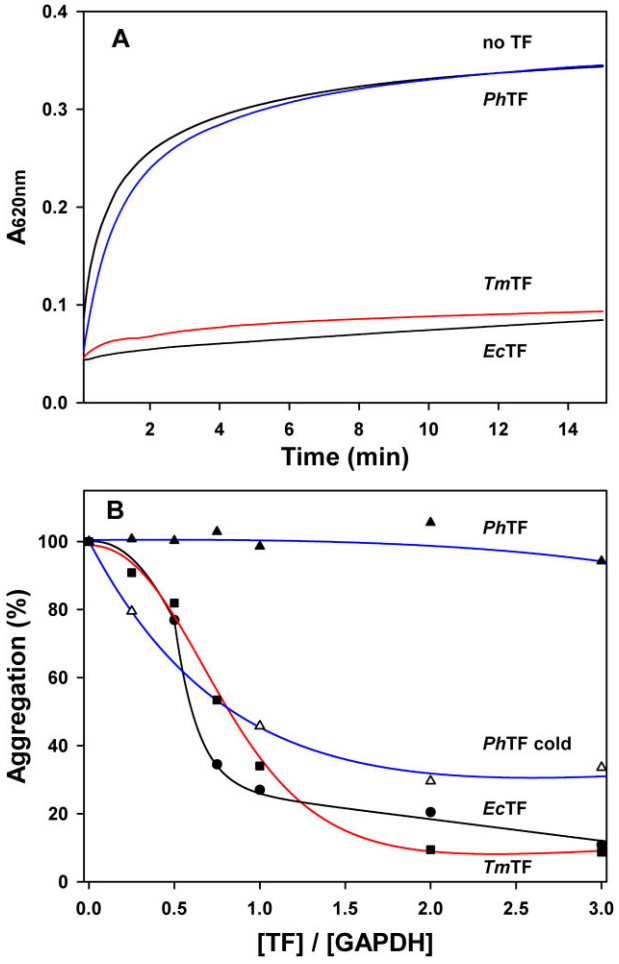

Fig. 1. Effect of trigger factors on GAPDH aggregation.
A. Time courses of aggregation of GAPDH (2.5 μM) at 20°C in the presence of equimolar TF ratios.
B. Concentration dependence of the holdase activity on GAPDH aggregation for *Ec*TF (●), *Tm*TF (■) and *Ph*TF (▲) at 20°C (values after 15 min). *Ph*TF delays GAPDH aggregation at 15°C after ice-cold incubation (△, *Ph*TF cold).

GAPDH interferes with productive folding (Huang *et al.*, 2000). *Ph*TF exhibits a weaker foldase activity, reaching a maximal reactivation of 10%. This reactivation yield is maintained even at high *Ph*TF concentrations, as already noted for another cold-adapted TF (Robin *et al.*, 2009). The binding of *Ph*TF to non-native forms of GAPDH is presumably weak and therefore does not compete with productive folding, as observed for *Ec*TF. In contrast, *Tm*TF has a poor foldase activity, and productive folding of GAPDH is suppressed at high molar ratios. This suggests that its holdase activity (Fig. 1) overcomes its foldase activity: bound aggregating GAPDH might not be released in a folded, active conformation. These three structurally homologous TFs thus display distinct patterns of foldase activity that reflect probably the strength of their interactions with non-native proteins.

Green fluorescent protein refolding assisted by TFs

The refolding of acid-denatured GFP (green fluorescent protein, mut2) can be followed in a wide range of temperatures, from 5°C to 50°C (upper limit for reproducible refolding). Furthermore, it does not involve residual denaturant concentrations and the intrinsic fluorescence of added TF does not interfere with the chromophore fluorescence of GFP. Following dilution in a neutral pH buffer, acid-denatured GFP spontaneously refolds and the appearance of chromophore fluorescence is monitored at 510 nm. Under our conditions, refolding of GFP was about 50% reversible.

The time course of GFP refolding was recorded at 15°C and at temperatures more relevant with respect to environmental conditions of the source bacteria (i.e. 5°C for *Ph*TF, 37°C for *Ec*TF and 50°C for *Tm*TF). Figure 3A illustrates the distinct effects of a 10-fold molar excess of each chaperone on the folding kinetics of GFP at 15°C. *Ph*TF and, in particular, *Ec*TF increased the final yield of refolded GFP. By contrast, *Tm*TF decreased both the rate and the final yield of GFP refolding. The extrapolated GFP refolding yields are reported in Fig. 3B and C as a function of TF/GFP molar ratio. At near-environmental temperatures (Fig. 3B), the yields of refolded GFP vary in a similar fashion as the yields of GAPDH reactivation (Fig. 2). *Ec*TF improves the refolding yield by nearly 50% up to a molar ratio of 2, followed by a decrease at higher ratios. *Ph*TF slightly improves the refolding yield by about 10%, whereas *Tm*TF in excess inhibits GFP refolding. At 15°C (Fig. 3C), similar patterns were observed, with the noticeable exception of *Ec*TF, which continues to improve

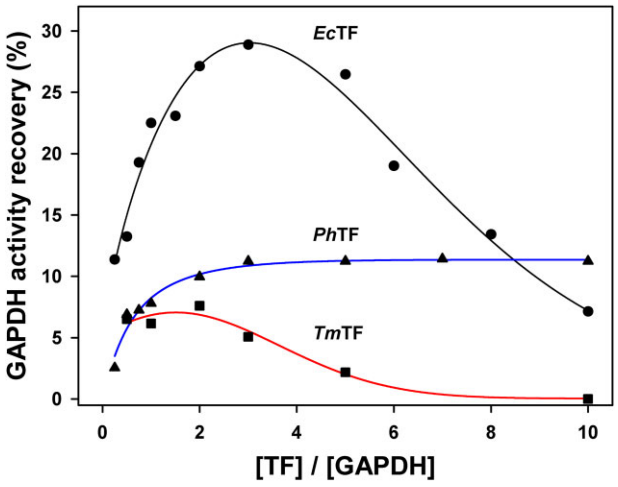

Fig. 2. Reactivation of denatured GAPDH in the presence of increasing concentrations of trigger factors at 15°C. Symbols: *Ec*TF (●), *Tm*TF (■) and *Ph*TF (▲). The percentage of GAPDH activity recovered is given with respect to enzymatic activity without GdmCl unfolding. In the absence of TFs, GAPDH aggregation precludes activity measurement.

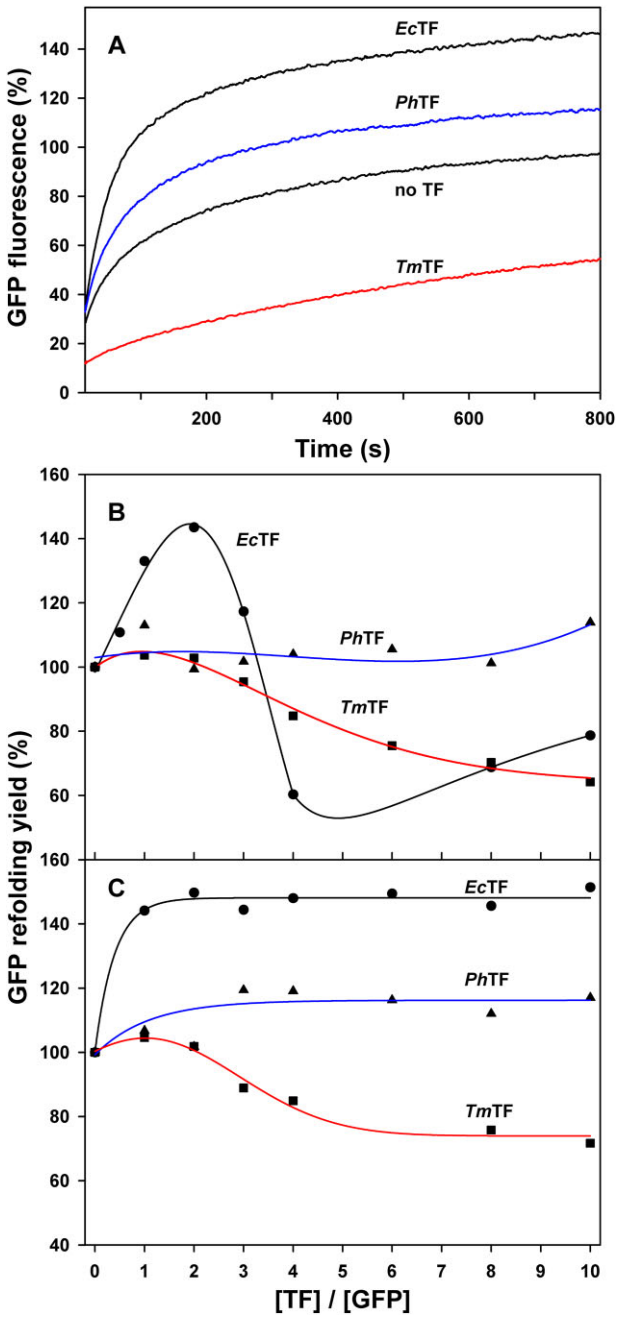

Fig. 3. GFP refolding assisted by trigger factors.
A. Refolding time courses of acid-denatured GFP (0.5 μM) at 15°C recorded by fluorescence monitored at 510 nm. TFs were added to a final 10-fold molar excess. Fluorescence intensity (extrapolated to the infinite) of spontaneously refolded GFP is taken as 100%.
B. Refolding yield of acid-denatured GFP at near-environmental temperatures: 5°C for *Ph*TF (▲), 37°C for *Ec*TF (●) and 50°C for *Tm*TF (■). Fluorescence intensity (extrapolated to the infinite) of spontaneously refolded GFP is taken as 100%.
C. Refolding yield of acid-denatured GFP at 15°C in the presence of increasing concentrations of *Ec*TF, *Tm*TF and *Ph*TF. Symbols as in B.

the refolding yield even at high concentration. This indicates that a decrease in temperature modifies the interaction between *Ec*TF and GFP during its refolding.

The GFP refolding kinetics recorded here after manual mixing can be described by the sum of a fast and a slow exponential phase ($R^2 > 0.998$), allowing to calculate the associated rate constants (k_{fast}, k_{slow}), normalized amplitudes (A_{fast}, A_{slow}) and extrapolated refolding yields F^{∞}. As shown in Table 1 and Table S2, for selected molar ratios, the three TFs affect GFP refolding kinetics differently. The increase in refolding yield in the presence of *Ec*TF at 15°C (Table 1) is accompanied by an increase of the amplitude of the fast phase, while the rate constants are slightly decreased. This points to a chaperone-like activity (Huang *et al.*, 2000) as chaperones improve productive folding but do not accelerate the folding rate. A similar effect is observed in the presence of *Ph*TF at 5°C (Table S2) and 15°C (Table 1) but to a lower extent as expected from its weaker foldase activity (Fig. 3B and C). At 37°C, *Ec*TF increases the rate and the amplitude of the fast folding reaction (Table S2) when present at equimolar concentration. At a 10-fold molar excess, rate and amplitude of this reaction decrease again, in agreement with the effects observed for the refolding yields (Fig. 3B). *Tm*TF decreased the folding yield of GFP (Fig. 3B and C) and retarded GFP refolding at both 15°C and 50°C mainly by changes in the fast folding reaction. Its rate constants and amplitudes are reduced whereas amplitudes of the slow phases are increased.

These kinetic data indicate that the three TFs are mainly involved in the folding events of GFP occurring during the fast phase. However, they exhibit quite distinct behaviours. *Ec*TF promotes or inhibits GFP folding depending on its concentration and on temperature. *Ph*TF displays a weak foldase activity whereas *Tm*TF is essentially a holdase.

Cooperation of TFs with E. coli *chaperonin*

The peculiar proteomic context of the Antarctic bacterium (overexpression of *Ph*TF, downregulation of heat shock chaperones, except GroEL) and the unexpected strong holdase activity of *Tm*TF prompted us to analyse possible cooperation between TFs and the *E. coli* GroEL/GroES chaperonin complex (GroELS or Hsp60/Hsp10) during the refolding of GFP at 15°C and at representative TF/GFP ratios.

As shown in Fig. 4A, GroELS alone improves the refolding yield of GFP by about 11% ($F^{\infty} = 111\%$) and induces a faster refolding of GFP as the fluorescence recovery already reaches 60% during the dead time of manual mixing. However, the GFP refolding yield is much higher in the presence of *Ec*TF at a twofold molar excess ($F^{\infty} \sim 150\%$) than in the presence of both *Ec*TF and

Table 1. Effects of TFs on kinetic parameters of GFP refolding at 15°C.

		Rate constants ($s^{-1} \times 10^{-3}$)		Amplitudes (%)		
	[TF]/[GFP]	k_{fast}	k_{slow}	A_{fast}	A_{slow}	F^{∞} (%)
GFP	0	37 ± 3	3.5 ± 0.4	41 ± 5	53 ± 2	100 ± 6
GFP + *Ph*TF	1	36 ± 2	3.5 ± 0.2	42 ± 4	59 ± 2	107 ± 2
	10	30 ± 0.3	3.9 ± 0.0	52 ± 5	52 ± 2	117 ± 7
GFP + *Ec*TF	1	29 ± 2	2.9 ± 0.2	86 ± 6	53 ± 4	144 ± 10
	10	27 ± 1	2.8 ± 0.0	101 ± 2	51 ± 1	151 ± 3
GFP + *Tm*TF	1	31 ± 7	2.9 ± 0.1	41 ± 9	59 ± 4	105 ± 11
	6	21 ± 4	1.7 ± 0.1	15 ± 2	70 ± 4	91 ± 6

Rate constants *k*, normalized amplitudes A and extrapolated refolding yields F^{∞} (values are mean ± SD, $n = 3$).

GroELS (F^{∞} = 127%). Indeed, the concomitant addition of GroELS and *Ec*TF in the dilution buffer decreases the rate constant and the amplitude of the fast phase of GFP refolding. Therefore, under these conditions, *Ec*TF is more efficient than GroELS as a foldase. Furthermore, these data suggest that GroELS competes with *Ec*TF for the same substrate during GFP refolding and decreases the refolding yield.

Individually, *Ph*TF (F^{∞} = 119% at TF/GFP = 3) and GroELS (F^{∞} = 111%) display a similar weak foldase activity on GFP refolding and, when present simultaneously, a 125% increase is observed (Fig. S2). The effects are thus almost additive, suggesting that both chaperones do not compete but catalyse GFP refolding independently.

*Tm*TF leads to a significant inhibition of GFP refolding (F^{∞} = 76% at TF/GFP = 8). However, the simultaneous addition of GroELS restores GFP refolding and doubles the refolding yield to 150% (Fig. 4B). In order to mimic the sequential involvement of both chaperones in the bacterial cell, *Tm*TF was added in the refolding buffer, followed by addition of GroELS after 5 min. This latter addition results in the fast resumption of GFP refolding and to a similar enhancement of the refolding yield. This demonstrates that GroELS efficiently competes for GFP bound to *Tm*TF and suggests cooperation of *Tm*TF as a holdase with downstream chaperones such as GroELS. Furthermore, the markedly improved yield in the presence of both *Tm*TF and GroELS suggests that initial binding to *Tm*TF is beneficial to GFP refolding by GroELS.

8-Anilinonaphthalene-1-sulfonic acid titration of TFs

The fluorescence quantum yield of ANS (8-anilinonaphthalene-1-sulfonic acid) increases when it binds to solvent-accessible hydrophobic regions of proteins. An ANS derivative has been reported to bind within the C-terminal domain of *Ec*TF bearing the chaperone activity (Shi *et al*., 2007). In order to detect possible differences in the solvent-exposed surfaces of the chaperone cavity, the investigated TFs were titrated with ANS. As shown in Fig. S3, the three TFS bind ANS with similar affinities ($K_d \sim$ 50 μM). However, the higher fluorescence intensities recorded for *Tm*TF indicates an increased hydrophobic environment of the binding site in the chaperone cavity.

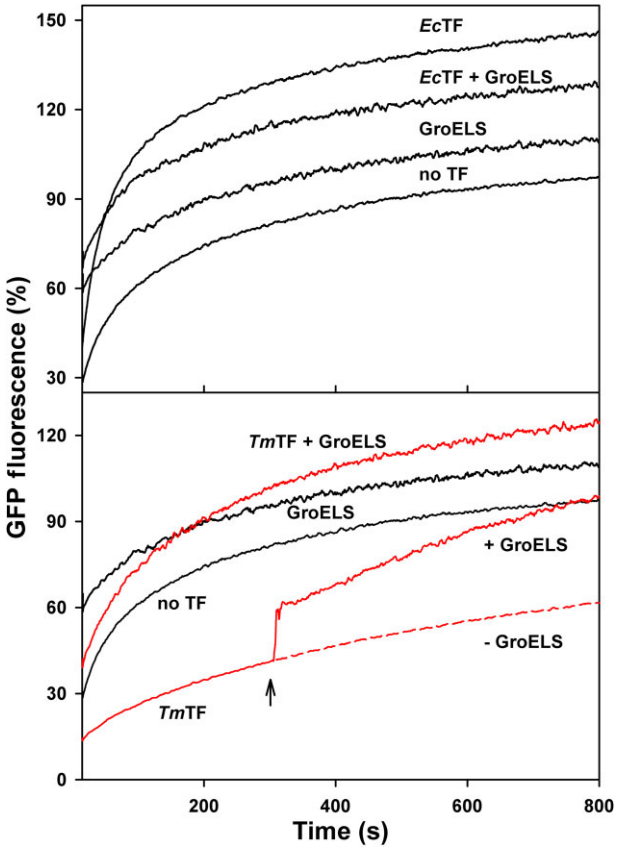

Fig. 4. GFP refolding in the presence of TFs and GroELS at 15°C.
A. GFP refolding in the presence of *Ec*TF and GroELS. Fluorescence time courses of GFP (0.5 μM) and in the presence of GroELS (9 μM, 9 mM ATP), *Ec*TF (1 μM) or *Ec*TF + GroELS.
B. GFP refolding in the presence of *Tm*TF and GroELS. Fluorescence time courses of GFP (0.5 μM) and in the presence of GroELS (9 μM, 9 mM ATP), *Tm*TF (4 μM) or *Tm*TF + GroELS. In the sequential experiment, addition of GroELS after 300 s is indicated by an arrow.

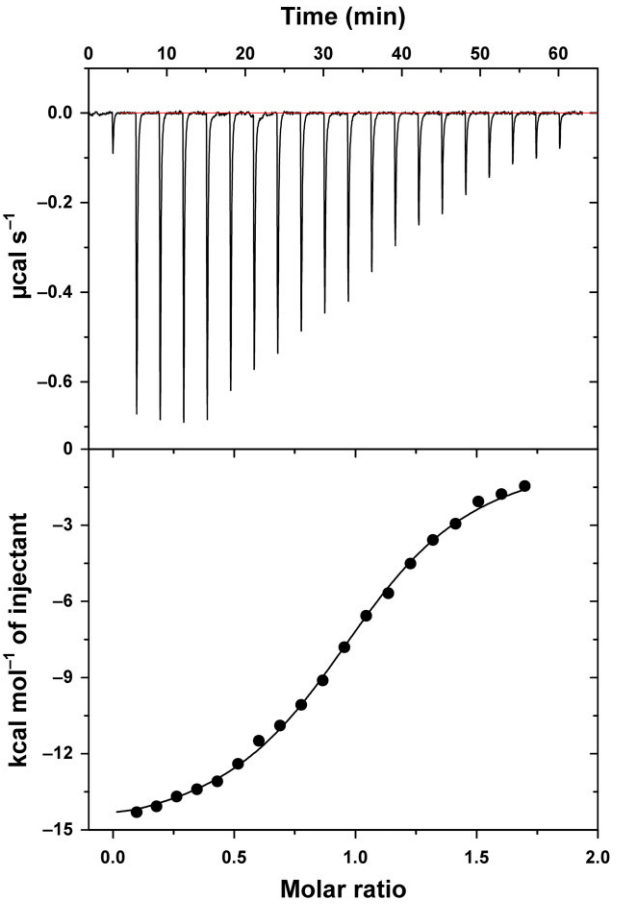

Fig. 5. ITC of *Ec*TF binding to α-casein in reverse titration at 37°C. Upper panel: exothermic microcalorimetric traces of *Ec*TF (280 µM) injections into α-casein (35 µM). Lower panel: Wiseman plot of heat releases versus molar ratio of injectant/protein in the cell and nonlinear fit of the binding isotherm for *n* equivalent binding sites.

Binding of TFs to an unstructured protein

In order to monitor the binding of TFs to an unfolded substrate, a new method was devised that used ITC (isothermal titration calorimetry) to record the binding to the natively unstructured α-casein (Uversky *et al*., 2000). As shown in Fig. 5 and Fig. S4, the binding of α-casein to *Ec*TF and *Tm*TF proceeds via detectable heat release providing high-quality thermograms. By contrast, the binding of α-casein to *Ph*TF was not detectable, even at low temperature (5°C) and high protein concentrations. This suggests that *Ph*TF binds unstructured proteins in solution with a very low affinity.

In reverse titrations (injections of TFs into initial excess of α-casein), *Ec*TF displays a 1/1 stoichiometry (Table 2) demonstrating equilibrium conditions under this experimental setup. The affinity constant K_a recorded by ITC is in excellent agreement with those reported for *Ec*TF using other unfolded substrates (Maier *et al*., 2001). The binding to α-casein proceeds via a large and negative enthalpy change, ΔH°_b (heat release), that reflects favourable interactions such as H-bonding or van de Waals contacts. However, this is compensated by an unfavourable entropic contribution, ΔS°_b, to the free energy of binding, ΔG°_b, generally regarded as a conformational change and reduction of degree of freedom upon binding (Velazquez Campoy and Freire, 2005). Interestingly, the binding parameters of *Tm*TF differ in many respects. The binding stoichiometry $n = 1.5$ (molar ratio of injectant/protein in the cell at mid saturation) indicates that more than one molecule of *Tm*TF bind to one molecule of α-casein. In the simplest model, this stoichiometry suggests the occurrence of two equal populations with one bound *Tm*TF ($n = 1$) and with two bound *Tm*TF ($n = 2$) per α-casein molecule. Although the K_a and ΔG°_b values are similar to those of *Ec*TF, the enthalpic and entropic contributions are drastically different. The binding by *Tm*TF is accompanied by a much weaker enthalpy change and with a favourable entropy change. The latter is generally interpreted as resulting from the favourable water molecule release from apolar binding surfaces (Velazquez Campoy and Freire, 2005), in agreement with the increased hydrophobicity shown by ANS titration. These parameters indicate that the hydrophobic effect dominates the binding of *Tm*TF to unfolded proteins.

Direct titrations (injections of α-casein into initial excess of TFs; Fig. S4) are closer to physiological conditions as the intracellular TF concentration exceeds that of ribosomes by a twofold to threefold molar excess (Hoffmann *et al*., 2010). In this experimental setup, several *Ec*TF and *Tm*TF molecules can bind to one α-casein molecule (Table S3). In the case of *Ec*TF, the stoichiometry $n = 0.6$ suggests the occurrence of three populations with one ($n = 1$), two ($n = 0.5$) and three ($n = 0.3$) TF molecules bound to α-casein, in the simplest model. Here again, more molecules of *Tm*TF than of *Ec*TF can bind to one α-casein molecule. At 25°C, up to four *Tm*TF molecules are bound ($n = 0.25$). These complex stoichiometries highlight the non-specific recognition of substrates by TF and explain why *Tm*TF is more efficient than *Ec*TF as a holdase in the chaperone tests.

Table 2. Thermodynamic parameters of binding from reverse titrations of TFs into α-casein at 37°C.

Protein	*n*	K_a (10^5 M^{-1})	ΔG°_b (kcal mol^{-1})	ΔH°_b (kcal mol^{-1})	$T\Delta S^\circ_b$ (kcal mol^{-1})
*Ec*TF	1.01	3.93	–7.9	–15.3	–7.4
*Tm*TF	1.50	3.94	–7.9	–7.1	0.8

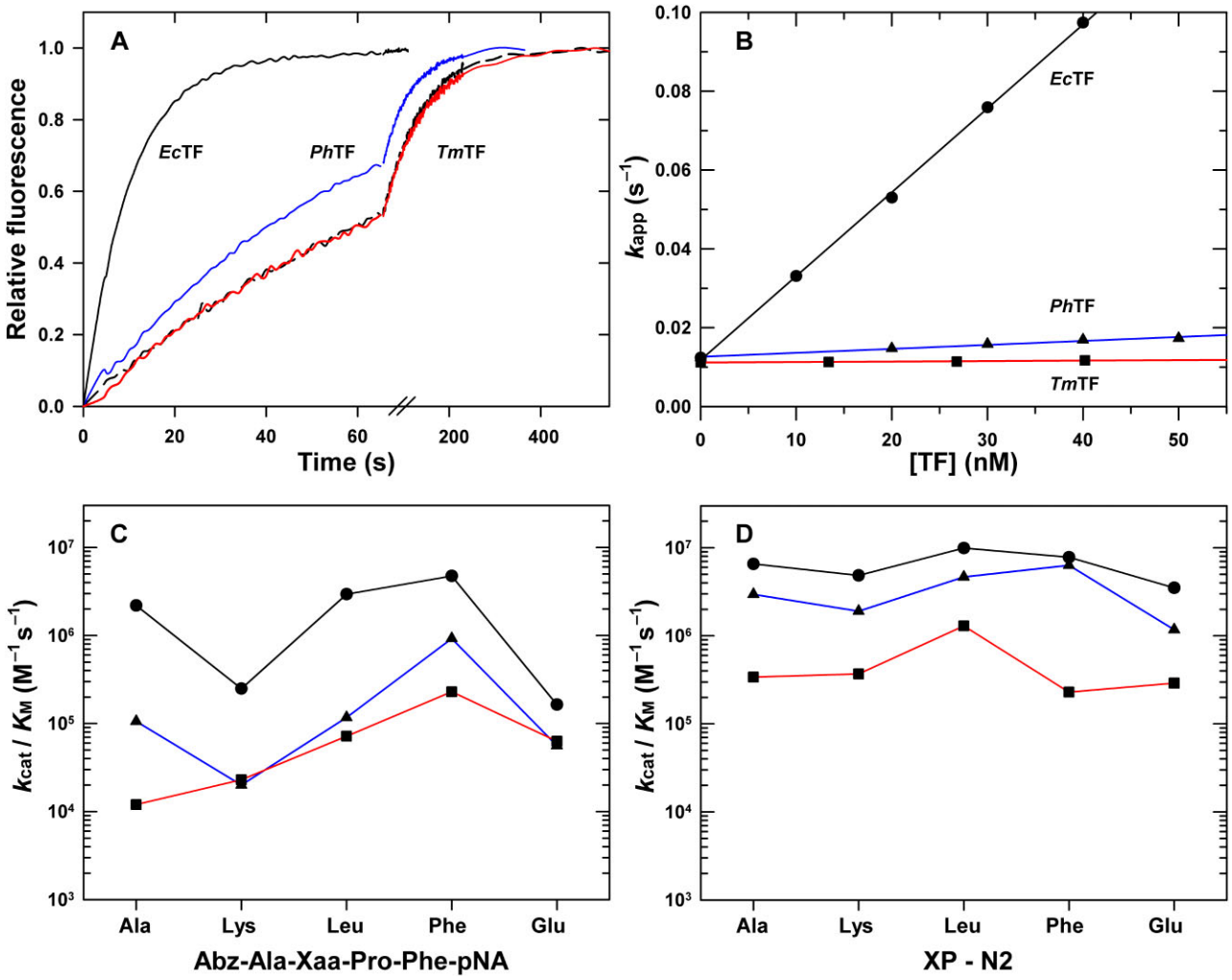

Fig. 6. Sequence specificity of trigger factors towards the residue preceding proline at 15°C.
A. Relative signal change upon *cis*/*trans* prolyl isomerization within Abz-AAPF-pNA in the absence of prolyl isomerase (dashed line) and in the presence of 40 nM *Ph*TF, *Ec*TF and *Tm*TF.
B. Apparent rate constant of prolyl isomerization (s^{-1}) as function of the TF concentration is illustrated for the peptide Abz-AAPF-pNA and *Ph*TF (▲), *Ec*TF (●) and *Tm*TF (■).
C. Catalytic efficiencies (k_{cat}/K_M) of the three TFs towards the residue preceding the proline in short peptides Abz-AXPF-pNA. The residue before Pro is indicated. Symbols as in B.
D. Catalytic efficiencies (k_{cat}/K_M) of the three TFs towards the residue preceding the proline within a refolding protein (XP-N2). The residue before Pro is indicated. Symbols as in B.

Sequence specificity of the PPIase domain of the three TFs

TFs contains a prolyl isomerase domain of the FKBP family and therefore shows a characteristic sequence specificity towards the residue P1 preceding the proline (Harrison and Stein, 1990; Zoldak *et al.*, 2009). The catalytic activity (k_{cat}/K_M) of prolyl isomerases can be measured with proline-containing tetrapeptides or during proline-limited folding reactions of proteins. FKBP domains often show high activity towards peptide substrates, but the catalysis of proline-limited protein folding is usually weak. The folding activity can be dramatically increased in the presence of a chaperone domain (Knappe *et al.*, 2007; Jakob *et al.*, 2009; Schmidpeter *et al.*, 2011; Geitner and Schmid, 2012).

We determined the sequence specificities of the three TFs by using five fluorescent tetrapeptides of the type aminobenzoyl-Ala-Xaa-Pro-Phe-p- nitroanilide (Abz-AXPF-pNA) (Zoldak *et al.*, 2009) with amino acids of different chemical character preceding the proline (i.e. Ala, Lys, Leu, Phe, Glu). At 15°C, the uncatalysed *cis*→*trans* isomerization of prolyl bonds is slow and the three TFs accelerate this isomerization (Fig. 6A), albeit with different

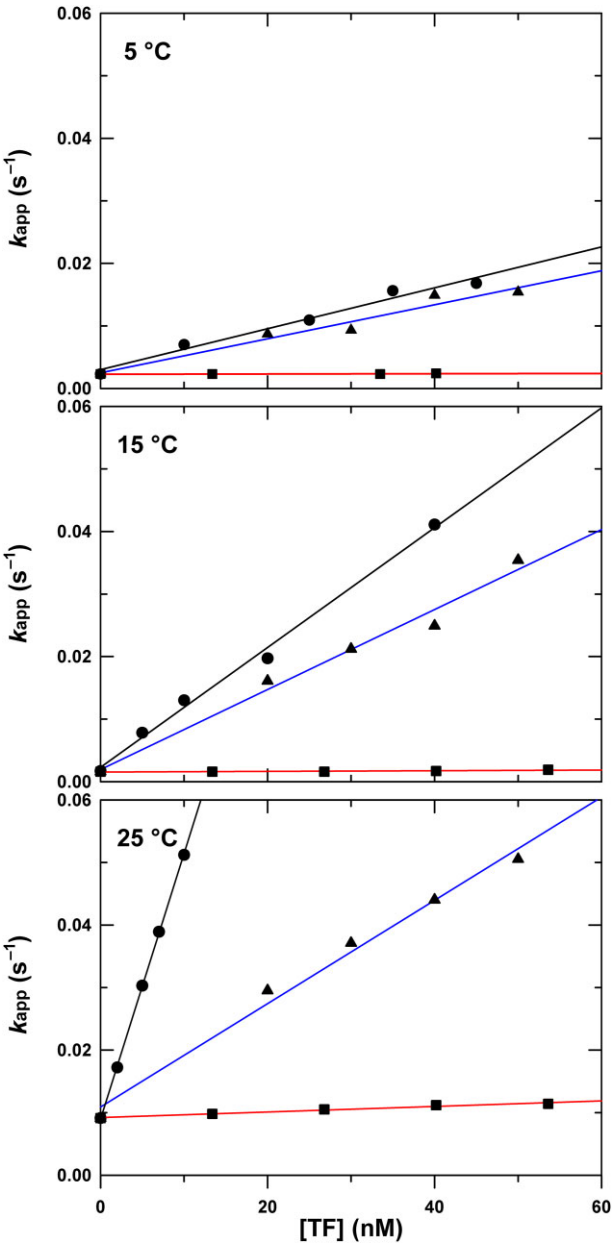

Fig. 7. Catalysis of refolding of RCM-T1 at different temperatures. The apparent rate constant of prolyl isomerization during refolding of RCM-T1 is plotted as function of the TF concentration for *Ph*TF (▲), *Ec*TF (●) and *Tm*TF (■) at 5°C, 15°C and 25°C.

catalytic efficiencies. The specificity constant k_{cat}/K_M is derived from the increase of the apparent rate of isomerization as a function of the TF concentration (Fig. 6B). *Ec*TF shows a 20- and 200-fold higher activity than *Ph*TF and *Tm*TF, respectively, in the assay with the Abz-AAPF-pNA peptide (Table S4). All three TFs show high sequence specificity with respect to the amino acid preceding the proline (Fig. 6C) and prefer hydrophobic residues (Phe and Leu) over charged amino acids (Lys and Glu) before the proline, as shown previously for *Ec*TF (Jakob *et al.*, 2009).

The differences between the three TFs are less pronounced when the activity towards a refolding protein is measured. For these assays, the N2 domain of the gene-3-protein of phage fd was used. Its refolding is limited in rate by the isomerization of Pro161 (Jakob and Schmid, 2008; 2009), and we employed five variants that differed at position 160 before Pro161 (XP-N2) (Fig. 6D and Table S5). Because of its chaperone domain, TF recognizes partly folded protein chains very well, regardless of the amino acid preceding the proline (Jakob *et al.*, 2009). Binding to the chaperone domain leads to a high local concentration of refolding protein molecules in close proximity to the active site of the prolyl isomerase domain (Zoldak and Schmid, 2011). The enrichment of protein substrates near the prolyl isomerase site thereby abolishes the intrinsic substrate specificity of the catalytic FKBP domain and renders refolding proteins good substrates for TF. *Ph*TF and *Ec*TF show almost identical catalytic efficiencies for all variants of the model protein N2. The activity of *Tm*TF is about 10-fold lower but also largely independent of the residue preceding the proline (Fig. 6D and Table S5).

Effects of the reaction temperature on the activity of TF

To test if the PPIase activity is adapted to temperature in a similar fashion as the chaperone activity, the specific activities of the three enzymes were determined at 5°C, 15°C and 25°C (Table S4). Above 25°C, uncatalysed *cis*/*trans* isomerization becomes too fast to be monitored after manual mixing.

The results of the peptide assays at 5°C and at 25°C are shown in Fig. S5. The sequence specificity of the three enzymes is similar at all temperatures with a strong preference for hydrophobic residues before Pro, and *Ec*TF being the most active enzyme.

The activity towards a protein substrate was recorded at different temperatures using reduced and carboxymethylated ribonuclease T1 (RCM-T1) (Fig. 7 and Table S6). This variant cannot form disulfide bonds because the Cys residues are modified. RCM-T1 is permanently unfolded in the absence of salt but refolds spontaneously in 2 M NaCl, where electrostatic repulsion between negatively charged groups is decreased. In unfolded proteins, most of the proline residues adopt the *trans* conformation. Pro39 is *cis* in folded RCM-T1, but in the unfolded state 85% of the molecules contain a *trans* Pro39. As a consequence, the refolding of 85% of all molecules is limited in rate by *trans*→*cis* isomerization at Pro39. The major part of the fluorescence change during refolding reflects this isomerization and makes RCM-T1 a good substrate for assaying the folding activity of

PPIases. (Mayr *et al*., 1994; 1996; Mucke and Schmid, 1994). As observed for the refolding of XP-N2 at 15°C (Fig. 6D), RMC-T1 is also a good substrate for TFs. *Ec*TF catalyses prolyl isomerization during the refolding of RCM-T1 with the highest activity at all temperatures tested. *Ph*TF is also active between 5 and 25°C whereas *Tm*TF always shows a very low activity, which is close to the detection limit. The three enzymes become less active at lower temperatures, and near 5°C, *Ph*TF is virtually as active as *Ec*TF. In the peptide assay such a convergence was not observed.

Discussion

Adaptations of the chaperone function to environmental temperatures

The adaptation of enzyme activity to extreme temperatures has been well documented and involves adjustments of the kinetic parameters in order to optimize the rate of enzyme reactions at environmental temperatures (Vieille and Zeikus, 2001; Feller and Gerday, 2003; Siddiqui and Cavicchioli, 2006; Feller, 2010). By contrast, how chaperones and folding enzymes adapt their function over the whole temperature range where life occurs has been largely unexplored. This report using TF as a model system clearly highlights significant differences in the chaperone activity that can be related to the bacterial environmental temperature.

In the Antarctic bacterium *P. haloplanktis*, *Ph*TF is a cold acclimation protein continuously overexpressed at low temperature, whereas all heat shock chaperones are strongly downregulated, with the noticeable exception of GroELS (Piette *et al*., 2010; 2011b). The persistence of GroELS expression at low temperature can tentatively be explained by the fact that this chaperonin is essential because a number of essential proteins are crucially GroELS dependent for folding, like in *E. coli* (Kerner *et al*., 2005; Hartl and Hayer-Hartl, 2009). In this respect, we found that *Ph*TF and GroELS do not compete and catalyse independently the *in vitro* refolding of GFP, in contrast to both *Ec*TF and *Tm*TF. This lack of *in vitro* synergy possibly reflects the essential function of both *Ph*TF and GroELS in *P. haloplanktis* growing at low temperature, when the expression of other folding helpers is severely repressed. Cold adaptation of *Ph*TF is well illustrated by its lack of holdase activity at 20°C, which resumes after ice-cold incubation (Figs 1 and 2). This points to a possible adaptation in particular of the chaperone domain of *Ph*TF for an optimal binding of protein substrates at low temperatures. But surprisingly, *Ph*TF displays only moderate foldase activity *in vitro* on both GAPDH and GFP, even at low temperature. Furthermore, its very low affinity towards an unstructured protein (α-casein) suggests a poor foldase function. Therefore, it can be proposed that *Ph*TF mainly retains the primary function of TFs as a ribosome-bound chaperone delaying premature compaction of nascent polypeptides. Its foldase activity in the unbound state is presumably not essential because low temperature slows down protein folding and prevents misfolding and aggregation.

Proteins synthesized by the ribosome in the hyperthermophile *T. maritima* are subjected to high environmental temperatures (85–90°C) that are expected to speed up protein folding, to favour misfolding and to promote heat-induced aggregation. The *in vitro* properties of *Tm*TF appear well suited to protect nascent polypeptides from these unproductive pathways. GAPDH reactivation (Fig. 2), GFP refolding (Fig. 3A) and ITC titrations indicate a strong holdase activity associated with a weak foldase activity of *Tm*TF. This improved binding to proteins during their folding provides a protective mechanism against misfolding and aggregation of nascent polypeptides at high temperature. Because of its strong binding, *Tm*TF drastically decreased the folding rate of GFP (Fig. 3) and arrested GAPDH refolding (Fig. 2). Therefore, *Tm*TF counteracts the unfavourable fast folding rate induced by high temperature. These properties suggest a crucial function of *Tm*TF in the adaptation of protein folding at high temperature. ANS titration (Fig. S3) and ITC (Table 2) indicate a more hydrophobic chaperone cavity in comparison with *Ph*TF and *Ec*TF. It is worth mentioning that thermophilic proteins tend to increase the global hydrophobicity of the protein core in order to improve thermal stability (Kumar and Nussinov, 2001; Vieille and Zeikus, 2001; Chakravarty and Varadarajan, 2002). Accordingly, the higher hydrophobicity of *Tm*TF chaperone cavity appears well suited to bind non-native thermophilic proteins during their folding. Furthermore, the cooperation observed with GroELS suggests an additional function of *Tm*TF. The *E. coli* chaperonin GroELS efficiently competes for GFP bound to *Tm*TF and promotes its refolding (Fig. 4B). This indicates that *Tm*TF might also be a carrier of partially folded polypeptides that are delivered to downstream chaperones such as DnaK and GroELS for final maturation or remodelling. Moreover, the high GFP refolding yield recorded in the simultaneous or sequential addition of *Tm*TF and GroELS (Fig. 4B) also suggests that *Tm*TF induces a folding-competent conformation of bound GFP because GroELS alone is unable to provide similar high refolding yields. These observations deserve further investigations using native chaperones from *T. maritima*.

The properties of *Ec*TF have been extensively investigated in previous studies (Hoffmann *et al*., 2010; 2012; Lakshmipathy *et al*., 2010), but the temperature context was generally overlooked. Indeed, *Ec*TF is a cold shock protein (Kandror and Goldberg, 1997) and is

overexpressed in *E. coli* by temperature downshifts from 37°C to 15°C (Inouye and Phadtare, 2004). Interestingly, GFP-assisted refolding by *Ec*TF changes its properties at 15°C, maintaining a high foldase activity without inhibition at high concentrations (Fig. 3C). It can be proposed that persistence of its chaperone activity at low temperature and high molar ratios might contribute to its chaperone function after a cold shock. As part of the cold shock response in *E. coli*, *Ec*TF thus changes its chaperone (and isomerase) characteristics and converges with the behaviour of *P. haloplanktis* at low temperature. Furthermore, the high enthalpic contribution to binding revealed by ITC (Fig. 5; Table 2) results in an increase of the affinity of *Ec*TF for unstructured proteins at low temperature, which should also favour its cold shock protein function.

PPIase activity in TFs

In addition to its chaperone function, TF also catalyses peptidyl-prolyl *cis*/*trans* isomerization, which is a rate-limiting step in the folding of numerous proteins (Schmid, 2005). Because of the high activation energy, prolyl isomerization is an intrinsically slow process and is strongly temperature dependent (Schmid, 2005). Accordingly, it has been suggested that prolyl isomerization strongly decelerates protein folding in psychrophiles at low temperature (Piette *et al*., 2011a). *Ph*TF resembles *Ec*TF in its substrate specificity but shows about 10-fold lower activity towards proline-containing tetrapeptides (Figs 6 and S5). However, in proline-limited folding reactions of two model proteins (XP-N2 and RCM-T1), the catalytic efficiency of *Ph*TF is similar to that of *Ec*TF, in particular at low temperature (Fig. 7). In this respect, the PPIase activity of *Ph*TF does not display the usual traits of cold-adapted enzymes, i.e. a higher activity at low temperature. However, overexpression of *Ph*TF in *P. haloplanktis* (38-fold at 4°C versus 18°C) noted in proteomic studies (Piette *et al*., 2010) is accompanied by the same substantial increase of the cellular PPIase activity that evidently contribute to accelerate prolyl isomerization. This is one of the very rare cases where the decrease of enzyme activity at low temperature is compensated by an increased amount of the enzyme catalyst and not by a higher catalytic constant, as observed in cold-adapted enzymes. It is also worth mentioning that psychrophilic proteins frequently display a reduced proline content (Feller and Gerday, 2003; Siddiqui and Cavicchioli, 2006). This has been related to the improvement of structural dynamics required for function in the cold, but it may also be the result of a selective pressure to reduce the number of proline-dependent rate-limiting steps in the folding of cold-adapted proteins.

The FKBP domains of *Tm*TF (Martinez-Hackert and Hendrickson, 2009) and *Ec*TF (Ferbitz *et al*., 2004; Kramer *et al*., 2004a) show similar structures, although they are only distantly related in amino acid sequence. *Tm*TF displays a weak but significant prolyl isomerase activity. Hyperthermophilic enzymes are often almost inactive at room temperature, and therefore *Tm*TF PPIase probably shows much higher activity at elevated temperatures, which currently are not accessible to PPIase assays. On the other hand, the rate of uncatalysed prolyl isomerization increases strongly with temperature (Schmid, 2005) and a high catalytic activity of *Tm*TF is possibly not essential for survival. Finally, *Ec*TF ensures the folding of newly synthesized proteins at 37°C. At this temperature, prolyl isomerization is often still rate limiting during protein folding and therefore a TF with both a high chaperone and a high PPIase activity (as observed here for TF from *E. coli*) would be of advantage. It is also worth mentioning that the genome of *P. haloplanktis* contains 14 annotated prolyl isomerases, as compared with eight PPIases in *E. coli* and only one putative PPiase in *T. maritima*, in addition to the single TF gene copy. This correlation between the number of genome-encoded PPIases and the environmental temperatures further suggests adaptations in the psychrophile to overcome rate-limiting steps in protein folding driven by prolyl isomerization, whereas this isomerization appears non-detrimental in the hyperthermophile.

The present report highlights the sophisticated mechanisms involved in microbial adaptation to temperature at the chaperone level. Moreover, the distinct properties displayed by the investigated TFs open new perspectives when considering the diversity of chaperones actively contributing to protein folding in a microbial cell and the wide range of environmental temperatures.

Experimental procedures

Production and purification of TFs

Production and purification of *Ph*TF, *Ec*TF and *Tm*TF were performed essentially as described (Piette *et al*., 2010; Struvay *et al*., 2013). The genes of TFs were cloned into pET22b expression vectors (Novagen) without His-tag. The proteins were expressed in *E. coli* BL21 (DE3) grown in Terrific Broth (100 μg ml^{-1} ampicillin) at 18°C. Expression was induced by 1 mM IPTG. The cells were harvested by centrifugation for 30 min at 20 000 *g* and 4°C. The pellets were re-suspended in 35 mM HEPES pH 7.6 supplemented with benzonase and a protease inhibitor cocktail (Complete, Roche) and disrupted on an EmulsiFlex-C3 homogenizer (Avestin). Cell debris were removed by 30 min centrifugation. The supernatants were brought to 90% saturation in $(NH_4)_2SO_4$, centrifuged, and the resulting pellets were re-suspended in 35 mM HEPES, pH 7.6, 30% saturation in $(NH_4)_2SO_4$ (or 10% for *Tm*TF). TFs were purified by a combination of Phenyl Sepharose (GE Healthcare) and DEAE-

agarose column chromatography as described (Piette *et al*., 2010). An additional step was required to complete the purification of *Tm*TF. The pool was dialysed against 5 mM phosphate, 1 mM PMSF, pH 7, loaded on a CHT hydroxyapatite column (BioRad) equilibrated in the same buffer and eluted with a linear gradient (1 l) from 5 to 200 mM phosphate. Purified protein samples (5–10 mg ml^{-1}) were stored at −70°C. Prior experiments, samples were filtered on 0.22 μm membranes and protein concentration was determined at 280 nm on an Uvikon XS spectrophotometer using extinction coefficients of 14 440, 17 420 and 31 860 M^{-1} cm^{-1} for *Ph*TF, *Ec*TF and *Tm*TF respectively.

Prevention of GAPDH aggregation

Prevention of GAPDH aggregation by TFs was performed essentially as described previously (Huang *et al*., 2000). GAPDH from rabbit muscle (G2267, Sigma) was denatured overnight by 3.0 M GdmCl at 4°C in 0.1 M potassium phosphate, 1 mM EDTA, 5 mM DTT, pH 7.5. Aggregation of denatured GAPDH was induced by a 50-fold dilution (2.5 μM final concentration) in the phosphate buffer containing increasing concentrations of TFs. The dilution was performed in a spectrophotometer cuvette thermostated at 20°C, and the time course of aggregation was monitored the increase of turbidity at 620 nm. Alternatively, the dilution was performed with ice-cold solutions in an ice bath, followed by a 30 min incubation in this bath. The reaction mixture was then transferred into a cuvette thermostated at 15°C to monitor the time course of aggregation. The concentration of tetrameric GAPDH was used for molar ratio calculation.

GAPDH refolding assay

GAPDH was denatured by 3.0 M GdmCl and diluted with potassium phosphate buffer, as described above. Mixtures were incubated for 30 min in melting ice and for a further 3 h at 15°C. The recovered enzymatic activity was measured after a 10-fold dilution of re-natured GAPDH to final conditions of 0.025 μM GAPDH, 700 μM β-nicotinamide adenine dinucleotide and 700 μM DL-glyceraldehyde 3-phosphate in the phosphate buffer. The synthesis of NADH at 15°C was followed spectrophotometrically at 340 nm. The extent of recovery of GAPDH activity was calculated by dividing the rate constants by the rate constant measured in a control with native GAPDH (Huang *et al*., 2000; Kramer *et al*., 2004a).

GFP refolding assay and refolding kinetics

For this assay, a highly fluorescent mutant of the GFP (GFP-mut2) (Chirico *et al*., 2002) was used as substrate. The gene was cloned in a pET28a expression vector and expressed in *E. coli* BL21 (DE3) grown in LB medium (50 μg ml^{-1} kanamycin) at 18°C. Protein expression was induced by 0.5 mM IPTG. The cells were harvested by centrifugation, re-suspended in 10 mM Tris buffer, 1% NaCl, pH 7.5, and centrifuged again. The cell pellet was re-suspended in 20 mM Tris, 10 mM $MgSO_4$, 100 mM KCl, pH 8, before addition of benzonase and a protease inhibitor cocktail (Halt, Thermo Scientific). The cells were disrupted on an EmulsiFlex-C3 homogenizer (Avestin), and cellular debris was removed by centrifugation. The supernatant was dialysed against 20 mM Tris, pH 8, loaded on a Q Sepharose High Performance column (28 ml, GE Healthcare) and eluted with a linear gradient (280 ml) from 0 to 300 mM NaCl. The fractions containing GFP-mut2 were pooled, dialysed against 20 mM Tris, pH 8, and purified on a HiTrap Heparin High Performance column (10 ml, GE Healthcare) using a linear gradient (30 ml) from 0 to 2 M NaCl. The last chromatographic step was a Sephacryl S-100 High Resolution gel filtration (120 ml, Amersham Biosciences), using 20 mM Tris, 250 mM NaCl, pH 8 as elution buffer.

GFP refolding assay were performed in triplicate essentially as described previously (Suno *et al*., 2004). GFP 10 μM (10 μl) was unfolded by mixing with 90 μl 50 mM glycine-H_2SO_4, pH 2, for 2 min at room temperature. The refolding of GFP was initiated by a 20-fold dilution with 50 mM Hepes-KOH, 200 mM KCl, pH 7.5 (pre-equilibrated at the experimental temperature). The refolding buffer contained the appropriate concentrations of TFs and, when indicated, 9 μM *E. coli* GroELS (Sigma) and 9 mM ATP. Fluorescence emission was recorded for 800 s (and up to 2400 s for slow kinetics) at 510 nm after excitation at 395 nm, using a thermostated spectrofluorometer Perkin-Elmer LS50. Kinetic data were fitted to a double exponential equation (Xie and Zhou, 2008)

$$F = A_{fast} \cdot e^{(-k_{fast} \cdot x)} + A_{slow} \cdot e^{(-k_{slow} \cdot x)} + F^{\infty} \quad (1)$$

to obtain the rate constants k_{fast} and k_{slow}, the amplitudes A_{fast} and A_{slow} of the corresponding phases and the maximal fluorescence intensity at infinite time F^{∞}.

ANS titration

ANS fluorescence measurements were performed at 20°C on a Jasco FP-8300 spectrofluorometer as described (Schonbrunn *et al*., 2000). The buffer used was 35 mM Hepes, 250 mM NaCl, pH 7.6, containing 5 μM TF. The excitation wavelength was 380 nm and emission spectra were recorded between 420 and 600 nm at various concentrations of ANS ranging from 0 to 180 μM. Fluorescence intensities at the maximum emission wavelength of 490 nm were plotted as a function of adjusted ANS concentrations. Data were fitted to the following equation to determine the dissociation constant K_d of ANS binding:

$$F = F_{max} \cdot [ANS]/(K_d + [ANS] - (K_{ife} \cdot [ANS]) \quad (2)$$

where K_{ife} accounts for the inner filter effect of ANS on fluorescence.

ITC

ITC experiments were performed on an ITC200 instrument (GE) fitted with a 200 μl sample cell. TFs and α-casein (Sigma-Aldrich) were dialysed against 50 mM Hepes-NaOH, 100 mM NaCl, 1 mM EGTA, pH 7.6. In a standard experiment, TFs (50–100 μM) were titrated by 20 injections (2 μl) of α-casein (200–250 μM) at an interval of 3 min using a syringe rotating at 1000 r.p.m. Reverse titrations were performed under the same conditions by injections of TFs (180–280 μM)

into α-casein (20–35 μM). The data so obtained were fitted via non-linear least squares minimization method to determine binding stoichiometry (*n*), association constant (K_a) and change in enthalpy of binding (ΔH°_b) using ORIGIN 7 software v.7 (OriginLab). The Gibbs free energy of binging, ΔG°_b, was calculated from K_a and the entropic term, $T\Delta S^\circ_b$, was derived from the Gibbs–Helmholtz equation using the experimental ΔH°_b value.

Assaying PPIase activity towards peptides

The PPIase activity of TFs towards fluorescent peptides was determined as previously described (Zoldak *et al*., 2009). The peptides were of the general formula aminobenzoyl-Ala-Xaa-Pro-Phe-4-nitroanilide (Abz-AXPF-pNA) with different proteinogenic amino acids at position Xaa. Peptides were dissolved in a mixture of 0.55 M anhydrous LiCl/tri-fluoroethanol at a concentration of 750 μM. Under these conditions, the *cis*-content of the peptidyl-prolyl bond within the tetra-peptide is increased and the quencher (pNA) is in close proximity to the fluorophore (Abz). The TF enzymes (1–60 nM) were pre-equilibrated in 1 cm cuvettes under constant stirring at the desired temperature for 2 min. The reactions were started by adding the peptide substrate to reach a final concentration of 3 μM. In aqueous solutions, the *trans* conformation of the prolyl bond is preferred and therefore *cis*→*trans* isomerization can be monitored by the increase in Abz fluorescence. The kinetics is accelerated in the presence of a PPIase, and its catalytic efficiency (k_{cat}/K_M) is derived from a plot of the measured rate constant of isomerization as a function of the PPIase concentration. This assay is also suitable for PPIases with chaperone domains such as TF (Jakob *et al*., 2009). All experiments were performed at a Jasco FP-6500 fluorescence spectrophotometer in 100 mM potassium phosphate, 1 mM EDTA, pH 7.5. Fluorescence emission of the aminobenzoyl moiety was recorded at 416 nm (bandwidth 5 nm) after excitation at 316 nm (bandwidth 3 nm).

Monitoring PPIase activity during refolding of a protein substrate

To assay the PPIase activity of TF in a protein folding reaction, the isolated N2 domain of the gene-3-protein of phage fd with the stabilizing substitution Q129H (N2) was used. N2 carries a Pro at position 161, at the tip of a loop region. Pro161 is *cis* in about 90% of all native molecules. An increase in fluorescence accompanies the *trans* to *cis* isomerization of this prolyl bond during refolding (Jakob and Schmid, 2008; 2009). We used variants of N2 with different amino acids at the position preceding Pro161 (XP-N2, with X being any proteinogenic amino acid). For these folding assays, XP-N2 was unfolded in 5.0 M urea for at least 1 h. Dilution to 0.16 M urea at a final protein concentration of 0.33 μM initiated the refolding of XP-N2. The slow fluorescence phase, which reflects prolyl isomerization at Pro161, is well catalysed by prolyl isomerases. To decrease background fluorescence from the PPIase, a tryptophan-free variant of *Ec*TF (*Ec*TF W151F) denoted as *Ec*TF* was used. This variant essentially shows the same activity as the wild-type protein (J. Koch, unpubl. obs.).

To perform the PPIase assays, 100 mM potassium phosphate, 1 mM EDTA, pH 7.5, was equilibrated in the cuvette at constant temperature and PPIase concentrations ranging from 0 to 60 nM were added. The assays were started by adding denatured XP-N2 under constant stirring. Fluorescence was monitored at 340 nm (bandwidth 5 nm) after excitation at 280 nm (bandwidth 3 nm) using a Jasco FP-6500 fluorescence spectrophotometer. In all cases, the fluorescence time courses were analysed by fitting mono-exponential functions to the data.

Because the N2 protein is already destabilized at 25°C and shows only a small signal change upon *trans*→*cis* isomerization at Pro161, another protein substrate, RNaseT1, was also used to analyse the dependence on temperature of TF-catalysed folding. Specifically, reduced and carboxymethylated RNaseT1 with the substitutions S54G P55N (RCM-T1) was employed. This variant is unfolded in aqueous buffer. Refolding is induced by transfer to high concentrations of NaCl (2.0 M) and is limited by the isomerization of Pro39. TF at the desired concentration was equilibrated in 100 mM Tris/HCl, $pH^{15°C}$ 7.5, 2.0 M NaCl. Refolding was initiated by diluting RCM-T1 100-fold to a final concentration of 0.1 μM. The increase of the fluorescence at 320 nm (10 nm bandwidth) after excitation at 268 nm (3 nm bandwidth) followed a monoexponential function.

Analysis of the PPIase activity

The obtained apparent rate constants for prolyl isomerization from the peptide and the protein assays increased linearly with PPIase concentration and followed the relation:

$$k_{app} = k_0 + [E] \times k_{cat}/K_M \quad (3)$$

where k_0 is the rate of the uncatalysed reaction, [E] is the enzyme concentration in the cuvette and k_{cat} and K_M are the turnover number and the Michaelis constant respectively.

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Supporting information

Additional Supporting information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Time courses of GAPDH aggregation at 15°C. GAPDH (2.5 μM) was diluted with *Ec*TF (2.5 μM), *Tm*TF (2.5 μM) or *Ph*TF (5 μM) and incubated at 0°C for 30 min. The mixture was loaded in a spectrophotometer cell at 15°C.

Fig. S2. GFP refolding in the presence of *Ph*TF and GroELS. Fluorescence time courses of GFP (0.5 μM) and in the presence of GroELS (9 μM, 9 mM ATP), *Ph*TF (1.5 μM) or *Ph*TF + GroELS at 15°C.

Fig. S3. ANS titration of trigger factors. Fluorescence increases of ANS bound to *Tm*TF (■), *Ec*TF (●) and *Ph*TF (▲). Solid lines are fits to a binding isotherm corrected for ANS inner filter effect.

Fig. S4. ITC of TFs binding to α-casein in direct titration. Upper panel: exothermic microcalorimetric traces of α-casein injections into TFs. Lower panel: Wiseman plot of heat releases versus molar ratio of injectant/protein in the cell and non-linear fit of the binding isotherm for n equivalent binding sites.

A. Injections of α-casein (260 μM) into *Ec*TF (47 μM) at 25°C.

B. Injections of α-casein (260 μM) into *Tm*TF (99 μM) at 25°C.

Fig. S5. Catalytic efficiencies (k_{cat}/K_M) of trigger factors towards short peptides at 5°C and 25°C. Symbols: *Ph*TF (▲), *Ec*TF (●) and *Tm*TF (■). The residue before Pro in the peptide Abz-AXPF-pNA is indicated.

Table S1. General properties of the investigated trigger factors.

Table S2. Effects of TFs on GFP refolding kinetic parameters at near-environmental temperatures. Rate constants *k*, normalized amplitudes A and extrapolated refolding yields F^{∞} (values are mean ± SD, $n = 3$).

Table S3. Thermodynamic parameters of binding from direct titrations of α-casein into TFs.

Table S4. Catalytic efficiencies of the three TFs towards short peptides at different temperatures.

Table S5. Catalytic efficiencies of the three TFs towards the refolding protein substrate XP-N2 at 15°C.

Table S6. Catalytic efficiencies of the three TFs towards the refolding protein substrate RCM-T1 at different temperatures.

Supplementary Material to:

Functional adaptations of the bacterial chaperone trigger factor to extreme environmental temperatures

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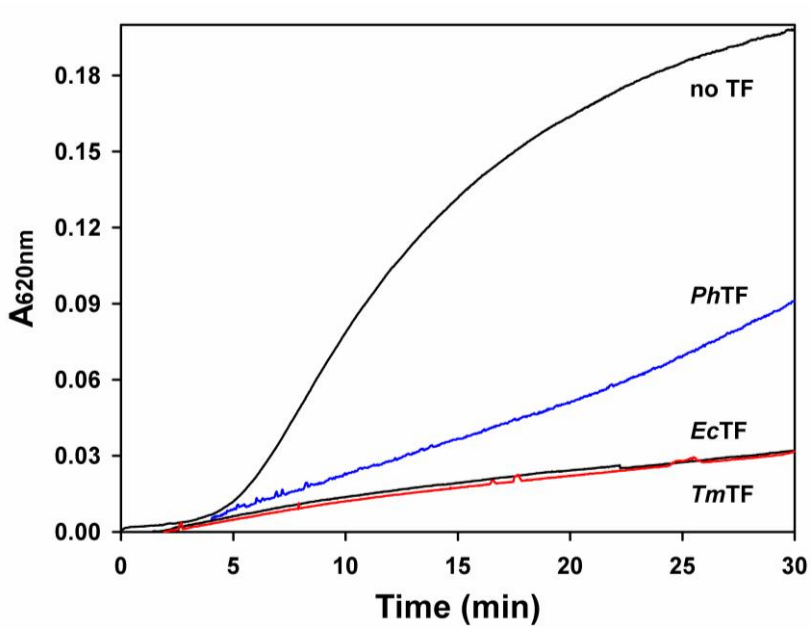

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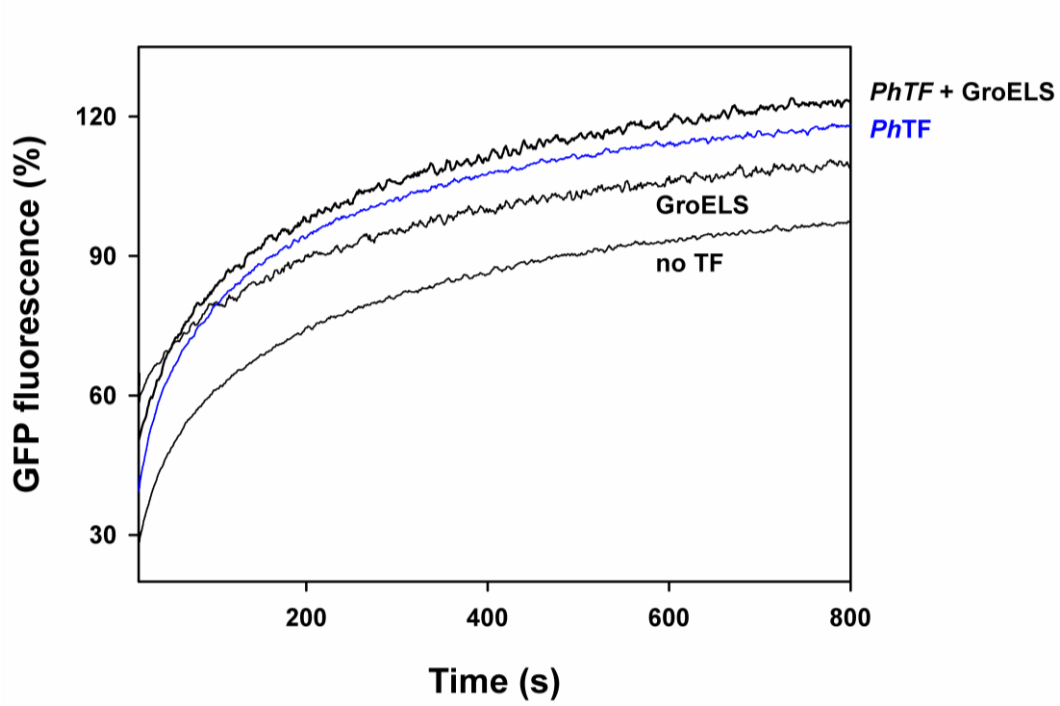

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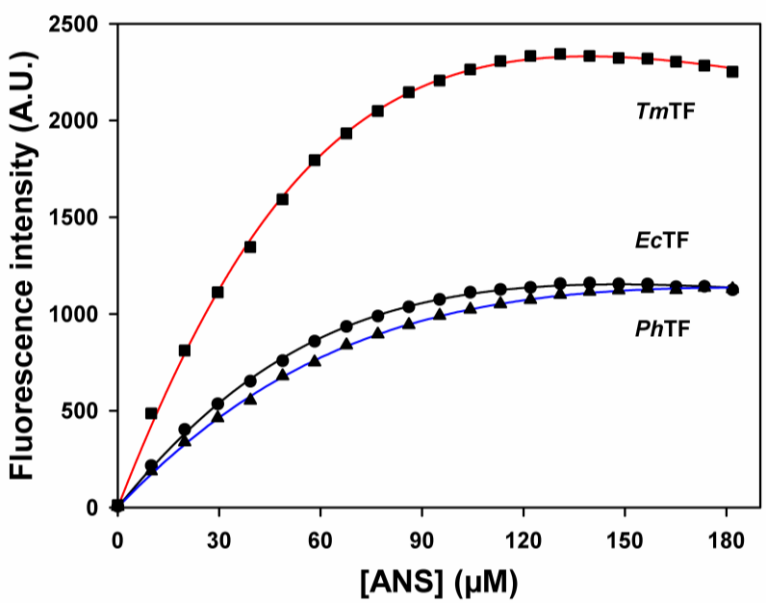

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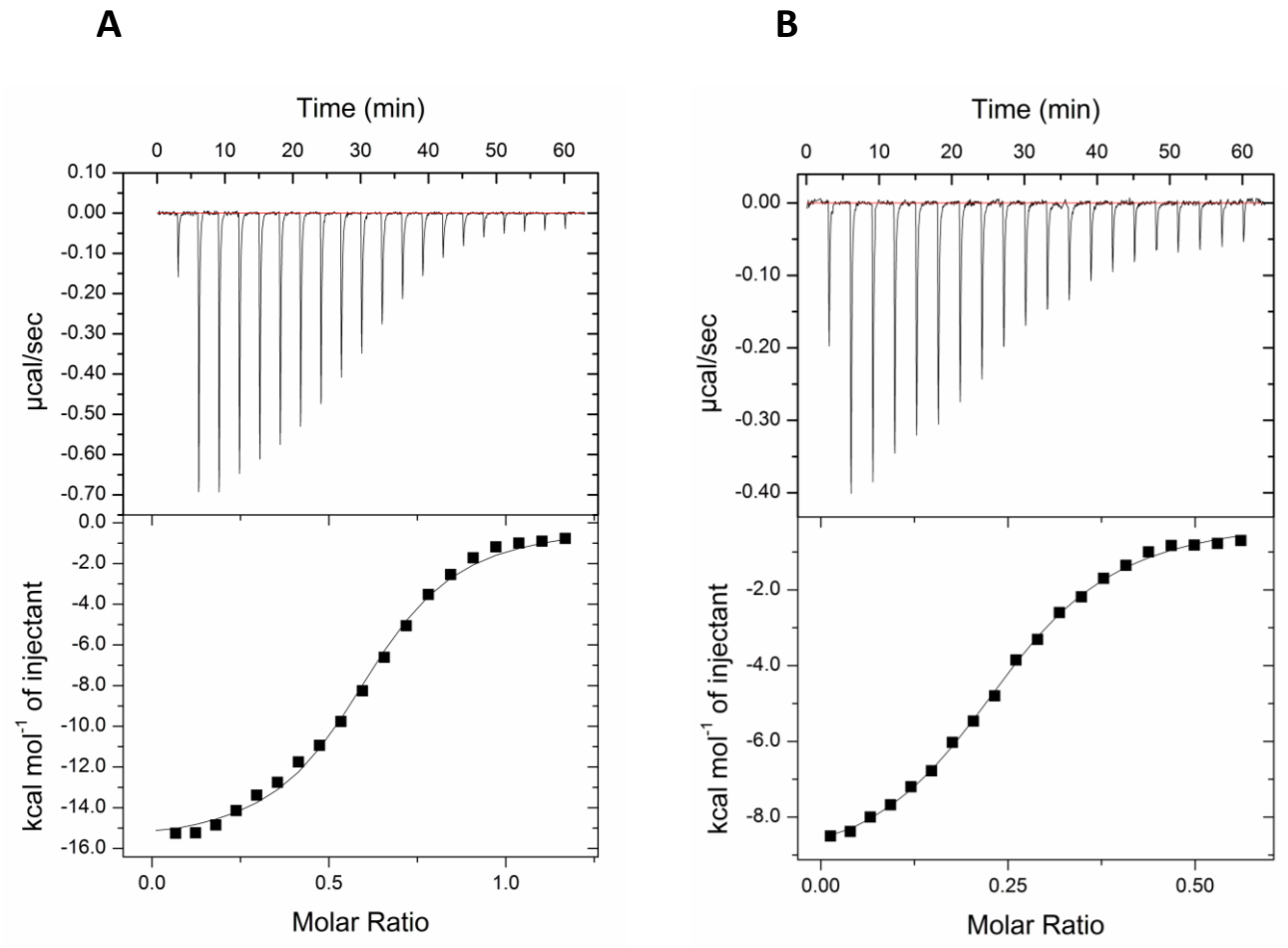

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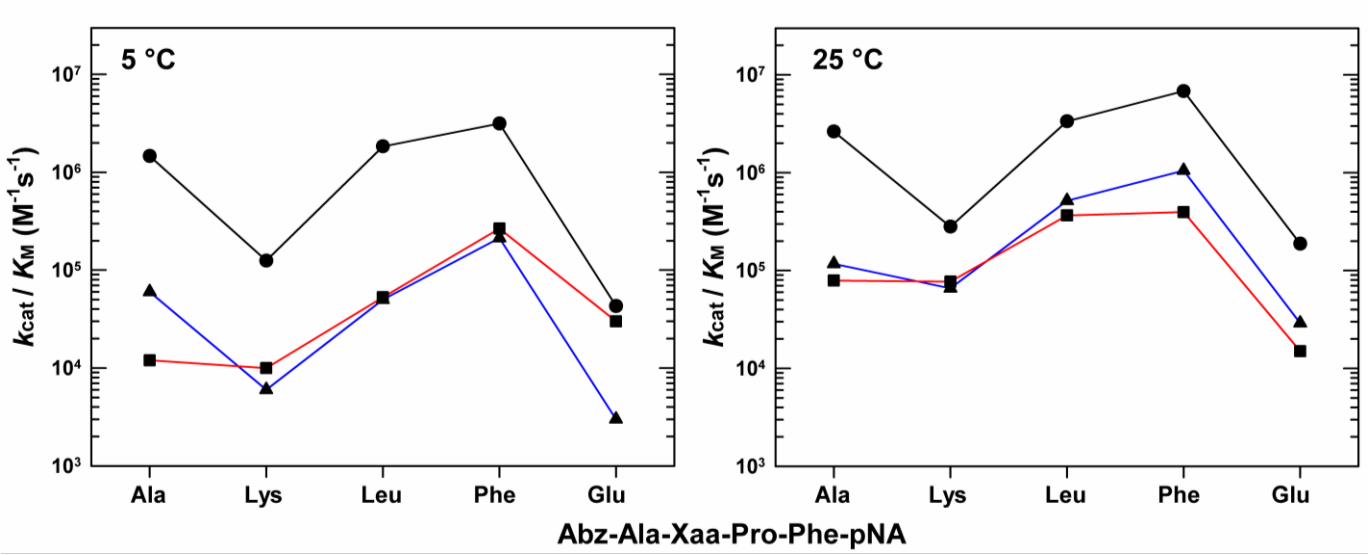

Supplementary Fig. S5. Catalytic efficiencies (k_{cat}/K_M) of trigger factors towards short peptides at 5 °C and 25 °C.

Symbols: *Ph*TF (▲), *Ec*TF (●) and *Tm*TF (■). The residue before Pro in the peptide Abz-AXPF-pNA is indicated.

Supplementary Table S1. General properties of the investigated Trigger Factors

Protein	Source	T_{env}[a]	GenBank	Residues (n)	Mr (Da) Theoretical	Mr (Da) ESI-Q-TOF	PDB
*Ph*TF	*Pseudoalteromonas haloplanktis* TAC125	< 0 °C	CR954246.1	434	47,534.5	47,540	-
*Ec*TF	*Escherichia coli* RR1	37 °C	M34066.1	432	48,192.6	48,227	1W26
*Tm*TF	*Thermotoga maritima* DSM3109	85-90 °C	EHA61365.1	425	49,897.7	49,900	3GU0

[a] Estimated average environmental temperatures

Supplementary Table S2. Effects of TFs on GFP refolding kinetic parameters at near-environmental temperatures. Rate constants *k*, normalized amplitudes A and extrapolated refolding yields F^{∞} (values are mean ± SD, n = 3).

			Rate constants (s^{-1} x 10^{-3})		Amplitudes (%)		F^{∞} (%)
	Temp	[TF]/[GFP]	k_{fast}	k_{slow}	A_{fast}	A_{slow}	
GFP	5 °C	0	33 ± 6	2.9 ± 0.7	43 ± 8	55 ± 3	100 ± 6
GFP + *Ph*TF		1	27 ± 2	2.7 ± 0.2	54 ± 1	55 ± 1	113 ± 2
		10	24 ± 2	3.0 ± 0.2	53 ± 2	55 ± 2	114 ± 4
GFP	37 °C	0	76 ± 4	8.2 ± 0.3	76 ± 1	21 ± 1	100 ± 1
GFP + *Ec*TF		1	110 ± 6	8.2 ± 0.0	102 ± 4	29 ± 2	133 ± 6
		10	95 ± 5	8.1 ± 0.2	58 ± 6	20 ± 2	79 ± 8
GFP	50 °C	0	125 ± 25	3.7 ± 0.5	85 ± 3	14 ± 2	100 ± 1
GFP + TmTF		1	73 ± 2	6.5 ± 1.2	82 ± 5	17 ± 2	104 ± 3
		6	70 ± 9	8.8 ± 1.0	35 ± 4	36 ± 6	75 ± 1

Supplementary Table S3. Thermodynamic parameters of binding from direct titrations of α-casein into TFs

Temperature	Protein	*n*	K_a 10^5 M^{-1}	$\Delta G°_b$ kcal mol^{-1}	$\Delta H°_b$ kcal mol^{-1}	$T\Delta S°_b$ kcal mol^{-1}
25 °C	*Ec*TF	0.63	7.19	-8.0	-15.5	-7.5
	*Tm*TF	0.25	3.32	-7.5	-9.5	-2.0
37 °C	*Ec*TF	0.64	5.42	-8.1	-26.3	-18.2
	*Tm*TF	0.39	2.12	-7.5	-9.9	-2.3

Supplementary Table S4. Catalytic efficiencies of the three TFs towards short peptides at different temperatures

TF	Temperature	k_{cat}/K_M (mM^{-1} s^{-1}) Abz-AXPF-pNA				
		Ala	Lys	Leu	Phe	Glu
*Ph*TF	5 °C	60	6	50	213	3
	15 °C	106	20	117	924	56
	25 °C	117	66	517	1052	29
Ec TF	5 °C	1467	125	1840	3153	43
	15 °C	2190	249	2950	4740	164
	25 °C	2630	280	3342	6820	188
*Tm*TF	5 °C	12	10	53	266	30
	15 °C	12	23	72	230	63
	25 °C	79	77	336	396	15

Given are the numerical values of k_{cat}/K_M as they are presented in Figs 8C and 9 for *Ph*TF, *Ec*TF and *Tm*TF towards the short peptides (Abz-AXPF-pNA). The amino acid preceding the proline and therefore determining the specificity is indicated as well as the reaction temperature. All PPIase assays were performed in 100 mM potassium phosphate, 1 mM EDTA, pH 7.5. Enzyme concentrations ranging from 0 nM to 60 nM were used.

Supplementary Table S5. Catalytic efficiencies of the three TFs towards the refolding protein substrate XP-N2 at 15 °C

	k_{cat}/K_M (mM^{-1} s^{-1}) XP-N2				
	Ala	Lys	Leu	Phe	Glu
*Ph*TF	2962	1908	4666	6325	1175
*Ec*TF	6566	4844	9928	7810	3508
*Tm*TF	340	370	1300	230	290

Given are the numerical values of k_{cat}/K_M as they are presented in Fig. 8D for *Ph*TF, *Ec*TF and *Tm*TF towards the refolding protein XP-N2. The amino acid preceding the proline is indicated. All PPIase assays were performed in 100 mM potassium phosphate, 1 mM EDTA, pH 7.5, 15 °C. Enzyme concentrations ranging from 0 nM to 60 nM were used.

Supplementary Table S6. Catalytic efficiencies of the three TFs towards the refolding protein substrate RCM-T1 at different temperatures

		k_{cat}/K_M (mM^{-1} s^{-1})
	Temperature	RMC-T1
*Ph*TF	5 °C	272
	15 °C	639
	25 °C	826
*Ec*TF	5 °C	327
	15 °C	958
	25 °C	4236
*Tm*TF	5 °C	2
	15 °C	5
	25 °C	44

Given are the k_{cat}/K_M values (mM^{-1} s^{-1}) derived from the plots in Fig. 10 for *Ph*TF, *Ec*TF and *Tm*TF towards the refolding protein RCM-T1. The reaction temperature is indicated. All PPIase assays were performed in 100 mM Tris/HCl, $pH^{15\ °C}$ 7.5, 2.0 M NaCl. Enzyme concentrations ranging from 0 nM to 60 nM were used.