Review

Protein folding at extreme temperatures: Current issues

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Georges Feller

Laboratory of Biochemistry, Center for Protein Engineering-InBioS, University of Liège, Institute of Chemistry B6a, 4000 Liège-Sart Tilman, Belgium

A R T I C L E I N F O	**A B S T R A C T**

The range of temperatures compatible with life is currently estimated from −25 °C, as exemplified by metabolically active bacteria between sea ice crystals, and up to 122 °C in hydrothermal vents as exemplified by the archaeon *Methanopyrus kandleri*. In the context of protein folding, as soon as a polypeptide emerges from the ribosome, it is exposed to the effects of environmental temperatures. Recent investigations have shown that the rate of protein folding is not adapted to extreme temperatures and should be very fast at high temperature and low in cold environments. This lack of adaptation is driven by kinetic constraints on protein stability. To counteract the deleterious effects of fast protein folding in hyperthermophiles, chaperones such as the Trigger Factor hold and slow down the rate of folding intermediates. Prolyl isomerization, a rate-limiting step in the folding of many proteins, is strongly temperature-dependent and impairs folding of psychrophilic proteins in the cold. This is compensated by reduction of the proline content in cold-adapted proteins, by an increased number of prolyl isomerases encoded in the genome of psychrophilic microorganisms and by overexpression of prolyl isomerases under low temperature cultivation. After folding, the native state is reached and although extremophilic proteins share the same fold, dramatic differences in stability have been recorded by differential scanning calorimetry.

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Contents

1. Introduction

In recent years, microbiological investigations of environments previously regarded as abiotic have considerably expanded the

spectrum of biological temperatures. Metabolically active bacteria have been found at −20 °C, thriving in the liquid brine veins between sea ice crystals [1,2]. More recently, the bacterium *Planococcus halocryophilus* isolated from Arctic permafrost was found to grow and to divide at −15 °C and to display residual metabolic activity at −25 °C [3], which possibly represents the lower temperature limit before dormancy. These low temperature extremophiles are termed psychrophiles. At the other extremity of the temperature range compatible with life, thermophiles are known for decades but hyperthermophiles have pushed the

limit above 100 °C. Hyperthermophilic bacteria such as *Thermotoga maritima* [4] or *Aquifex aeolicus* [5] isolated from geothermally heated seafloor sediments are limited to 90–95 °C, whereas the most extreme environments such as deep sea hydrothermal vents, are exclusively colonized by Archaea. For many years, the archaeon *Pyrolobus fumarii* growing at 113 °C was regarded as the most heat-resistant microorganism [6], but more recently *Methanopyrus kandleri*, thriving at 122 °C and enduring exposure up to 130 °C, has established the current upper temperature limit for life [7]. It should be noted that such high temperature for cell proliferation is reached under *in situ* high pressure.

Microbial life under these extreme environmental temperatures obviously requires a vast array of adaptations at all cellular levels, including macromolecules. In this respect, proteins and enzymes play a pivotal role as they drive the cell cycle and metabolism. At low temperature, enzymes have to remain active and proteins have to maintain adequate functional dynamics at temperatures that slow down molecular motions. This is reached by adopting a mobile and flexible structure through reduction of all types of weak interactions involved in the shaping of protein conformation such as hydrogen bonds, salt bridges, van der Waals contacts, helix dipole, etc. and the hydrophobic effect. But the price to pay for such an improved structural dynamics is the pronounced heat-lability of psychrophilic proteins [8–12]. In contrast, thermophilic and hyperthermophilic proteins have to maintain a functional native state at elevated temperatures that would otherwise unfold mesophilic proteins. Their robust and heat-stable structure arises from the improvement in strength and number of all types of weak interactions and structural factors stabilizing the protein conformation [13–15].

Here, we will focus on protein folding, a crucial topic for the biophysical understanding of life at extreme temperatures. Indeed, as soon as a nascent polypeptide emerges from the ribosome, it is exposed to the effects of environmental temperatures. Recent investigations have addressed some essential questions: *i)* what is the effect of extreme environmental temperatures on the protein folding rate; *ii)* the Trigger Factor (TF) is the first chaperone interacting with nascent chains: how does it help protein folding at extreme temperatures; *iii)* how do prolyl isomerases catalyze prolyl isomerization, a rate-limiting step in protein folding and *iv)* what are the properties of the final native state of protein adapted to these temperatures? Many of the reviewed results have been obtained with TF as a model protein. In Bacteria, TF is a ribosome-bound chaperone interacting with virtually all synthesized polypeptides and it also possesses a peptidyl-prolyl *cis/trans* isomerase (PPiase) activity [16]. This chaperone has been isolated from model bacteria: the Antarctic psychrophile *Pseudoalteromonas haloplanktis*, a fast growing strain at low temperature [17], the mesophilic reference *Escherichia coli* and the hyperthermophile *Thermotoga maritima* [4].

2. The folding rate is not adapted to extreme temperatures

In order to determine the folding rate constant, the unfolded protein (usually in urea or guanidinium chloride) is diluted with a buffer and its refolding is monitored by a stopped-flow spectrophotometer as the process is completed within a few seconds. Similar experiments are performed for unfolding, starting with the native state (Fig. 1).

By using a range of final denaturant concentrations, a "Chevron plot" is constructed (see Fig. 2 as an example).

The left arm of the plot corresponds to folding kinetics and the right arm depicts unfolding kinetics. Finally, the most relevant parameters, the microscopic rate constants for folding k_f (H$_2$O) and for unfolding k_u (H$_2$O) are obtained by extrapolation on the Y axis, *i.e.* in the absence of denaturant, in H$_2$O.
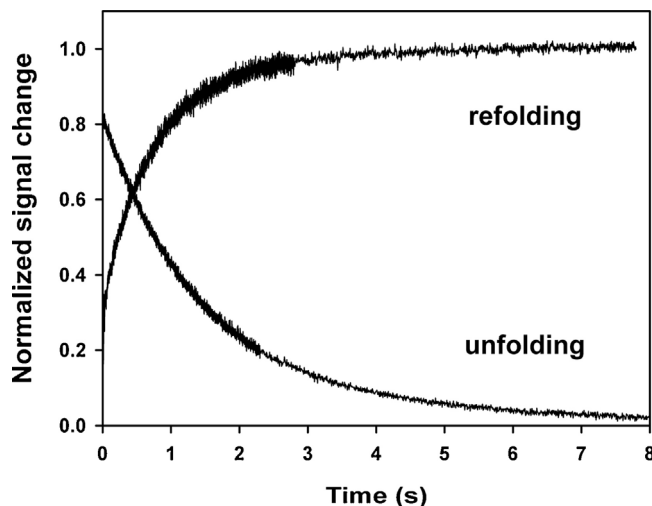

Fig. 1. Representative folding and unfolding fluorescence traces recorded by stopped-flow. Rate constants are obtained by adjusting one or several exponential functions to the kinetic traces. Unpublished data from [18] using a psychrophilic TF.
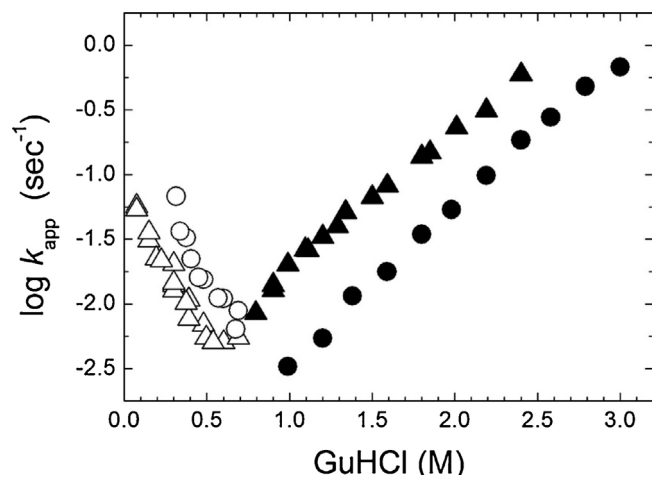

Fig. 2. Chevron plots of the rate constants k_{app} for unfolding (closed symbols, right arms) and refolding (open symbols, left arms) of tryptophan synthase α subunit from the psychrophile *S. frigidimarina* (▲,△) and from the mesophile *E. coli* (●,○) at 25 °C.
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Because these rate constants strongly differ in proteins and are also affected by the experimental setup, it is essential to compare series of homologous extremophilic proteins under the same experimental conditions [20]. This has been performed using the above-mentioned TF [18]. For the sake of clarity, Fig. 3 is a simplified version of the original work (only the main phases are depicted) that allows one to draw several conclusions. As far as folding is concerned, at the same temperature, the psychrophilic TF (Fig. 3, left panel, blue line) folds slower than its mesophilic homologue (black line) and the hyperthermophilic protein (red line) folds slightly faster. In the narrow window of temperature accessible to stopped-flow experiments, the temperature dependence of this folding rate was similar for the three proteins. Accordingly, one has to conclude that the folding rate is not adapted to extreme temperatures because under environmental conditions the hyperthermophilic protein should fold extremely fast, whereas the psychrophilic protein should fold even slower in the cold. The same observation was made for a psychrophilic tryptophan synthase subunit (Fig. 2) as compared with its *E. coli* homologue [19] and suggests a common behavior of cold-adapted proteins. On the other hand, proteins from
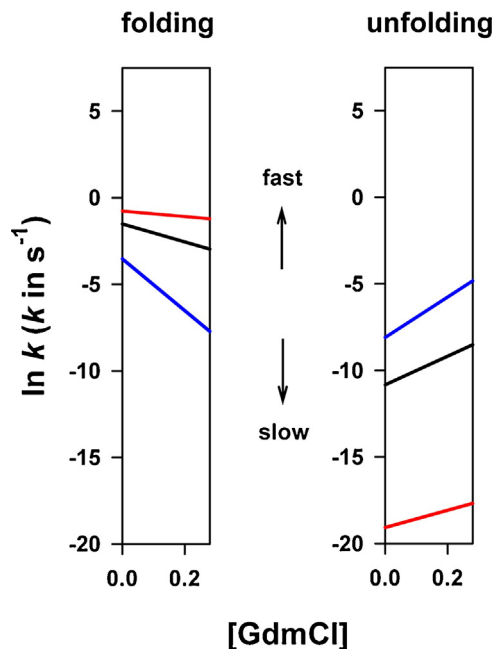
Fig. 3. Close-up of ordinate extrapolations from Chevron-plots. Data are shown for psychrophilic (blue), mesophilic (black) and hyperthermophilic (red) TFs at 9 °C. Extrapolations of the rate constants for the determination of the microscopic rate constants for folding k_f (H$_2$O) and unfolding k_u (H$_2$O) in the absence of denaturant. Adapted from [18].

Fig. 4. GFP refolding assisted by trigger factors. Data are shown for psychrophilic (blue), mesophilic (black) and hyperthermophilic (red) TFs. Refolding time courses of acid-denatured GFP at 15 °C recorded by fluorescence. Fluorescence intensity (extrapolated to the infinite) of spontaneously refolded GFP (no TF) is taken as 100%. Adapted from [33].

thermophiles and hyperthermophiles generally fold slightly faster [21–23] or with similar rate constants [21,24–26] as compared with their mesophilic homologues.

In contrast to folding, the differences in the unfolding rates of extremophilic TF are much larger (Fig. 3, right panel). The hyperthermophilic protein unfolds very slowly, whereas the psychrophilic protein unfolds faster as compared with the mesophilic counterpart. In fact, the slow unfolding of hyperthermophilic proteins has been previously identified as the main kinetic contribution to the unusually high stability of these proteins [24–29]. Indeed, in a simple two-state equilibrium between the unfolded state U and the native state N shown in Eq. (1),

$$U \underset{k_u}{\overset{k_f}{\rightleftharpoons}} N \tag{1}$$

a forward displacement of the equilibrium towards N, *i.e.* an increase of stability, is obtained by a slower unfolding rate k_u, as observed in heat-stable proteins. Conversely, a backward displacement of the equilibrium, *i.e.* a decrease of stability, is reached by a faster unfolding rate k_u and this is precisely what is observed for the natively unstable psychrophilic proteins (Figs. 2 and 3) [18,19] and also predicted by molecular dynamics simulations [30]. Very significantly, stabilization of a psychrophilic alpha-amylase by site-directed mutagenesis results in a slower unfolding rate of the mutants [31], underlining the kinetic strategy leading to the natively unstable conformation of psychrophilic proteins. Therefore, there is a fine tuning of the unfolding rate values in order to adjust protein stability to the environmental temperature. This results in a continuum from fast to slow unfolding rates in unstable to hyperstable proteins.

However, the same rationale can be applied to the folding rate k_f. In Eq. (1), an increase in stability is obtained by a fast folding rate k_f, whereas a lower stability is reached by a slower folding rate. Again, this is exactly what is observed for hyperthermophilic and psychrophilic proteins, respectively (Figs. 2 and 3). Significantly also, all mutants of a hyperthermophilic enzyme destabilized
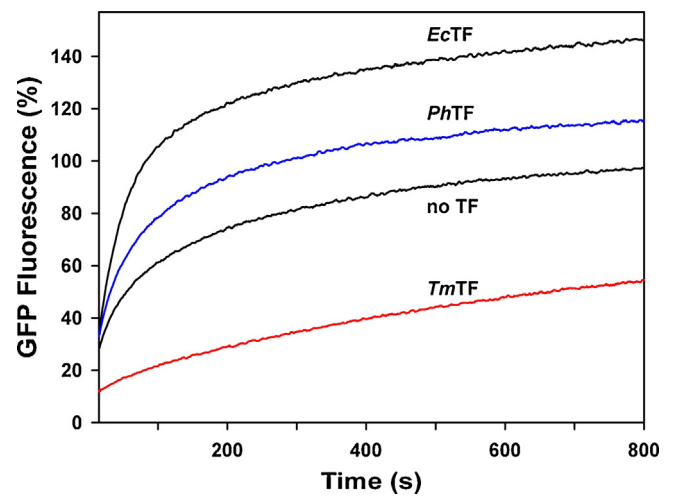
by site-directed mutagenesis displayed both faster unfolding rates and slower folding rates than the wild-type protein [25,32]. In summary, the natively unstable structure of psychrophilic proteins is gained via both a fast unfolding rate and a slow folding rate. Conversely, hyperthermophilic proteins are characterized by a slow unfolding rate and, to a lower extent, by a fast or unchanged folding rate. It follows that the folding rate cannot be adapted to extreme temperatures because adjustments of protein stability to environmental temperature is under kinetic control. Lowering the folding rate in hyperthermophiles would result in protein destabilization, whereas accelerating the folding rate in psychrophiles would increase protein stability.

What are the physiological consequences for the nascent polypeptide? Intuitively, the slow folding rate of psychrophilic proteins should not be a concern, except that it contributes to slow down the cell cycle, as observed in the environment but resulting from many other temperature sensitive cellular events. In contrast, both the fast folding rate of hyperthermophilic proteins and high temperature should have deleterious effects for the nascent polypeptide because they increase the probability of misfolding events and aggregation of misfolded species. This aspect is analyzed in the next section.

3. The trigger factor rescues protein folding at high temperature

If the folding rate is not adapted to extreme temperatures in the test tube, chaperones can have a pivotal role to rescue protein folding *in vivo*. TF is the first folding assistant acting co-translationally on synthesized polypeptides in bacteria. This is an obvious candidate to test the effects of chaperones and this has been performed using the above-mentioned extremophilic TFs [33]. Fig. 4 illustrates a classical refolding experiment in which acid denatured green fluorescent protein (GFP) is allowed to refold after buffer neutralization, either alone or in the presence of added TF. The fluorescence intensity of GFP is directly proportional to the concentration of native GFP in the experiment. The mesophilic TF from *E. coli* is a very efficient foldase that can improve the yield of native GFP up to 150%. The psychrophilic TF is also a foldase with however a weaker chaperone activity. This can be tentatively related to the fact that low temperature slows down protein folding and reduces the probability of misfolding and aggregation. As a matter of fact, low
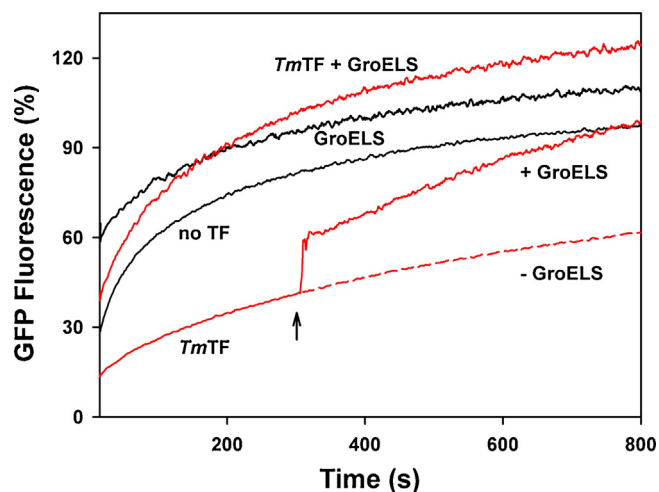
Fig. 5. GFP refolding in the presence of TF from the hyperthermophile *T. maritima* and GroELS at 15 °C. Fluorescence time courses of GFP alone and in the presence of GroELS, *Tm*TF or *Tm*TF + GroELS. In the sequential experiment, addition of GroELS after 300 s is indicated by an arrow.
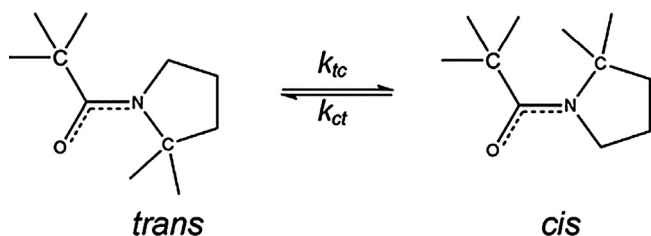Adapted from [33].

Fig. 6. *Trans* and *cis* isomers of a peptidyl-prolyl peptide bond.
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temperature cultivation of *E. coli* frequently avoids the formation of insoluble inclusion bodies of recombinant proteins [34], as well as expression in a psychrophilic host at low temperature [35]. Furthermore, low temperature weakens the hydrophobic effect. Therefore, an efficient foldase is possibly not required by psychrophiles in cold conditions. But the most insightful result was obtained with the hyperthermophilic TF. As shown in Fig. 4, TF from *T. maritima* is not a foldase (it does not promote the spontaneous GFP refolding) but instead it is a holdase: it binds to the refolding GFP and drastically decreases its folding rate. Accordingly, the holdase function of this TF should be regarded as the main adaptation in hyperthermophilic bacteria in order to counteract the deleterious effects of high temperature on protein folding.

Interestingly, when the well-known GroELS chaperone is added to the refolding mixture, a burst in refolded GFP is observed (Fig. 5). A similar result has been reported for the thermophilic TF from *Thermus thermophilus* [36]. This strongly suggests that the hyperthermophilic TF acts as a carrier of folding intermediates for delivery to downstream chaperones and final maturation. Furthermore, the strongly improved yield of native GFP in the presence of both TF and GroELS suggests that the hyperthermophilic TF maintains intermediate species in a folding-competent state which favors the action of downstream chaperones.

To explore the holdase function, ANS titration and isothermal titration calorimetry have revealed a hydrophobic chaperone cavity which potentially binds apolar components of folding intermediates, therefore lowering their rate of internalization and consequently the folding rate of the client protein [33]. The stoichiometry of interaction with α-casein, an intrinsically disordered protein (natively unfolded), indicated a higher number of

hyperthermophilic TF molecules bound to the unfolded state as compared with the mesophilic homologue, which also contributes to the strong holdase activity.

Further studies are awaited in order to unravel the complex interplay between nascent polypeptides and chaperones at high temperature. This is especially relevant for hyperthermophilic archaea which possess a specific chaperone network, distinct from that found in bacteria [37,38].

4. Prolyl isomerization impairs protein folding in psychrophiles

Prolyl isomerization is a rate-limiting step in the folding of many proteins [39]. This arises from the weak steric constraints exerted by the pyrrolidine ring of proline to favor either the *cis* or the *trans* conformation in the unfolded state and to the slow isomerization involving the rotation about the peptide bond, which has a partial double-bond character (Fig. 6). It follows that the time spent by folding intermediates to explore the conformational space and to adopt the required prolyl *cis* or *trans* conformation is longer than for other amino acid side chains which are almost invariably constrained in *trans*. Prolyl isomerization intrinsically possesses a high activation energy and is therefore strongly temperature dependent [40]. In the context of extreme temperatures, one can intuitively assume that prolyl isomerization is very fast in hyperthermophiles and should not be a concern for protein folding. In contrast, slow prolyl isomerization at low temperature should impair the folding of psychrophilic proteins. Several lines of evidence indicate that this is indeed the actual situation.

4.1. The proline content in proteins is positively correlated with environmental temperature

The first observation refers to the proline content which is low in psychrophilic proteins and high in heat-stable polypeptides. For instance, in homologous alpha-amylases (∼50 kDa) the psychrophilic enzyme contains 13 prolines, the mesophilic homologue has 19 prolines and the heat-stable enzyme has 25 prolines, *i.e.* nearly twice the content of the cold-adapted protein. These differences have been related to adjustments of protein stability [8,10,11]. Indeed, in the proline residue, the structure of the pyrrolidine ring bonded to the main chain nitrogen locks a dihedral angle and severely restricts rotations of the backbone. This local rigidity induced by the prolyl residue is a well-known structural factor improving protein stability [14,15,42]. Consequently, heat-stable proteins display a high proline content, whereas psychrophilic proteins tend to decrease this content in order to reach a natively unstable conformation. In the context of protein folding, the high proline content of hyperthermophilic proteins should not be a concern as a result of fast prolyl isomerization at high temperature, whereas the low proline content of psychrophilic proteins reduces the probability of slow and rate-limiting folding steps. Therefore, the low proline content in psychrophilic polypeptides has two distinct contributions: it avoids prolyl-limited folding events and it destabilizes the native state.

4.2. The number of prolyl isomerases in extremophilic genomes is correlated with temperature

Cells are equipped with specialized catalysts, peptidyl-prolyl *cis/trans* isomerases (PPiases or rotamases), which speed-up prolyl isomerization in proteins. Fig. 7 illustrates a classical experiment in which increasing amounts of PPiase proportionally accelerate the folding of a proline-limited protein, revealing a true enzymatic activity [43]. The second observation indicating
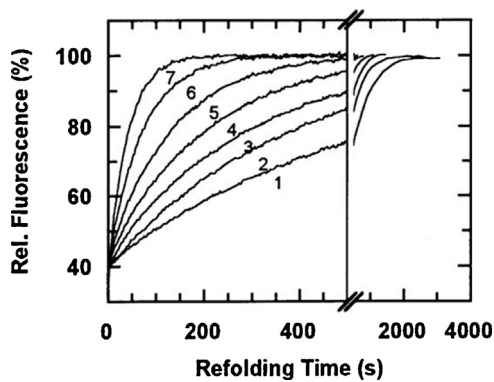
Fig. 7. Catalytic activity of PPiases on protein folding. The refolding rate of a model protein (RCM-T1, reduced and carboxymethylated ribonuclease T1) limited by pro-line isomerization can be slow (1). Addition of increased amounts of PPiase (2–7) accelerates the folding rate of the model protein, as shown by the increases of fluorescence.

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Table 1
Prolyl isomerases encoded in the genome of representative extremophilic and mesophilic Gram-negative bacteria and their environmental temperatures.

Strain	Temperature	PPiase genes
Colwellia psychrerythraea 34H	< 0 °C	18
Pseudolateromonas haloplanktis TAC 125	< 0 °C	15
Pseudomonas extremaustralis 14-3	< 0 °C	14
Escherichia coli K12	37 °C	10
Thermus thermophilus HB8/HB27	65 °C	4
Thermotoga maritima MSB8	85–90 °C	1
Aquifex aeolicus VF5	85–95 °C	1

that prolyl isomerization is influenced by extreme temperatures is the number of genes encoding PPiases in extremophilic genomes. Various types of PPiases are expressed in cells (FKBP-type, cyclophilin-type, parvulin-type) and all have been screened in representative extremophilic genomes using MaGe (www.genoscope.cns.fr/agc/microscope/home/index.php).

Table 1 shows that when compared with *E. coli*, the Gram-negative mesophilic reference, psychrophilic genomes contain a higher number of PPiase genes, nearly twice for *C. psychrerythrae*. Conversely, when the growth temperature increases, the number of PPiases gradually drops to 4 genes at 65 °C and to 1 gene at 85–95 °C. Gram-positive bacteria encode a lower number of PPiases but the same trend is observed. The strains *Planococcus antarcticus* and *P. halocryophilus* thriving around 0 °C encode 7 and 6 PPiases, respectively, whereas the mesophiles *B. subtilis* and *Enterococcus* species all contain 4 PPiases. In contrast *Geobacillus* and *Thermin-cola* species thriving at 60 °C only contain 3 PPiases. But the most convincing evidence that the PPiase genomic content is correlated to temperature is provided by methanogenic archaea because they have colonized the largest range of environmental temperatures. As shown in Table 2, mesophilic archaea encode 4–5 PPiases, whereas in the 65–85 °C range only 2 PPiases are found. Significantly, *M. kandleri*, the most heat-resistant organism known to date, only encodes one PPiase. In addition, the genome of most hyperther-mophilic archaea such as *Pyrococcus furiosus*, *Sulfolobus solfataricus* or *Thermococcus* species also only encodes one PPiase.

Overall, this analysis is a strong indication that prolyl isomerization is sufficiently fast at high temperature, whereas in psychrophiles this isomerization requires powerful catalytic assistance by a full set of PPiases. Furthermore, the persistence of only one PPiase in hyperthermophilic archaea suggests that the folding of a subset of essential proteins is limited by prolyl isomerization. Alternatively, other functions of archaeal PPiases, such as

Table 2
Prolyl isomerases encoded in the genome of representative extremophilic and mesophilic methanogenic archaea and their environmental temperatures.

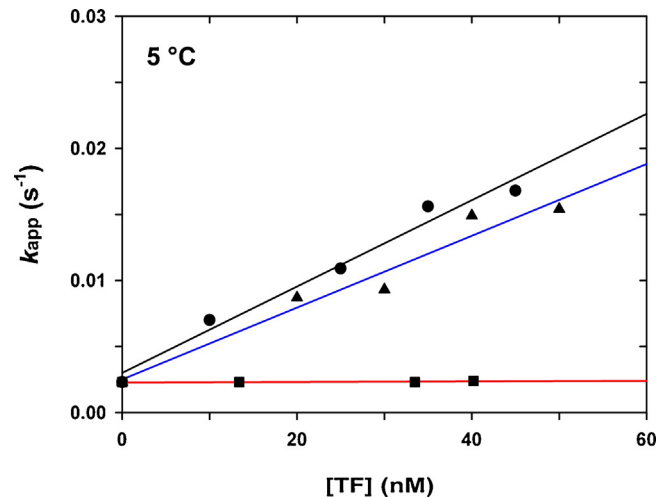| Strain	Temperature	PPiase genes
Methanosarcina barkeri	25 °C	5
Methanosarcina acetivorans	37 °C	5
Methanosarcina mazei	37 °C	5
Methanococcus maripaludis	37 °C	4
Methanococcus aeolicus	45–50 °C	3
Methanothermobacter thermautotrophicus	65–70 °C	2
Methanocaldococcus jannaschii	85 °C	2
Methanopyrus kandleri	122 °C	1

Fig. 8. Catalysis of refolding of RCM-T1 (reduced and carboxymethylated ribonuclease T1). Data for the psychrophilic TF (▲, blue), *E. coli* TF (●, black) and the hyperthermophilic TF (■, red). The apparent rate constant of prolyl isomerization during refolding of RCM-T1 is plotted as function of the TF concentration.
Adapted from [33].

chaperone or anti-aggregation activities, could be responsible for their genomic persistence [44]. It should be mentioned that the above analysis depicts a general trend but not an absolute rule as exceptions occur in the database. This analysis strongly relies on the quality of genome annotations and other factors, such as the microorganism life style (salinity, pressure, ecological niche...), are possibly involved.

4.3. Prolyl isomerases are overexpressed in psychrophiles

Enzyme activity displays typical adaptive traits to environmental temperature [8,45] and it was therefore of interest to check PPiases activity in extremophiles. There is only one PPiase shared by model extremophilic bacteria and this is the TF again. The PPiase activity was analyzed using newly developed substrates and methodologies [33]. Fig. 8 depicts such an experiment in which the increase of the folding rate of a proline-limited protein substrate is plotted as a function of the PPiase concentration in order to calculate the catalytic efficiency k_{cat}/K_m of the isomerization reaction. It can be seen that the PPiase activity of the hyperther-mophilic protein is extremely low and close to the detection limit. This was expected because hyperthermophilic enzymes require high temperature to become fully activated and their rigid structure precludes significant activity, even at room temperature. More surprising was the same PPiase activity shared by both the psy-chrophilic and mesophilic TFs. Indeed, in most cases the activity of psychrophilic enzymes is very high in order to compensate for the decrease of reaction rates inherent to low temperature [10–12]. The psychrophilic PPiase escapes this rule but the reasons
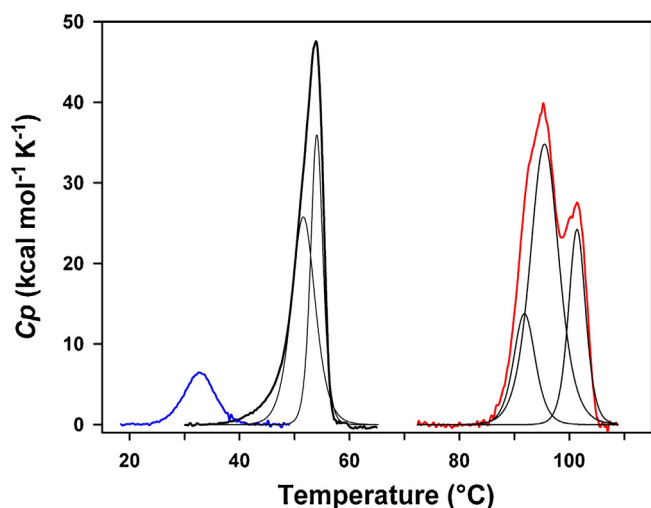
Fig. 9. Thermal unfolding of extremophilic proteins. Thermograms of DNA-ligases recorded by differential scanning microcalorimetry showing, from left to right, psychrophilic (blue), mesophilic (black) and hyperthermophilic (red) proteins. Adapted from [52].

remain unclear. This can be tentatively related to the peculiar but still hypothetical reaction mechanism of PPiases [46] which possibly precludes cold adaptation. However, proteomic studies have brought an unsuspected answer to this paradox [47]. Indeed, it was found that this TF is overexpressed nearly 40 times by the psychrophile *P. haloplanktis* under low temperature cultivation. Apparently, if the PPiase specific activity cannot be improved at low temperature, the microorganism adapts by dramatically increasing the enzyme concentration and therefore the available cellular PPiase activity. Moreover, overexpression of PPiases has been reported in almost all proteomic studies of psychrophiles so far [48] and this is probably the most firmly established result shared by proteomics of psychrophiles. Again, this points to the negative effect of prolyl isomerization on folding of psychrophilic proteins and to the requirement of catalytic assistance by PPiases.

5. Thermodynamic stability of extremophilic proteins

After all folding events, the native state is reached and although extremophilic proteins share the same fold and 3D structures, they display dramatic differences in terms of stability. The energetics of structure stability was essentially analyzed by differential scanning calorimetry (DSC) of homologous extremophilic proteins [18,45,49–53].

5.1. Microcalorimetric studies

A demonstrative example of microcalorimetric records for the heat-induced unfolding of extremophilic proteins is illustrated in Fig. 9. These proteins clearly show distinct stability patterns that evolve from a simple unfolding profile in the unstable psychrophilic protein to a more complex profile in the stable hyperthermophilic counterpart. The unfolding of the cold-adapted protein occurs at much lower temperatures as indicated by the temperature of half-denaturation T_m = 33 °C, given by the top of the transition (Table 3). Accordingly, this protein spontaneously unfolds at a mesophilic temperature of 37 °C. By contrast, the hyperthermophilic protein unfolds around 100 °C. Melting point values up to 150 °C have been reported for hyperthermophilic archaeal proteins [54,55]. The calorimetric enthalpy ΔH_{cal} (area under the curves in Fig. 9) corresponds to the total amount of heat absorbed during unfolding, but it also reflects the enthalpy of disruption of bonds involved in

Table 3
Microcalorimetric parameters of thermal unfolding shown in Fig. 9.

DNA-ligases	T_m (°C)	ΔH_{cal} (kcal mol^{-1})
Psychrophilic	33	46
Mesophilic	54	253
Hyperthermophilic	95–101	413

maintaining the protein compact structure. There is a clear trend for increasing ΔH_{cal} values in the order psychrophile < mesophile < hyperthermophile (Table 3). On this basis, the hyperthermophilic protein possesses a nearly ten times higher stability provided by its various enthalpy-driven weak interactions when compared with the cold-adapted one. The psychrophilic protein unfolds according to a cooperative, all-or-none process, revealing a uniformly low stability of its architecture. By contrast, both homologous proteins display two to three transitions (indicated by deconvolution of the heat capacity function in Fig. 9). Therefore, the conformation of these mesophilic and hyperthermophilic proteins contains structural blocks or units of distinct stability that unfold independently.

From these observations, it can be concluded that psychrophilic proteins possess a fragile molecular edifice that is uniformly unstable and stabilized by fewer weak interactions than homologous mesophilic proteins. By contrast, hyperthermophilic proteins are robust molecules, made of various stability domains and stabilized by a high number of enthalpy-driven weak interactions.

5.2. Stability curves

The thermodynamic stability of a protein that unfolds reversibly according to a two-state mechanism (between the native state N and the unfolded state U) is described by the classical Gibbs-Helmholtz relation:

$$\Delta G_{N-U} = \Delta H_{N-U} - T\Delta S_{N-U} \qquad (2)$$

The latter relation can be rewritten for any temperature (T) using the parameters determined experimentally by DSC:

$$\Delta G_{N-U}(T) = \Delta H_{cal}(1-T/T_m) + \Delta Cp(T-T_m) - T\Delta Cp\ln(T/T_m) \qquad (3)$$

where ΔCp is the difference in heat capacity between the native and the unfolded state. Computing Eq. (3) in a temperature range where the native state prevails in solution provides the protein stability curve [56], *i.e.* the free energy of unfolding as a function of temperature (Fig. 10). In other words, this is the work required to disrupt the native state at any given temperature [57] and is also referred to as the thermodynamic stability. By definition, ΔG_{N-U} is zero at T_m, at equilibrium. At temperatures below T_m, the stability increases, as expected, but perhaps surprisingly for the non-specialist, the stability reaches a maximum then it decreases at lower temperatures (Fig. 10). In fact, this function predicts a temperature of cold-unfolding, which is generally not observed because it occurs below 0 °C [58]. Increasing the stability of a protein is essentially obtained by lifting the curve towards higher free energy values [59,60], as exemplified by the hyperthermophilic protein (Fig. 10), whereas the low stability of a psychrophilic protein is reached by a global collapse of its curve. In all cases, there is no significant shift of the curves towards high or low temperatures. As far as extremophiles are concerned, one of the most puzzling observations is that most proteins obey this pattern, *i.e.* whatever the microbial source, either from hot springs or from sea ice, the maximal stability of their proteins is clustered around room temperature [59,61,62]. This indicates that the various and sometimes opposites forces driving folding are optimally balanced in this temperature range. It should be noted that when stability curves are computed from equilibrium unfolding by chemical denaturants, shifts of the temperature for maximal stability towards higher or lower temper-
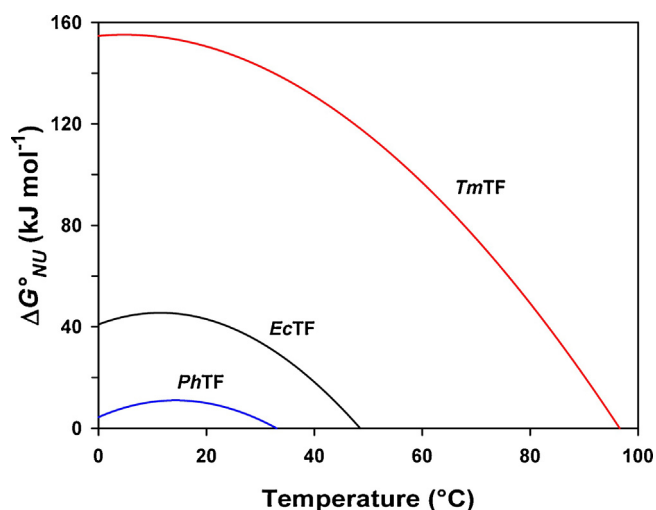
Fig. 10. Gibbs free energy of unfolding, or conformational stability, of homologous proteins. The work required to disrupt the native state is plotted as a function of temperature for psychrophilic (blue, *Ph*TF), mesophilic (black, *Ec*TF) and hyperthermophilic (red, *Tm*TF) proteins.
Adapted from [18].

atures have been reported [24,63]. Such discrepancies remain to be clarified but possibly originate from the distinct unfolding mechanisms and the nature of the unfolded states. The ΔCp parameter in Eq. 3 contributes to the curvature of the stability curve and a decrease of this value induces a flattening of the function. Reduction of the ΔCp value has been reported for some thermophilic and hyperthermophilic proteins [18,23,64–66]. It has been shown that the difference in heat capacity between the native and unfolded states decreases with temperature and vanishes at around $120\,^{\circ}C$ for most mesophilic proteins [67]. It follows that the higher the melting temperature, the lower the ΔCp value.

According to the bell-shaped stability curve, the environmental temperatures for mesophiles and hyperthermophiles lie on the right limb of the curve and obviously, the thermal dissipative force is used to promote molecular motions in these molecules. By contrast, the environmental temperatures for psychrophiles lie on the left limb of the stability curve. It follows that molecular motions in proteins at low temperatures are gained from the factors ultimately leading to cold-unfolding [49], *i.e.* the hydration of polar and non-polar groups and the weakening of the hydrophobic effect [68]. The origin of flexibility in psychrophilic proteins at low temperatures is therefore drastically different from mesophilic and hyperthermophilic proteins, the latter taking advantage of the conformational entropy rise with temperature to gain in mobility.

A surprising consequence of the free energy function for the psychrophilic protein shown in Fig. 10 is its weak stability at low temperatures when compared with mesophilic and thermophilic proteins, whereas it was intuitively expected that cold-adapted proteins should also be cold stable. This protein is in fact both heat and cold labile. As a result, cold unfolding of psychrophilic proteins has been experimentally recorded at the temperature predicted by the stability curve [18,49], providing validation of the free energy function.

6. Conclusions

The literature on protein folding in temperature extremophiles is still scarce but this should improve rapidly because, besides fundamental aspects, the topic is significantly relevant in biotechnology, such as for the expression of soluble recombinant proteins at low temperature by psychrophiles [35] or for the synthesis of robust enzyme catalysts used in harsh industrial conditions [69]. The topic is also of interest for astrobiology as life might have evolved on cold or hot planets [70]. The available results underline the necessity of investigating series of homologous extremophilic and mesophilic proteins in order to screen the largest spectrum of biological temperatures. Current studies using extremophilic proteins which unfold fully reversibly according to a perfect two-state mechanism should refine the available data. Multidisciplinary approaches are also needed because, for instance, cold adaptation is not always the converse of hot adaptation. In addition, multiple factors can be involved as illustrated here by chaperones or by the effect of prolyl isomerization.

Finally, the main drawback in protein folding studies of extremophiles is the fact that the related experiments cannot be performed at the environmental temperatures. At low temperatures, condensation on optics strongly perturbs spectroscopic signals and requires abundant nitrogen flushing, which is not always technically feasible. At high temperatures, folding events become so fast that they cannot be recorded and all model protein substrates used in these experiments are mesophilic, such as GFP, and unfold or aggregate at temperatures well below those encountered by thermophiles. In all cases, results obtained in a limited window of temperatures, as well as the associated values, have to be extrapolated to either low or high temperatures, which can be controversial. Reviewers are sometimes reluctant to take these limitations into account.

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