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TOPICAL REVIEW

Protein stability and enzyme activity at extreme biological temperatures

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Abstract

Psychrophilic microorganisms thrive in permanently cold environments, even at subzero temperatures. To maintain metabolic rates compatible with sustained life, they have improved the dynamics of their protein structures, thereby enabling appropriate molecular motions required for biological activity at low temperatures. As a consequence of this structural flexibility, psychrophilic proteins are unstable and heat-labile. In the upper range of biological temperatures, thermophiles and hyperthermophiles grow at temperatures >100 °C and synthesize ultra-stable proteins. However, thermophilic enzymes are nearly inactive at room temperature as a result of their compactness and rigidity. At the molecular level, both types of extremophilic proteins have adapted the same structural factors, but in opposite directions, to address either activity at low temperatures or stability in hot environments. A model based on folding funnels is proposed accounting for the stability–activity relationships in extremophilic proteins.

(Some figures in this article are in colour only in the electronic version)

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1. Introduction

The range of natural temperatures encountered on Earth spans from −89.2 °C, a temperature recorded in 1983 at the Russian research station Vostok in Antarctica, up to ∼2 000 °C as monitored in active volcanoes. The latter are of course incompatible with life and it is generally assumed that the upper limit should be 140–150 °C, considering the rate of thermally induced destruction of essential biomolecules such as ATP, the universal energy carrier [1]. As a matter of fact, the most hyperthermophilic microorganisms isolated to date are *Pyrolobus fumarii* growing at 113 °C [2] and more recently *Methanopyrus kandleri* still growing at 122 °C but enduring prolonged exposure to 130 °C [3]. Both are Archaea (the third domain of life besides Bacteria and Eukarya) that possess an unsurpassed ability to colonize harsh environments. They have been isolated from black smokers, i.e. hydrothermal chimneys expelling hot water at ∼300 °C and found on the deep ocean floor where continental crusts are generated. Other very hot environments include volcanic solfataras or mud pots hosting, for instance, the archaeon *Sulfolobus solfataricus*

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and hot springs from which the well-known thermophilic bacterium *Thermus aquaticus* has been isolated. The latter species has provided the first thermostable DNA polymerase that has revolutionized modern molecular biology by enabling the polymerase chain reaction or PCR.

At the other extremity of the biological temperature scale, low temperatures are less detrimental to cellular structures. As a result, psychrophiles, i.e. organisms living permanently at temperatures close to or below the freezing point of water, in thermal equilibrium with the medium, include a large range of representatives from all three domains of life such as bacteria, yeasts, algae, marine invertebrates or polar fish. These fish synthesize antifreeze proteins, allowing them to thrive at −2.2 °C in cold seawater [4]. While hydrothermal locations are sparsely distributed on our planet surface, it is frequently overlooked that most biotopes on Earth are cold and permanently exposed to temperatures below 5 °C. Such low mean temperatures mainly arise from the fact that ∼70% of the Earth's surface is covered by oceans that have a constant temperature of 4 °C below 1000 m depth, irrespective of the latitude. The polar regions account for another 15%, to which the glacial and alpine regions must be added, as well as permafrost representing more than 20% of terrestrial soils. There is no formal lower temperature limit for life under natural conditions, as most microbial species can be maintained for extended periods of time in low temperature freezers at −80 °C, then revived under appropriate conditions. Bacterial survival has been reported in frozen samples up to half a million years old and such viability was correlated with the capacity to slowly repair DNA, therefore preserving the cell's genetic programme [5]. Arctic permafrost has revealed a high biodiversity in cryopegs, i.e. salty water pockets that have remained liquid for about 100 000 years at −10 °C [6]. Polar regions possess unusual microbiotopes such as porous rocks in Antarctic dry valleys hosting microbial communities surviving at −60 °C [7] or the liquid brine veins between sea ice crystals harbouring metabolically active microorganisms at −20 °C [8]. These examples illustrate the unsuspected ability of microorganisms to adapt to low temperatures but also imply that psychrophiles are the most abundant extremophiles in terms of biomass, diversity and distribution [9].

Life at these extreme biological temperatures obviously requires a vast array of adaptations. This review mainly focuses on protein stability and enzyme activity, with special reference to psychrophilic proteins because, unlike thermophilic proteins, cold-adapted polypeptides have been only recently investigated. Comparison will be made with proteins from mesophiles (organisms thriving at mild temperatures such as the bacterium *Escherichia coli* or warm-blooded animals) that have been extensively studied. Thermophilic proteins have to maintain their architecture at elevated temperatures and are therefore very stable. As a result of their compact and rigid conformation, thermophilic enzymes are nearly inactive at room temperature as catalysis requires appropriate molecular motions. On the other hand, psychrophilic enzymes have to compensate for the exponential decrease of chemical reaction rates induced by temperature lowering. These proteins have improved the dynamics or the flexibility of their structures to promote catalysis and in turn are unstable and heat-labile. Although these extremophilic proteins have been subjected to two distinct selective pressures (stability for thermophiles and activity for psychrophiles), it will be shown here that both parameters have been intricately linked in the course of evolution and a model is proposed accounting for the stability–activity relationships in extremophilic proteins.

2. Stability of extremophilic proteins

2.1. Defining protein stability

Following its linear synthesis on the ribosome, a polypeptide has to adopt its final and biologically active three-dimensional conformation. The forces driving protein folding are essentially the same that those driving the formation of oil micelles in water: the entropic cost of encaging non-polar groups in the water molecule network is high and the system evolves towards the burial of these groups in a globular structure, away from the water molecules in the solvent. This hydrophobic effect is a primary determinant of protein stability. Once folded, stability is further modulated by interactions between groups that have been brought into contact. In proteins, van der Walls interactions and H-bonds are the most abundant but salt bridges (or ion pairs), aromatic (or cation-π) interactions and some structural disulfide bonds make a substantial contribution to stability. Structural factors are also involved such as the occurrence of glycine residues that allow large dihedral rotation around the alpha-carbon or proline that, in contrast, induces a local point of rigidity in the polypeptide chain.

For the experimentalist, protein stability can be defined as the energetics of the unfolding reaction, from the native state N to the fully unfolded state U, which occurs via two basic pathways: unfolding can be either irreversible or reversible. In the irreversible unfolding pathway, as in (1), the protein cannot fold back when the denaturing agent (chemical or physical) is removed:

$$\mathrm{N} \xrightarrow{k} \mathrm{U}. \tag{1}$$

Such irreversibility should be regarded as an *in vitro* artefact if one remembers the spontaneous folding of nascent polypeptides *in vivo*. Irreversibility frequently arises from aggregation, misfolding, chemical modification or from the lack in the test tube of chaperones that assist folding in the cell. In the simplest model, the irreversible unfolding reaction is characterized by a first-order rate constant k that can be varied by temperature. Such data analysed in the Eyring formalism, assuming a transition state intermediate, give access to the Gibbs energy of activation, $\Delta G^{\#}$. As irreversible unfolding is kinetically driven, this is sometime referred to as the kinetic stability.

Reversible unfolding of proteins, as in (2), is less frequently obtained *in vitro* but is much more informative as equilibrium thermodynamic equations can be applied:

$$\mathrm{N} \overset{K}{\rightleftharpoons} \mathrm{U}. \tag{2}$$

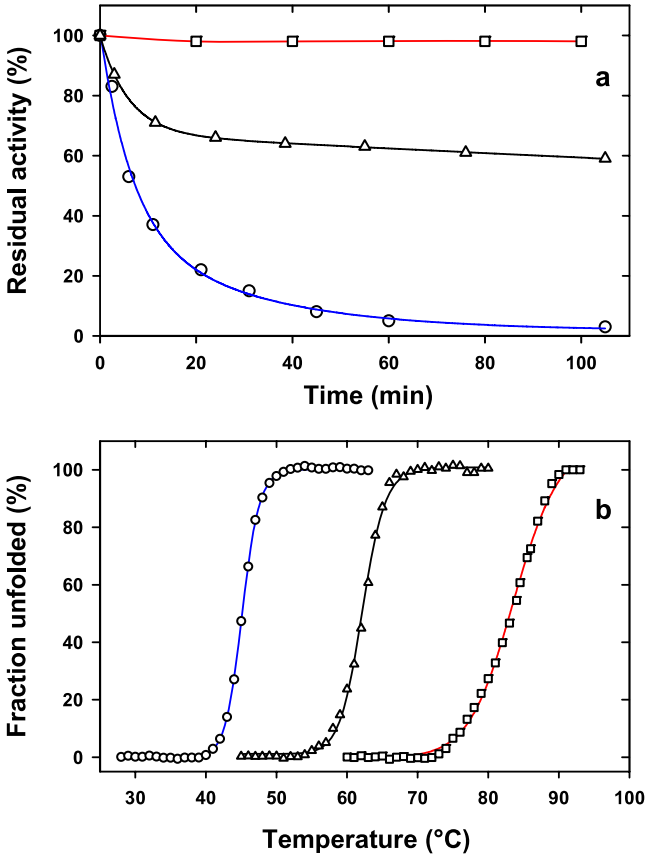

Figure 1. Stability of psychrophilic (circles, blue), mesophilic (triangles, black) and thermophilic (square, red) enzymes. In this selected example, the homologous enzymes are α-amylases that hydrolyze starch as a substrate. (a) Activity decay during incubation at 50 °C and (b) thermal unfolding recorded by fluorescence spectroscopy.

Indeed, the equilibrium constant K characterizing the reversible unfolding reaction provides access to the Gibbs free energy of unfolding, $\Delta G_{\mathrm{N-U}}$, referred to as the conformational stability or the thermodynamic stability. More complex unfolding pathways are also observed but will not be considered here.

The temperature dependence of a series of homologous psychrophilic, mesophilic and thermophilic enzymes is depicted in figure 1. Activity of the thermophilic enzyme is resistant to high temperature incubation whereas the psychrophilic enzyme is quickly and irreversibly inactivated. Heat-induced unfolding, as probed by intrinsic fluorescence (that monitors structural integrity), displays the same trends. As a rule, there is a gradual increase from psychrophiles to thermophiles of the protein resistance towards denaturing agents such as temperature, chemicals, detergents or proteolysis.

2.2. Chemical denaturation

Protein stability is commonly quantified by recording the unfolding curve induced by guanidinium chloride (GdmCl) or urea as these chemical denaturants frequently induce a reversible transition. Although the precise mechanism is still

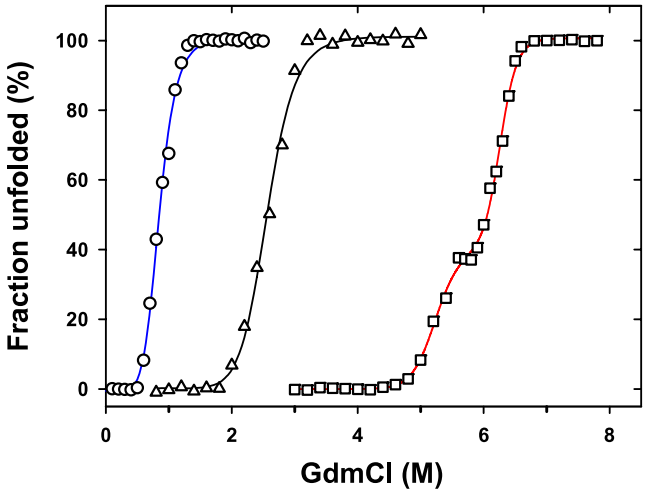

Figure 2. Equilibrium unfolding of psychrophilic (○, blue), mesophilic (△, black) and thermophilic (□, red) α-amylases as recorded by fluorescence emission. Thermodynamic parameters of GdmCl-induced unfolding at 20 °C for the psychrophilic protein: $C_{1/2} = 0.9$ M, $m = 4.3$ kcal mol^{-1} M^{-1}, $\Delta G_{\mathrm{H_2O}} = 3.7$ kcal mol^{-1}; for the mesophilic protein: $C_{1/2} = 2.6$ M, $m = 2.7$ kcal mol^{-1} M^{-1}, $\Delta G_{\mathrm{H_2O}} = 6.9$ kcal mol^{-1}; and for the thermophilic protein: $C_{1/2} = 6.0$ M, $\Delta G_{\mathrm{H_2O}} = 23.8$ kcal mol^{-1} (adapted from [10]).

under debate, the effect of these chemicals can be summarized by mentioning that most protein groups are more soluble in solutions of these denaturants than in water. Accordingly, the hydrophobic effect is weakened and even abolished at high concentrations.

The stability of homologous psychrophilic, mesophilic and thermophilic proteins is illustrated in figure 2. These enzymes unfold at distinct denaturant concentrations ($C_{1/2}$) and are characterized by a decrease of unfolding cooperativity (m value) and the appearance of unfolding intermediates (in the thermophilic protein) as the stability increases. Within the unfolding transition, an equilibrium constant K can be calculated for each denaturant concentration and a plot of the corresponding ΔG values is used to extrapolate the stability at zero denaturant concentration, $\Delta G_{\mathrm{H_2O}}$, as in (3):

$$\Delta G = \Delta G_{\mathrm{H_2O}} - m[\mathrm{GdmCl}]. \qquad (3)$$

The drawback of such an analysis with extremophilic proteins lies in the sharp and very cooperative transition of psychrophilic proteins, therefore inducing errors in $\Delta G_{\mathrm{H_2O}}$ arising from small imprecisions on the slope m. On the other hand, thermophilic proteins unfold at high denaturant concentrations, also promoting errors arising from the long range extrapolation of $\Delta G_{\mathrm{H_2O}}$. Nevertheless, the values reported in figure 2 indicate a ratio of 1/2/6 for the stability homologous psychrophilic, mesophilic and thermophilic proteins, respectively.

2.3. Microcalorimetric studies

Differential scanning calorimetry (DSC) provides a better characterization of the various parameters involved in protein stability. In a typical DSC experiment, a protein solution

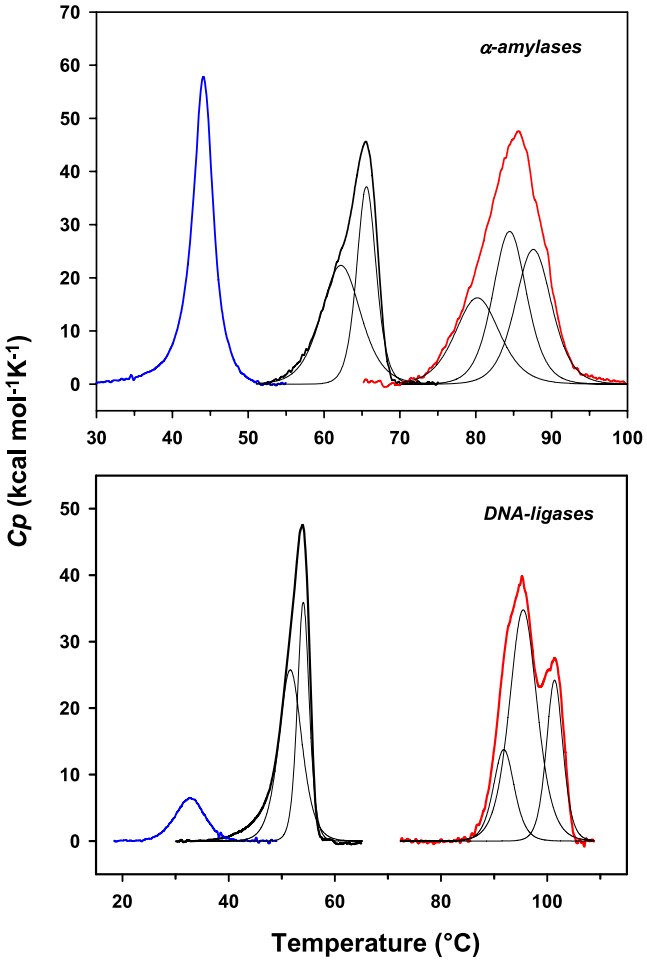

Figure 3. Thermal unfolding of extremophilic proteins. Thermograms of α-amylases and DNA-ligases recorded by differential scanning microcalorimetry showing, from left to right on each panel, psychrophilic (blue), mesophilic (black) and (hyper)thermophilic (red) proteins. The cold-adapted proteins are characterized by a lower T_m (top of the transition) and ΔH_{cal} (area under the transition), by a sharp and cooperative transition and by the lack of stability domains (indicated by thin lines in stable proteins). Adapted from [12, 14].

is heated in the microcalorimeter cell up to thermally induced unfolding. This is an endothermic process and the microcalorimeter records the heat absorption peak, or thermogram, between the native state (before the transition) and the unfolded state (after the transition). Figure 3 displays the calorimetric records of heat-induced unfolding for psychrophilic, mesophilic and thermophilic proteins. These enzymes clearly show distinct stability patterns that evolve from a simple profile in the unstable psychrophilic proteins to a more complex profile in very stable thermophilic counterparts.

- The unfolding of the cold-adapted proteins occurs at lower temperatures as indicated by the T_m values which correspond to the temperature of half-denaturation (for a two-state process) and is given by the top of the transition. For instance, T_m of the psychrophilic ligase is 33 °C: it follows that, at a typical mesophilic temperature of 37 °C, almost all the protein population is already in the unfolded state. In contrast, temperatures >100 °C are frequently required to fully unfold thermophilic proteins. As an example, rubredoxin from the hyperthermophile archeon *Pyrococcus furiosus* is one of the most stable proteins with an extrapolated T_m approaching 200 °C [11].
- The energetics of structural stability has been essentially revealed by microcalorimetry [12–15]. The area under the curves in figure 3 corresponds to the total amount of heat absorbed during unfolding and is given by the calorimetric enthalpy, ΔH_{cal}. This parameter reflects the enthalpy of the disruption of bonds involved in maintaining the compact protein structure. There is a clear trend for increasing ΔH_{cal} values in the order psychrophiles < mesophiles < thermophiles (table 1).
- The transition for psychrophilic proteins is sharp and symmetric whereas other proteins are characterized by a flattening of the thermograms. This is indicative of a pronounced cooperativity during unfolding of the psychrophilic protein: the structure is stabilized by fewer weak interactions and disruption of some of these interactions strongly influences the whole molecular edifice and promotes its unfolding. On the other hand, the transition for thermophilic proteins is much less cooperative and occurs over a broader range of temperatures: as a result, the structure of these heat-stable proteins resists against unfolding in a large temperature interval.
- The psychrophilic proteins unfold according to an all-or-nothing process, revealing a uniformly low stability of the architecture. In the examples depicted in figure 3, the structure of the cold-adapted proteins is made by only one cooperative unit. By contrast, all other homologous enzymes display two to three transitions (either observable or indicated by deconvolution of the heat capacity function in figure 3). Therefore, the conformation of these mesophilic and thermophilic proteins contains structural blocks or units of distinct stability that unfold independently.
- Finally, the unfolding of the psychrophilic proteins is frequently more reversible than that of other homologous enzymes that are irreversibly unfolded after heating. The weak hydrophobicity of the core clusters in cold-adapted proteins and the low melting temperature, at which hydrophobic interactions are restrained, certainly account for this reversible character because, unlike mesophilic

Table 1. Microcalorimetric parameters of thermal unfolding (shown in figure 3).

Proteins	T_m (°C)	ΔH_{cal} (kcal mol^{-1})
α-amylases		
Psychrophilic	44	214
Mesophilic	66	319
Thermophilic	86	487
DNA-ligases		
Psychrophilic	33	46
Mesophilic	54	253
Thermophilic	95–101	413

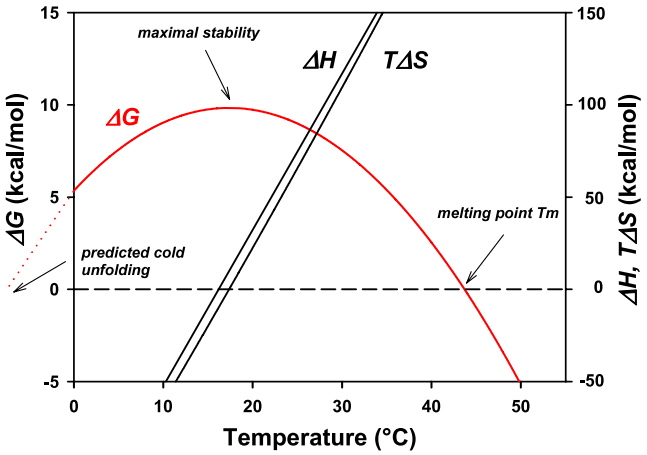

Figure 4. The main properties of a protein stability curve. Plot of the conformational stability ΔG and of the enthalpic (ΔH) and entropic ($T\Delta S$) contributions. Note the different scales used: the stability of a protein is marginal as it is only a small difference between two large and opposite contributions. The parabolic function of ΔG is limited by the temperature of cold denaturation (below 0 °C) and by the high temperature melting point T_m, at which $\Delta G_{N-U} = 0$. At temperatures below the maximal stability, entropy becomes the stabilizing contribution in the environmental thermal range of psychrophiles.

proteins, aggregation does not occur, or occurs to a lower extent.

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. In contrast, thermophilic proteins are robust molecules, made of various stability domains and stabilized by a high number of enthalpy-driven weak interactions.

2.4. Thermodynamic stability

The thermodynamic stability of a protein that unfolds reversibly according to a two-state mechanism, as in (2), is described by the classical Gibbs–Helmholtz relation:

$$\Delta G_{N-U} = \Delta H_{N-U} - T\Delta S_{N-U}. \quad (4)$$

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

$$\Delta G_{N-U}(T) = \Delta H_{cal}(1 - T/T_m) + \Delta Cp(T - T_m) - T\Delta Cp \ln(T/T_m) \quad (5)$$

where ΔCp is the difference in heat capacity between the native and the unfolded state. This parameter reflects the hydration of non-polar groups that are exposed to water upon unfolding and is determined by another set of calorimetric experiments. Computing equation (5) in a temperature range where the native state prevails provides the protein stability curve [16], i.e. the free energy of unfolding as a function of temperature (figure 4). In other words, this is the work required to disrupt the native state at any given temperature [17] and is also referred to as the conformational stability.

Some general properties of the stability curve are worth mentioning [16] before analysing the behaviour of extremophilic proteins. By definition, the conformational stability is nil at the equilibrium temperature, the melting point T_m (equilibrium constant $K = [U]/[N] = 1$). At temperatures below the melting point, the stability increases, as expected, but perhaps surprisingly for the non-specialist, the stability reaches a maximum close to room temperature, then it decreases at lower temperatures. In fact, this function predicts a temperature of cold-unfolding, which is generally not observed because it occurs below 0 °C. Nevertheless, cold-unfolding has been well demonstrated under specific conditions [18]. The enthalpic and entropic contributions to the free energy of unfolding are computed in figure 4. These functions have a slightly curved shape and cross each other, by definition, at both the low and the high temperature melting points (out of scale in figure 4). The different scale used in figure 4 should be mentioned: proteins are marginally stable from a thermodynamic point of view as the conformational stability is only a small difference between two large and opposite contributions. The maximal stability of a protein is reached when the entropic contribution cancels and therefore this maximal stability is purely enthalpic by nature. At temperatures below the maximal protein stability, both the enthalpy and the entropy change their sign: it follows that, in this region of the stability curve, the enthalpic term is a destabilizing factor whereas the entropic contribution becomes the stabilizing factor of the native state.

Referring again to figure 4, there are in principle three basic ways to increase the stability and to reach high T_m typical of thermophilic proteins (the reverse holds for unstable psychrophilic proteins): (i) to shift the whole stability curve towards high temperatures, (ii) to decrease the curvature of the plot so as to broaden the curve and (iii) to uplift the whole stability curve. Each scenario corresponds to modifications of parameters in equation (5), reflecting structural adaptations at the molecular level. Figure 5 illustrates representative stability curves for homologous psychrophilic, mesophilic and thermophilic proteins that provide valuable insights into these molecular adaptations in extremophilic proteins.

- Obviously, the distinct stability of extremophilic proteins mainly follows the third above-mentioned alternative: the high stability in thermophiles is reached by lifting the curve towards higher free energy values, whereas the low stability in psychrophiles corresponds to a global collapse of the bell-shaped stability curve [15, 19–21]. This arises from large differences in T_m and ΔH_{cal} values in equation (5), as also illustrated in figure 3 and table 1. It follows that enthalpy-driven interactions play a preeminent role in adjustments of the conformational stability.
- Although the number of reliable data is currently limited, a trend to decrease ΔCp from mesophilic to thermophilic proteins has been noted [21]. As ΔCp contributes to the curvature of the stability function, a lower value in thermophilic proteins results in a slightly broader plot. The possible structural origin of this modification of ΔCp in thermophiles remains controversial but

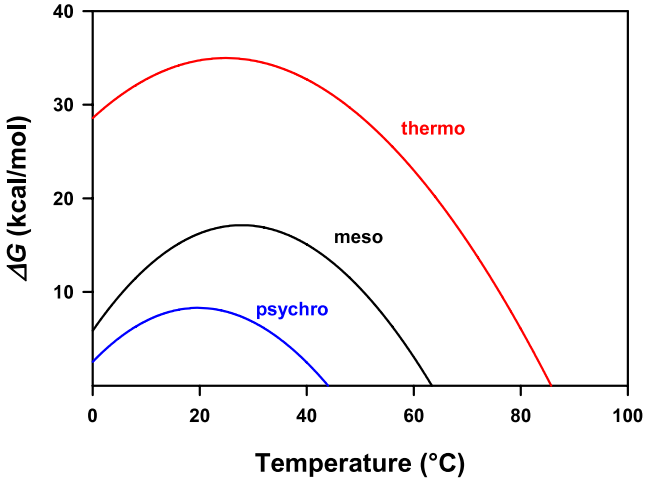

Figure 5. Gibbs free energy of unfolding, or conformational stability, of homologous extremophilic proteins. The work required to disrupt the native state is plotted as a function of temperature. The high stability of the thermophilic protein is reached by lifting the curve towards higher free energy values, whereas the low stability of the psychrophilic proteins corresponds to a global collapse of the bell-shaped stability curve. Adapted from [10].

polar → charged amino acid substitutions is the most consistent genomic correlate for hyperthermostability [22] and may indeed contribute to lower ΔCp values. But experimental artefacts cannot be excluded: if thermophilic proteins do not fully unfold at high temperatures and retain residual structures, this will decrease the observed ΔCp value [23].

- As shown in figure 5, the temperatures of maximal stability for the three proteins are similar. As far as extremophiles are concerned, this is a puzzling observation because whatever the microbial source, either from hydrothermal vents or from polar ice, the maximal stability of their proteins is clustered around room temperature. This has been explained by the fact that the hydrophobic effect, a major driving force in protein folding, is optimal around 20 °C [19, 24, 25].
- As a result of the nearly constant temperature of maximal stability, the environmental temperatures for mesophiles and thermophiles lie on the right limb of the bell-shaped stability 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 [26], i.e. the hydration of polar and non-polar groups [27]. The origin of flexibility in psychrophilic proteins at low temperatures is therefore drastically different from mesophilic and thermophilic 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 figure 5 is its weak stability at low temperatures when compared with mesophilic and thermophilic proteins, whereas it was intuitively expected that cold-active proteins should also be cold-stable. This protein is, in fact, both heat- and cold-labile. Assuming constant properties of the solvent below 0 °C (i.e. no freezing) and the absence of protective effects from cellular components, this protein should unfold at −10 °C. Therefore cold denaturation of some key enzymes in psychrophiles can be an additional, though unsuspected, factor limiting life at low temperatures.
- As mentioned in figure 4, the enthalpic contribution is the stabilizing factor for mesophilic and thermophilic proteins at their physiological temperatures. In sharp contrast, the entropic contribution becomes the stabilizing factor of cold-adapted proteins at low temperatures. It has been argued that this noticeable involvement of entropy reflects a relative disorder in psychrophilic macromolecules to maintain dynamics and function in the cold [28].

Table 2. Thermodynamic parameters for the irreversible unfolding of α-amylases. Activation data at an identical unfolding rate constant, $k = 0.05\ s^{-1}$. Adapted from [10].

	$\Delta G^{\#}$ (kcal mol^{-1})	$\Delta H^{\#}$ (kcal mol^{-1})	$T\Delta S^{\#}$ (kcal mol^{-1})
Psychrophile	20.2	109.7	89.2
Mesophile	21.5	84.7	63.2
Thermophile	22.9	74.2	51.3

2.5. *Kinetic stability*

Kinetically driven stability refers to irreversible unfolding according to equation (1). Comparison of psychrophilic, mesophilic and thermophilic proteins can be complex as they unfold in a narrow range of temperatures but separated by large temperature intervals (i.e. it is virtually impossible to record unfolding of an unstable psychrophilic protein at the unfolding temperature of a thermophilic protein). This has been overcome by comparing these proteins at the temperature at which they unfold at the same rate and to analyse the results in the formalism of the transition state theory [10, 13, 14, 29]. Table 2 reports an example of the thermodynamic characteristics for the irreversible unfolding of these proteins. The free energy of activation $\Delta G^{\#}$ represents the energy barrier that has to be mastered before protein denaturation. The lower the $\Delta G^{\#}$ value, the lower the kinetic stability. Although the extremophilic proteins are compared at an identical denaturation rate, the lower $\Delta G^{\#}$ value for the psychrophilic protein arises from the low temperature at which this rate is reached, and conversely for the thermophilic homologue. It is worth mentioning that the low kinetic stability of the psychrophilic protein is the result of the largest enthalpic and entropic contributions and, in contrast, the high kinetic stability of the thermophilic protein arises from much smaller contributions. The activation enthalpy $\Delta H^{\#}$ essentially reflects the cooperativity and temperature dependence of the system: the high value for the psychrophilic protein indicates that, for a given increase of temperature, the increase of the denaturation rate k will be much higher than for a thermophilic

protein. Hence, assuming no entropic contribution, the kinetic stability of the psychrophilic protein would be extremely high. This is not the case because of the large unfavourable entropic contribution. The positive value of $\Delta S^{\#}$ suggests that the randomness of the transition state increases before irreversible unfolding. One can propose that, when jumping the $\Delta G^{\#}$ barrier, the transition state of the psychrophilic protein is already very disordered before denaturation, whereas the transition state of the thermophilic protein resists the disorder before denaturation. A thermophilic protein can then be regarded as an intrinsically stable macromolecule that counteracts heat denaturation by a weak cooperativity of unfolding [10].

2.6. Structural flexibility

The previous sections provided a rather static picture of stability while the behaviour of these proteins in solution is biologically relevant. The protein native state prevailing under physiological conditions is not a single molecular species but is better described by an ensemble of statistically populated conformers. In this context, 'flexibility' refers to these fluctuating conformations of comparable energy that can be interconverted with a series of small changes. The larger the flexibility, the larger is the population of conformers and the lower is the energy barrier for interconversion between them [30]. Micro-unfolding of discrete regions of the protein participates in the so-called breathing of the macromolecular conformation. These transitions between different conformations are concerted motions of groups of side chains and segments of backbone involving hinge and rocking motions in a timescale ranging from 10^{-8} to 10^{3} s. Group fluctuations superimpose on the large structural motions: side chain and backbone motions originate from rotations about individual bonds, stretching and torsional motions at about 10^{-12} s. The lower limit on the rate constant for any motion in protein is determined by molecular vibration at about 10^{-13} s [31].

Determination of molecular flexibility is complex as it requires the definition of the types and amplitudes of atomic motions as well as a timescale for these motions. In this respect, fluorescence quenching of extremophilic proteins was used as this method averages most of these parameters into a single signal (figure 6). This technique utilizes increasing concentrations of a small quencher molecule (acrylamide in figure 6) in order to probe the accessibility of tryptophan residues within a protein. The decrease of fluorescence arising from diffusive collisions between the quencher and the fluorophore reflects the ability of the quencher to penetrate the structure and can be viewed as an index of protein permeability. It was found that the structure of a psychrophilic protein has an improved propensity to be penetrated by a small quencher molecule, whereas a thermophilic protein only displays a moderate quenching effect. Accordingly, the latter behaves as a compact molecule undergoing few internal motions whereas a psychrophilic protein is a flexible molecule displaying numerous opening and closing motions or frequent micro-unfolding events [10, 13, 14, 29]. Such

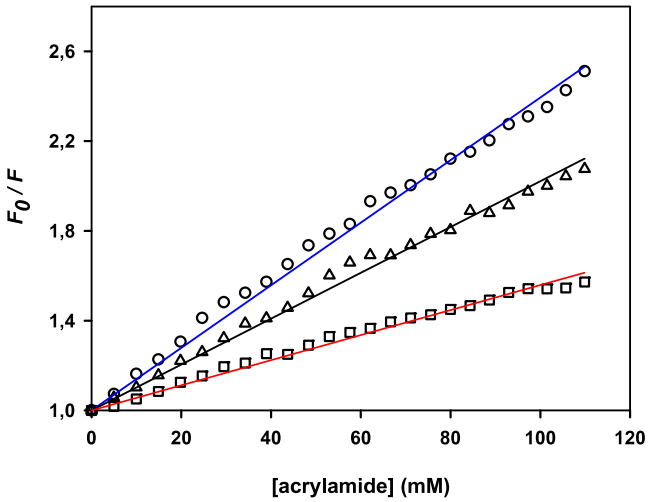

Figure 6. Permeability of the protein structure at room temperature. Fluorescence quenching experiments on psychrophilic (circles, blue), mesophilic (triangles, black) and thermophilic (squares, red) proteins. The steep slope recorded for the psychrophilic protein indicates that its structure is easily penetrated by a small quencher molecule (acrylamide), resulting in a larger attenuation of the intrinsic fluorescence (F0/F), whereas the thermophilic protein is more rigid and displays fewer internal motions. Adapted from [10].

differences in flexibility have received further support by the quantification of macromolecular dynamics in the whole protein content of psychrophilic, mesophilic, thermophilic and hyperthermophilic bacteria by neutron scattering [32]. Neutron spectroscopy provides a unique tool to study thermal atomic motions in macromolecules because neutron wavelengths and energies match motion amplitudes and frequencies, respectively [32]. These experiments have indeed revealed that the resilience (equivalent to macromolecular rigidity in terms of a force constant) increases with physiological temperatures. Furthermore, it was also shown that the atomic fluctuation amplitudes (equivalent to macromolecular flexibility) were similar for each microorganism at its physiological temperature. This is in full agreement with Somero's 'corresponding state' concept [33] postulating that protein homologues exhibit comparable flexibilities to perform their biological function at their physiologically relevant temperatures. In this respect, the first experimental approach is apparently the pioneering work of Privalov [34] reporting an extensive analysis of collagen structure from organisms adapted to different temperatures and including kinetics of hydrogen exchange. The author concluded that the correlation between physiological temperature and collagen stability can be explained, assuming that some definite level of motility of protein structure is required for its efficient functioning in living systems. Thus, it is very likely that the requirement of a definite motility and stability under physiological conditions is the primary one in the evolution of the collagen chemical structure.

2.7. Structural determinants of stability

Besides the large amount of thermophilic protein structures, the number of x-ray crystal structures from psychrophilic

Table 3. A selection of relevant psychrophilic proteins of known crystal structure.

Cold-adapted protein	Source	PDB entry
Alpha-amylase	Antarctic bacterium *Pseudoalteromonas haloplanktis*	1AQH
Xylanase	Antarctic bacterium *Pseudoalteromonas haloplanktis*	1H12
Cellulase	Antarctic bacterium *Pseudoalteromonas haloplanktis*	1TVN
Ca^{2+} Zn^{2+} protease	Antarctic bacterium *Pseudomonas* sp.	1G9K
Beta-lactamase	Antarctic bacterium *Pseudomonas fluorescens*	2QZ6
Citrate synthase	Antarctic bacterium *Arthrobacter* sp.	1A59
Beta-galactosidase	Antarctic bacterium *Arthrobacter* sp.	1YQ2
Subtilisin	Antarctic bacterium *Bacillus* sp.	2GKO
Alkaline phosphatase	Antarctic bacterium	2IUC
Aminopeptidase	Arctic bacterium *Colwellia psychrerythraea*	3CIA
Phenylalanine hydroxylase	Arctic bacterium *Colwellia psychrerythraea*	2V27
Malate dehydrogenase	Arctic bacterium *Aquaspirillium arcticum*	1B8P
Serine proteinase	Arctic bacterium *Vibrio* sp.	1SH7
Isocitrate dehydrogenase	Arctic bacterium *Desulfotalea psychrophila*	2UXQ
Aspartate carbamoyltransferase	Deep-sea bacterium *Moritella profunda*	2BE7
Adenylate kinase	Bacterium *Bacillus globisporus*	1S3G
Triose-phosphate isomerase	Marine bacterium *Vibrio marinus*	1AW1
Tyrosine phosphatase	Bacterium *Shewanella* sp.	1V73
Catalase	Bacterium *Vibrio salmonicida*	2ISA
Lipase	Bacterium *Photobacterium lipolyticum*	2ORY
Superoxide dismutase	Bacterium *Aliivibrio salmonicida*	2W7W
Alkaline phosphatase	Bacterium *Vibrio* sp.	3E2D
Alkaline phosphatase	Arctic shrimp *Pandalus borealis*	1K7H
Lactate dehydrogenase	Antarctic icefish *Champsocephalus gunnari*	2V65
Trypsin	Atlantic salmon *Salmo salar*	2TBS
Elastase	Atlantic salmon *Salmo salar*	1ELT
Pepsin	Atlantic cod *Gadus morhua*	1AM5
Uracil-DNA glycosylase	Atlantic cod *Gadus morhua*	1OKB

enzymes has increased dramatically to ∼40, demonstrating the growing interest for these peculiar proteins. Some of these cold-adapted proteins are listed in table 3. This has allowed a fine analysis of the factors modulating stability in these proteins displaying extreme and opposite behaviour. However, the interpretation of these structural data is frequently difficult for two main reasons. First, the structural adaptations are extremely discrete, when compared with mesophilic proteins, and can easily escape the analysis. Some examples are illustrated in figure 7 for a psychrophilic enzyme. Second, these structural adaptations are very diverse, reflecting the complexity of factors involved in the stability of a macromolecule at the atomic level. For instance, it was found that all structural factors currently known to stabilize the protein molecule could be attenuated in strength and number in the structure of cold-active proteins [35, 36]. An exhaustive description of all these factors is beyond the scope of this section but the essential features are summarized below. Previous review articles can be consulted for a comprehensive survey of this topic [35, 37–40].

The observable parameters related to protein stability include structural factors and mainly weak interactions between atoms of the protein structure. In psychrophilic proteins, this involves the clustering of glycine residues (providing local mobility) or the strategic location of glycine residues close to functional regions (active site, binding site, etc) while the global content in this residue is not modified. At the primary structure level, the number of proline residues in loops (providing reduced chain flexibility between secondary structures) increases from psychrophilic to thermophilic proteins, as well as the number of arginine residues which are capable of forming multiple salt bridges and H-bonds. The number of weak electrostatic interactions also increases from cold-adapted to heat-stable proteins and includes ion pairs, aromatic interactions or H-bonds. The size and relative hydrophobicity of non-polar residue clusters forming the protein core are frequently smaller in psychrophilic proteins, lowering the compactness of the protein interior by weakening the hydrophobic effect on folding, whereas thermophilic proteins frequently adopt the reverse strategy. The N-and C-caps of α-helices are also altered, weakening the charge–dipole interaction in psychrophilic proteins or strengthening this dipole in thermophilic polypeptides [41]. Loose or relaxed protein extremities appear to be preferential sites for unzipping in cold-adapted proteins whereas these extremities are buried or constrained by various interactions in thermophilic proteins. The binding of stabilizing ions, such as calcium, can be extremely weak in psychrophilic proteins or very strong in the thermophilic homologues, with binding constants differing from mesophiles by several orders of magnitude. Insertions and deletions are sometimes responsible for specific properties such as the acquisition of extra-surface charges or the weakening of subunit interactions. Calculation of the solvent-accessible area showed that some psychrophilic enzymes expose a higher proportion of non-polar residues to the surrounding medium [42, 43]. This is an entropy-driven destabilizing factor caused by the reorganization of water molecules around exposed hydrophobic side chains. This accessible hydrophobic surface tends to decrease from psychrophilic to thermophilic proteins [14]. Calculations

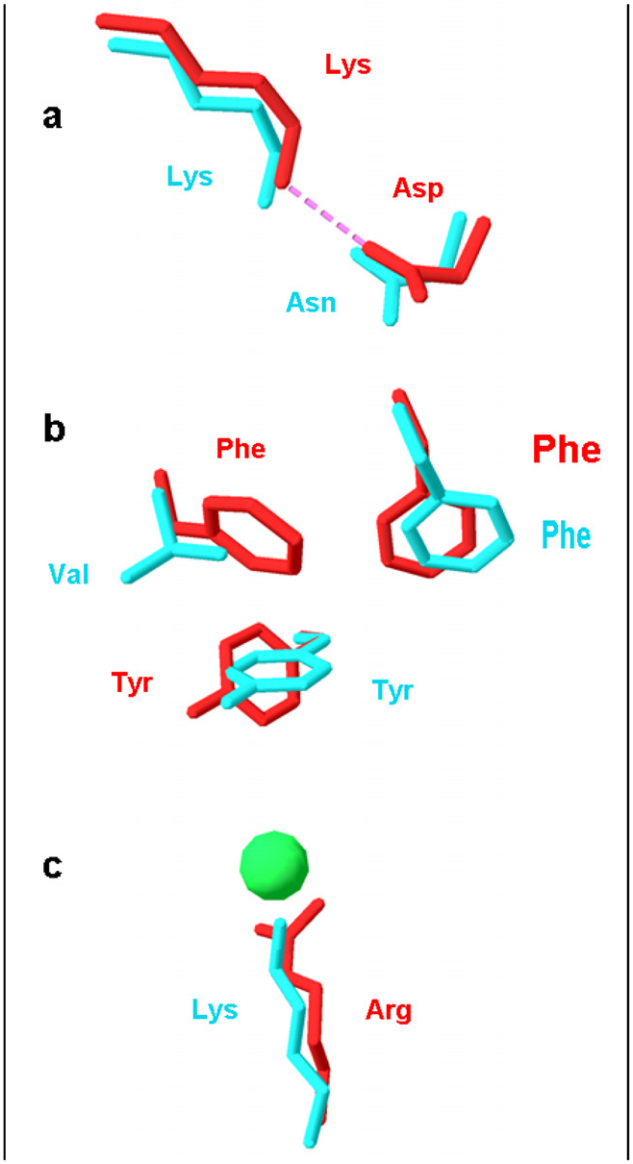

Figure 7. Weak interactions contributing to structural stability in homologous psychrophilic (blue, pale grey in the printed version) and mesophilic (red, dark grey in the printed version) α-amylases. (a) In the mesophilic enzyme, a salt bridge between an Lys–Asp pair connects two secondary structures; in the psychrophilic enzyme, the acidic residue is replaced by its amide derivative Asn devoid of net charge and therefore both secondary structures are not held together to the same extent. (b) The core of the mesophilic protein is stabilized by three aromatic side chains performing two face-to-edge weak electrostatic interactions; in the psychrophilic protein, the substitution Phe $\rightarrow$ Val disrupts this network. (c) The mesophilic protein coordinates a chloride ion in a bidentate mode via an Arg side chain whereas in the heat-labile protein Lys can only provides a unidentate coordination resulting in a weaker binding constant. The involvement of these weak interactions in stability has been demonstrated by site-directed mutagenesis [12].

of the electrostatic potential revealed in some instances an excess of negative or positive charges at the surface of cold-adapted proteins and, indeed, their pI is frequently more acidic or basic than that of their mesophilic or thermophilic homologues. This has been related to improved interactions with the solvent, which could be of prime importance in the acquisition of flexibility near zero degrees [26] as mentioned in section 2.4. Additionally, it has been proposed that these differences in pI values contribute to improve the protein solubility at low temperatures [44]. Besides the balance of charges, the number of salt bridges covering the protein surface is also modified. There is now a clear correlation between surface ion pairs and temperature adaptation, since these weak interactions significantly increase in number from psychrophiles to mesophiles and hyperthermophiles, the latter showing arginine-mediated multiple ion pairs and interconnected salt bridge networks [45, 46]. Such an altered pattern of electrostatic interactions at the molecular surface is thought to improve the dynamics or the 'breathing' of the external shell of cold-active enzymes and to provide resilience to the protein surface in thermophiles [47].

The above-mentioned factors are not found in all extremophilic proteins: each protein adopts its own strategy by using one or a combination of these altered structural factors in order to adapt the local or global mobility of the protein edifice. Comparative structural analyses of psychrophilic, mesophilic and thermophilic proteins indicate that each protein family displays different structural strategies to adapt to temperature. However, some common trends are observed: the number of ion pairs, the side chain contribution to the exposed surface and the apolar fraction of the buried surface show a consistent increase with increasing optimal temperatures [36, 48–50]. As a result of the great diversity of factors involved in protein stability, the bias in the amino acid composition observed in individual extremophilic proteins (variable proline or arginine content, etc) is not found when analysing the mean amino acid composition of the whole genome. Nevertheless, the primary message of these studies indicates that there is a continuum in structural adaptations of extremophilic proteins, whereas the selective pressure acts on an improved biological activity of psychrophilic proteins at low temperatures and on an improved thermal stability of thermophilic proteins at elevated temperatures. The following sections will show that both aspects have been intricately linked in the course of evolution.

3. Activity of extremozymes

3.1. Linking activity and flexibility

This section mainly focuses on psychrophilic enzymes as they are all faced with a main constraint, sometimes referred to as the thermodynamic challenge [51]: to be active at low temperatures. The activity of enzymes is strongly dependent on the surrounding temperature. The catalytic constant k_{cat} corresponds to the maximum number of substrate molecules converted to product per active site, per unit of time. In the simple Michaelis–Menten mechanism, the k_{cat} parameter is the first-order rate constant for the chemical conversion of the enzyme–substrate complex into enzyme and product. The transition state theory assumes the existence of a stable activated complex $ES^{\#}$ in equilibrium with the ground state ES:

$$E + S \rightleftharpoons ES \rightleftharpoons ES^{\#} \overset{k_{cat}}{\rightarrow} E + P. \quad (6)$$

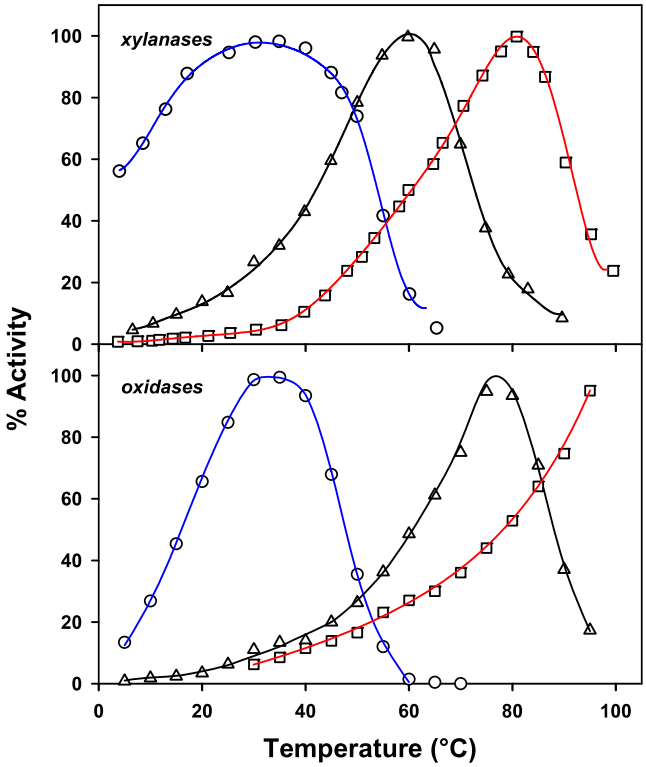

Figure 8. Temperature dependence of the relative activity in psychrophilic (circles, blue), mesophilic (triangles, black) and thermophilic (squares, red) enzymes.

The temperature dependence of the catalytic rate constant is given by the Eyring equation:

$$k_{\mathrm{cat}} = \kappa \frac{k_{\mathrm{B}} T}{h} \mathrm{e}^{-\Delta G^{\#}/RT} \tag{7}$$

where κ is the transmission coefficient generally close to 1, k_{B} is the Boltzmann constant (1.3805×10^{-23} J K^{-1}), h the Planck constant (6.6256×10^{-34} J s) and $\Delta G^{\#}$ the free energy of activation or the variation of the Gibbs energy between the activated enzyme–substrate complex ES$^{\#}$ and the ground state ES. Accordingly, the activity k_{cat} is exponentially dependent on the temperature. As a rule of thumb, for a biochemical reaction catalyzed by an enzyme from a mesophile, a 10 °C temperature increase (or decrease) results in a 2–3 times higher (or lower) activity ($Q_{10} = 2$–3). This is the main factor preventing the growth of non-adapted organisms, even the simplest microbial forms, at low temperatures but also preventing the growth of thermophiles and hyperthermophiles at room temperature.

The effect of temperature on the activity of extremophilic enzymes is illustrated in figure 8. It should be noted that equation (7) is only valid for the exponential rise of activity with temperature on the left limb of the curves. Deviation from this exponential rise occurs when thermally induced alterations of the enzyme catalyst itself compete with thermal activation of the reaction, giving rise to an apparent temperature optimum (apparent temperature because it is strongly dependent on the enzymatic assay conditions). This is followed by the activity decay at high temperatures, when enzyme inactivation dominates as a result of unfolding or aggregation. Models have been proposed to simulate the effects of these parameters on activity [52, 53] and to take the viscosity of the medium into account [37]. The comparison of the effect of temperature on the activity of extremophilic enzymes in figure 8 reveals some basic features of temperature adaptation. (i) In order to compensate for the slow reaction rates at low temperatures, psychrophiles synthesize enzymes having an up to tenfold higher specific activity (in terms of k_{cat}) in this temperature range. This is, in fact, the main physiological adaptation to cold at the enzyme level. In contrast, thermophilic enzymes are nearly inactive at room temperature. (ii) The temperature for apparent maximal activity for cold-active enzymes is shifted towards low temperatures, reflecting the weak stability of these proteins and their unfolding and inactivation at moderate temperatures. On the other hand, an apparent temperature optimum for thermophilic enzymes is sometimes not observed before the boiling point of the reaction medium as a result of their ultra-stable conformation. Such observations on various extremophilic enzymes have suggested relationships between the activity of the enzyme, the flexibility of the protein and its stability. Indeed, the high activity at low temperatures of cold-adapted enzymes seems to arise from an increased flexibility of the protein structure, especially at temperatures that strongly slow down molecular motions, but the consequence of this improved mobility of the protein structure is, of course, a weak stability. In the case of the rigid thermophilic enzymes, the conformational fluctuations required for catalytic function are restricted at room temperature, also linking flexibility and activity [54, 55]. This flexibility hypothesis has received strong experimental support, as discussed in section 2.6.

3.2. Structural adaptations at the active site

The first basic observation from crystal structures of extremophilic enzymes is that all reactive side chains as well as most side chains pointing towards the catalytic cavity are strictly conserved. This means that the overall catalytic mechanism and reaction pathway are not modified. This aspect is not really surprising as the specific reaction mechanism of enzymes is not prone to drastic variation. As far as psychrophiles are concerned, the pattern provided by the first structure of a cold-active enzyme [42, 56] was quite astonishing as it was found that all 24 residues forming the catalytic cleft and involved in substrate binding are strictly conserved in both the cold-active and the mesophilic homologues (figure 9). This outstanding example of active site identity demonstrates that the specific properties of psychrophilic enzymes can be reached without any amino acid substitution in the reaction centre. As a consequence, changes occurring elsewhere in the molecule are responsible for the optimization of the catalytic parameters (see below 3.6).

Nevertheless, significant structural adjustments at the active site of psychrophilic enzymes have been frequently reported. In many cases, the catalytic cavity appears to be larger and more accessible to ligands. This is achieved by various ways, including small deletions in loops bordering the active site [43] or by distinct conformation of these loops, and by replacement of bulky side chains for smaller groups at the entrance. In the case of a Ca^{2+}, Zn^{2+}-protease from

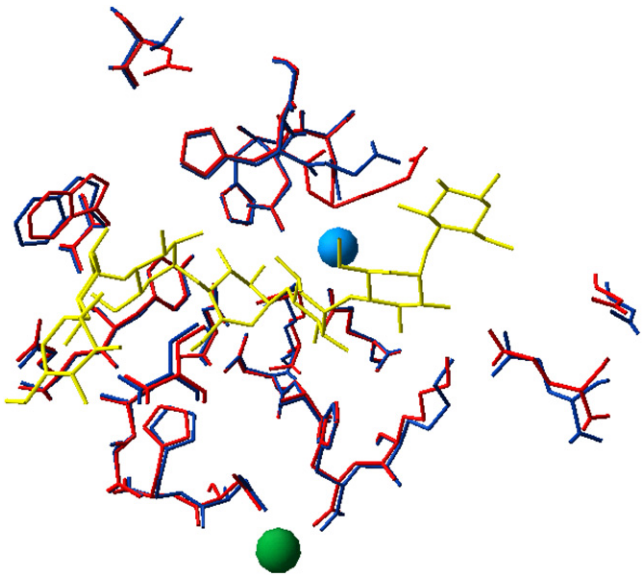

Figure 9. Superimposition of the active site residues in psychrophilic (blue) and mesophilic (red) α-amylases. The chloride and calcium ions are shown as blue and green spheres, respectively. The 24 residues performing direct or water-mediated interactions with the substrate analogue (yellow, pale grey in the printed version) are identical and superimpose perfectly within the resolution of the structures, demonstrating a structural identity in these psychrophilic and mesophilic enzymes.

a psychrophilic *Pseudomonas* species, an additional bound Ca^{2+} ion pulls the backbone forming the entrance of the site and markedly increases its accessibility when compared with the mesophilic homologue [57]. Furthermore, this active site conformation in the cold-active protease, without bound substrate, is close to the substrate-bound conformation of the mesophilic homologue: this suggests that the psychrophilic protease already adopts a catalytically competent conformation in its substrate-free structure. As a result of such better accessibility, cold-active enzymes can accommodate substrates at a lower energy cost, as far as the conformational changes are concerned, and therefore reduce the activation energy required for the formation of the enzyme–substrate complex. The larger active site may also facilitate easier release and exit of products and thus may alleviate the effect of a rate limiting step on the reaction rate. It was also shown that an opening of the active site takes place upon binding of substrate or product in a cold-active xylanase whereas similar large scale movements are not observed in mesophilic or thermophilic structural homologues [58]. This can be tentatively related to higher active site mobility in the psychrophilic enzyme.

In addition, differences in electrostatic potentials in and around the active site of psychrophilic enzymes appear to be a crucial parameter for activity at low temperatures. Electrostatic surface potentials generated by charged and polar groups are an essential component of the catalytic mechanism at various stages: as the potential extends out into the medium, a substrate can be oriented and attracted before any contact between enzyme and substrate occurs. Interestingly, the cold-active citrate synthase [43], malate dehydrogenase [59], uracil-DNA glycosylase [60] and trypsin [35, 61, 62] are characterized by marked differences in electrostatic potentials near the active site region compared to their mesophilic or thermophilic counterparts that may facilitate interaction with oppositely charged ligands. In all cases, the differences were caused by discrete substitutions in non-conserved charged residues resulting in local electrostatic potential differing in both sign and magnitude.

Finally, two last examples illustrate the unsuspected diversity of strategies used to improve the activity in psychrophilic enzymes. With few exceptions, β-galactosidases are homotetrameric enzymes bearing four active sites. However, the crystal structure of a cold-active β-galactosidase revealed that it is a homohexamer, therefore possessing six active sites certainly contributing to improving the activity at low temperatures [63]. Cellulases are microbial enzymes displaying a modular organization made of a globular catalytic domain connected by a linker to a cellulose binding domain. Psychrophilic cellulases were found to possess unusually long linkers, about five times longer than in mesophilic cellulases [64, 65]. The long linker adopts a large number of conformations and considering the cellulose binding domain anchored to the cellulose fibres and a rotation of the extended molecule around this axis, it was calculated that the catalytic domain has a 40-fold higher accessible surface area of substrate when compared with a mesophilic cellulase possessing a much shorter linker. Here also, increasing the available surface of the insoluble substrate to the catalytic domain should improve the activity of this enzyme at low temperatures.

3.3. Active site dynamics

Psychrophilic enzymes all share at least one property: a heat-labile activity, irrespective of the protein structural stability. Furthermore, the active site appears to be the most heat-labile structural element of these proteins [10, 13, 14]. Figure 10 illustrates this significant difference between the stability of the activity and the stability of the structure. The lower panel shows the stability of the structure as recorded by fluorescence. As expected, the structure of the cold-active enzyme is less stable than the mesophilic and thermophilic counterparts. In the upper panel, the activity is recorded under the same experimental conditions and it can be seen that mesophilic and thermophilic enzymes are inactivated when the protein unfolds. In contrast, activity of the cold-active enzyme is lost before the protein unfolds. This means that the active site is even more heat-labile than the whole protein structure. It was also shown that the active site of the psychrophilic α-amylase is the first structural element that unfolds in transverse urea gradient gel electrophoresis [66]. All these aspects point to a very unstable and flexible active site and illustrate a central concept in cold adaptation: localized increases in flexibility at the active site are responsible for the high but heat-labile activity [67], whereas other regions of the enzyme might or might not be characterized by low stability when not involved in catalysis [68].

The heat-labile activity of psychrophilic enzymes suggests that the dynamics of the functional side chains at the active site is improved in order to contribute to cold activity. This view is strongly supported by the enzymological properties

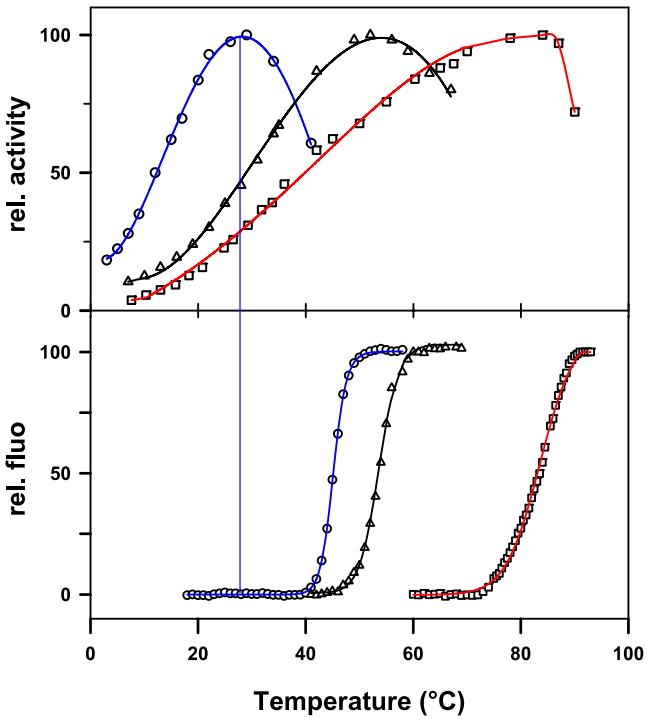

Figure 10. Inactivation and unfolding of extremophilic enzymes. The activity of psychrophilic enzymes (upper panel, circles) is inactivated by temperature before unfolding of the protein structure (lower panel) illustrating the pronounced heat-lability of the active site. In contrast, inactivation of mesophilic (triangles) or thermophilic (squares) enzymes closely corresponds to the loss of protein conformation. Adapted from [10].

Figure 11. Inhibition models of α-amylases. Reaction pathways for the competitive inhibition of starch hydrolysis by maltose for the mesophilic α-amylase PPA and of the mixed-type inhibition for the psychrophilic α-amylase AHA. Under identical experimental conditions, the cold-active enzyme forms the ternary complex ESI [70].

Table 4. Relative activity of the psychrophilic (AHA) and the mesophilic (PPA) α-amylases on macromolecular polysaccharides and on maltooligosaccharides. Adapted from [70].

	Relative activity (%)	
Substrate	AHA	PPA
Macromolecular substrates		
Starch	100	100
Amylopectin	96	68
Amylose	324	214
Dextrin	108	95
Glycogen	74	59
Short oligosaccharides		
Maltotetraose G4	17	22
Maltopentaose G5	69	145
Maltohexaose G6	94	147
Maltoheptaose G7	119	155
Maltooligosaccharides (G4 to G10 mix)	64	101

of cold-active enzymes. Non-specific psychrophilic enzymes accept various substrates and have a broader specificity than the mesophilic homologues, because substrates with slightly distinct conformations or sizes can fit and bind to the site [35, 69]. This active site flexibility of cold-active enzymes in solution is also well demonstrated by the psychrophilic α-amylase [70]. In this specific case, the above-mentioned structural identity of the catalytic cleft with its mesophilic homologue from pig (figure 9) precludes the involvement of adaptive mutations within the active site in the analysis of the results. As shown in table 4, both the psychrophilic and mesophilic α-amylases degrade large macromolecular polysaccharides made of glucose units linked by α-1, 4 bonds. These substrates have a complex structure and are generally branched. Taking the natural substrate, starch, as the reference, it can be seen that the psychrophilic enzyme is more active on all these large substrates. Being more flexible, the active site can accommodate easily these macromolecular polysaccharides. Considering the small substrates, the reverse situation is observed. Both enzymes are active on short oligosaccharides of at least four glucose units but in this case, the psychrophilic α-amylase is less active on all these small substrates. Apparently, the flexible active site accommodates less efficiently these short oligosaccharides.

The inhibition patterns provide additional insights into the specific properties of psychrophilic active sites (figure 11). Both the mesophilic and the psychrophilic α-amylases are inhibited by maltose, the end product of starch hydrolysis. In the case of the mesophilic enzyme, the enzyme can bind either the substrate (in a productive mode) or the inhibitor, but not both. In contrast, the cold-active enzyme can also bind either the substrate or the inhibitor but also both, forming the ternary complex ESI, once again suggesting a more accessible and flexible active site, although formed by identical amino acid side chains.

3.4. Adaptive drift of substrate binding

As a consequence of the improved active site dynamics in cold-active enzymes, substrates bind less firmly in the binding site (if no point mutations have occurred) giving rise to higher K_m values. An example is given in table 5 showing that the psychrophilic α-amylase is more active on its macromolecular substrates whereas the K_m values are up to 30-fold larger, i.e. the affinity for the substrates is up to 30-fold lower. Ideally, a functional adaptation to cold would mean optimizing both k_{cat} and K_m. However, a survey of the available data on psychrophilic enzymes [71] showed that optimization of the k_{cat}/K_m ratio is far from a general rule but, on the contrary, that the majority of cold-active enzymes improve the k_{cat} value at the expense of K_m, therefore leading to suboptimal values of the k_{cat}/K_m ratio, as also shown in table 5. There is, in fact, an evolutionary pressure on K_m to increase in order to maximize the overall reaction rate. Such adaptive drift of K_m has been well illustrated by the lactate dehydrogenases from Antarctic fish [67] and by the psychrophilic α-amylase [12]. In both cases, temperature adaptive increases in k_{cat} occur

Table 5. Kinetic parameters for the hydrolysis of polysaccharides at 25 °C by the psychrophilic (AHA) and the mesophilic (PPA) α-amylases. Adapted from [70].

	AHA			PPA		
Substrate	k_{cat} (s^{-1})	K_m (mg l^{-1})	k_{cat}/K_m (s^{-1} mg^{-1} l)	k_{cat} (s^{-1})	K_m (mg l^{-1})	k_{cat}/K_m (s^{-1} mg^{-1} l)
Starch	663	155	4.3	327	41	8.0
Amylopectin	636	258	2.5	222	53	4.2
Amylose	2148	178	12.1	700	36	19.4
Dextrin	716	586	1.2	311	61	5.1
Glycogen	491	1344	0.3	193	46	4.2

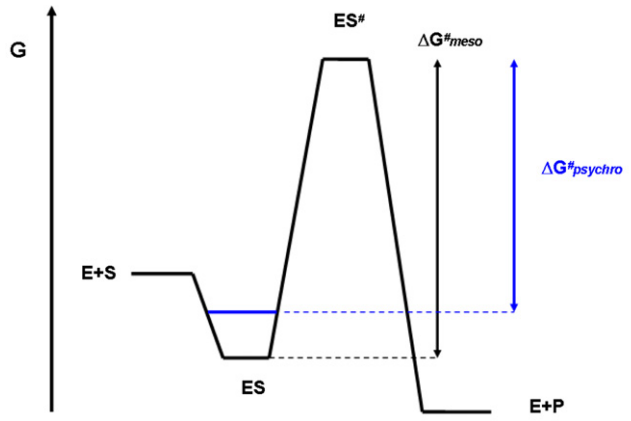

Figure 12. Optimization of activity by decreasing substrate affinity in psychrophilic enzymes. Reaction profile for an enzyme-catalyzed reaction with Gibbs energy changes under saturating substrate concentration. Weak substrate binding (in blue) decreases the activation energy ($\Delta G^{\#}$ *psychro*) and thereby increases the reaction rate.

concomitantly with increases in K_m in cold-active enzymes. As already mentioned, such identity of the sites also implies that adjustments of the kinetic parameters are obtained by structural changes occurring distantly from the reaction centre. It is worth mentioning that the low binding strength is not restricted to substrates but is also observed for most binding partners such as cofactors or ions.

Several enzymes, especially in some cold-adapted fish, counteract this adaptive drift of K_m in order to maintain or to improve the substrate binding affinity by amino acid substitutions within the active site [35]. The first reason for these enzymes to react against the drift is obvious when considering the regulatory function associated with K_m, especially for intracellular enzymes. The second reason is related to the temperature dependence of weak interactions. Substrate binding is an especially temperature-sensitive step because both the binding geometry and interactions between binding site and ligand are governed by weak interactions having sometimes opposite temperature dependences. Hydrophobic interactions form endothermically and are weakened by a decrease in temperature. In contrast, interactions of an electrostatic nature (ion pairs, hydrogen bounds, van der Waals interactions) form exothermically and are stabilized at low temperatures. Therefore low temperatures do not only reduce the enzyme activity (k_{cat}), but can also severely alter the substrate binding mode according to the type of interaction involved. The chitobiase from an Antarctic bacteria has nicely illustrated both aspects, as well as the extent of the kinetic optimization that can be reached during cold adaptation of enzymes [72].

Table 6. Activation parameters of the hydrolytic reaction of α-amylases at 10 °C. Adapted from [10].

	k_{cat} (s^{-1})	$\Delta G^{\#}$ (kcal mol^{-1})	$\Delta H^{\#}$ (kcal mol^{-1})	$T\Delta S^{\#}$ (kcal mol^{-1})
Psychrophile	294	13.3	8.3	−5.5
Mesophile	97	14.0	11.1	−2.9
Thermophile	14	15.0	16.8	1.8

3.5. Energetics of activity at low temperatures

Referring to equation (7), the high activity of cold-adapted enzymes corresponds to a decrease of the free energy of activation $\Delta G^{\#}$. Two strategies have been highlighted to reduce the height of this energy barrier. Figure 12 illustrates the first strategy where an evolutionary pressure increases K_m in order to maximize the reaction rate. According to the transition state theory, when the enzyme encounters its substrate, the enzyme–substrate complex ES falls into an energy pit. For the reaction to proceed, an activated state $ES^{\#}$ has to be reached, which eventually breaks down into the enzyme and the product. The height of the energy barrier between the ground state ES and the transition state $ES^{\#}$ is defined as the free energy of activation $\Delta G^{\#}$: the lower this barrier, the higher the activity, as reflected in equation (7). In the case of cold-active enzymes displaying a weak affinity for the substrate, the energy pit for the ES complex is less deep (dashed in figure 12). It follows that the magnitude of the energy barrier is reduced and therefore the activity is increased. This thermodynamic link between affinity and activity is valid for most enzymes (extremophilic or not) under saturating substrate concentrations and this link appears to be involved in the improvement of activity at low temperatures in numerous cold-active enzymes [67, 71].

The second and more general strategy involves the temperature dependence of the reaction catalyzed by cold-active enzymes. Table 6 reports the enthalpic and entropic contributions to the free energy of activation in extremophilic α-amylases. The free energy of activation $\Delta G^{\#}$ is calculated from equation (7) using the k_{cat} value at a given temperature and the enthalpy of activation $\Delta H^{\#}$ is obtained by recording

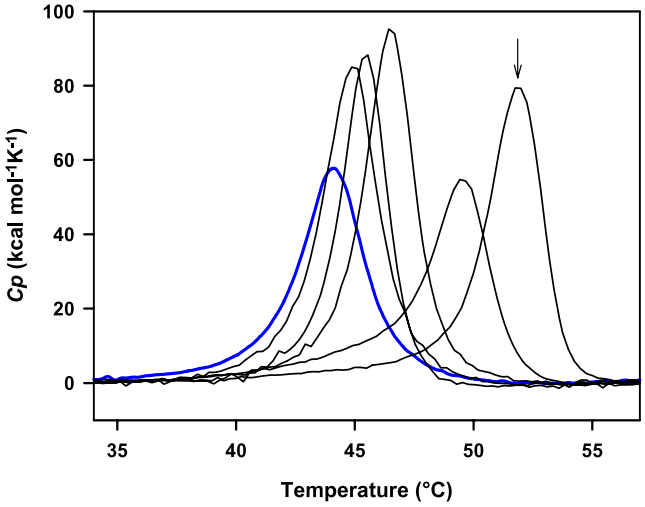

Figure 13. Engineering mesophilic-like stability in mutants of the psychrophilic α-amylase. Structure-stabilizing interactions have been introduced in the heat-labile enzyme (heavy line). As shown by the microcalorimetric thermograms, the resulting mutants (thin lines) display increased melting points and calorimetric enthalpies. The most stable mutant (arrow) bears six additional interactions. Adapted from [12, 75].

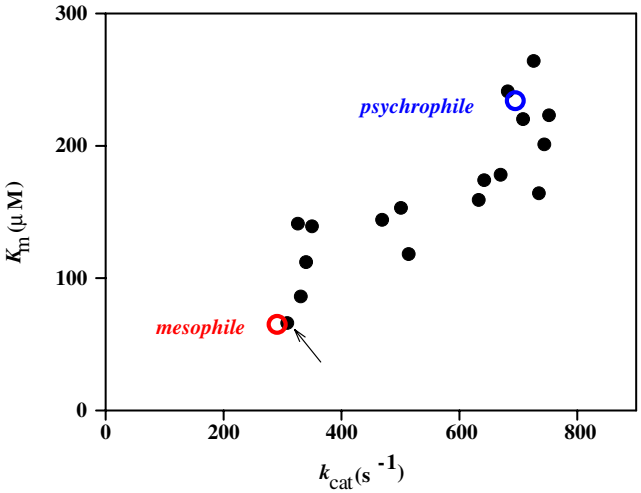

Figure 14. Engineering mesophilic-like activity in mutants of the psychrophilic α-amylase. This plot of the kinetic parameters for the stabilized mutants (filled symbols) shows that the general trend is to decrease the activity and to increase the affinity for the substrate of the wild-type psychrophilic enzyme (open symbol). The most stable mutant-bearing six additional interactions (arrow) displays kinetic parameters nearly identical to those of the mesophilic homologue (open symbol). Adapted from [12, 75].

the temperature dependence of the activity [73] as in figure 8. The enthalpy of activation $\Delta H^{\#}$ depicts the temperature dependence of the activity: the lower this value, the lower the variation of activity with temperature. The low value found for almost all psychrophilic enzymes demonstrates that their reaction rate is less reduced than for other enzymes when the temperature is lowered. Accordingly, the decrease of the activation enthalpy in the enzymatic reaction of psychrophilic enzymes can be considered as the main adaptive character to low temperatures. This decrease is structurally achieved by a decrease in the number of enthalpy-driven interactions that have to be broken during the activation steps. These interactions also contribute to the stability of the protein folded conformation and, as a corollary, the structural domain of the enzyme bearing the active site should be more flexible. It is interesting to note that such a macroscopic interpretation of the low activation enthalpy in cold-active enzymes fits with experimental data on active site dynamics mentioned in section 3.3. Table 6 shows that the entropic contribution $T\Delta S^{\#}$ for the cold-active enzyme is larger and negative. This has been interpreted as a large reduction of the apparent disorder between the ground state with its relatively loose conformation and the well-organized and compact transition state [73]. As a consequence of active site flexibility, the enzyme–substrate complex ES occupies a broader distribution of conformational states translated into increased entropy of this state, compared to that of the mesophilic or thermophilic homologues. Furthermore, a broader distribution of the ground state ES should be accompanied by a weaker substrate binding strength, as indeed observed for numerous psychrophilic enzymes. This assumption has received strong experimental support by using microcalorimetry to compare the stabilities of free extremophilic enzymes with the same enzymes trapped in the transition state conformation by a non-hydrolyzable substrate analogue [10].

3.6. Probing the activity–stability relationships

In order to check the validity of the proposed relationships between the activity and the stability in cold-active enzymes, the psychrophilic α-amylase has been used as a model because the identical architecture of its active site, when compared with a close mesophilic homologue (figure 9), indicates that structural adaptations affecting the active site properties occur outside from the catalytic cavity. Accordingly, the crystal structure [42, 56] has been closely inspected to identify structural factors involved in its weak stability, as shown in figure 7. On this basis, 17 mutants of this enzyme were constructed, each of them bearing an engineered residue forming a weak interaction found in mesophilic α-amylases but absent in the cold-active α-amylase, or a combination of up to six stabilizing structural factors [12, 74, 75]. As illustrated in figure 13, it was found that single and multiple amino acid side chain substitutions significantly increase the melting point T_m and the calorimetric enthalpy ΔH_{cal}. However, in the context of catalysis at low temperatures, the most significant observation was that these mutations tend to decrease both k_{cat} and K_m. As shown in figure 14, stabilizing the cold-active α-amylase tends to decrease the k_{cat} values and concomitantly the K_m values of the mutant enzymes, revealing the high correlation between both kinetic parameters illustrated in figure 12. In fact, in addition to an engineered mesophilic-like stability, the multiple-mutant bearing six stabilizing structural factors also displays an engineered mesophilic-like activity in terms of alterations in k_{cat} and K_m values and even in thermodynamic parameters of activation [75]. Considering the various available data on the psychrophilic α-amylase, it can be concluded that the improved molecular motions of the side chains forming the active site (motions responsible for the high activity, the low affinity and heat lability) originate from the

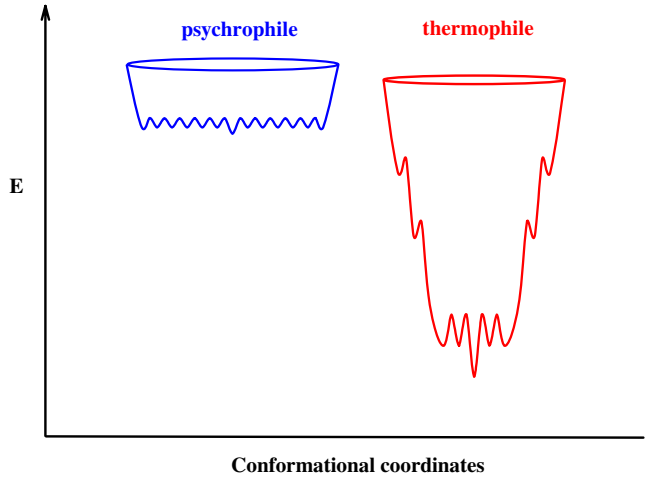

Figure 15. Folding funnel model of enzyme temperature adaptation. In these schematic energy landscapes for extremophilic enzymes, the free energy of folding (E) is depicted as a function of the conformational diversity. The height of the funnels is deduced from the determination of the conformational stabilities. The top of the funnels is occupied by the unfolded states in the numerous random coil conformations, whereas the bottom of the funnels corresponds to native and catalytically active conformations. The ruggedness of the bottom depicts the energy barriers for interconversion, or structural fluctuations of the native state [10].

lack of structure-stabilizing interactions in the vicinity, or even far from, the active site. This is another strong indication that structural flexibility is an essential feature related to catalysis at low temperatures in psychrophilic enzymes.

4. Conclusions

The various properties of extremophilic enzymes that have been presented in the previous sections can be integrated in a model based on folding funnels [76, 77] to describe the activity–stability relationships in these proteins. Figure 15 depicts the energy landscapes of psychrophilic and thermophilic enzymes. The top of the funnel is occupied by the unfolded state, adopting numerous available conformations and having a high free energy (considering the spontaneous folding reaction), whereas the bottom of the funnel is occupied by the stable (low free energy) native state adopting a limited number of catalytically active conformations. The height of the funnel, i.e. the free energy of folding, also corresponding to the conformational stability, has been fixed here in a 1–5 ratio according to the stability curves shown in figure 5. The upper edge of the funnels is occupied by the unfolded state in random coil conformations but it should be noted that psychrophilic proteins tend to have a lower proline content than mesophilic and thermophilic homologues, a lower number of disulfide bonds and a higher occurrence of glycine clusters [35–37, 78]. Accordingly, the edge of the funnel for the psychrophilic protein is slightly larger (broader distribution of the unfolded state) and is located at a higher energy level. When the polypeptide is allowed to fold, the free energy level decreases, as well as the conformational ensemble. However, thermophilic proteins pass through intermediate states corresponding to local minima of energy. These minima are responsible for the ruggedness of the funnel slopes and for the reduced cooperativity of the folding–unfolding reaction, as demonstrated by heat-induced unfolding (figure 3). In contrast, the structural elements of psychrophilic proteins generally unfold cooperatively without intermediates, as a result of fewer stabilizing interactions and stability domains [12, 26], and therefore the funnel slopes are steep and smooth. The bottom of the funnel, which depicts the stability of the native state ensemble, also displays significant differences between both extremophilic enzymes. The bottom for a very stable and rigid thermophilic protein can be depicted as a single global minimum or as having only a few minima with high energy barriers between them [79, 80] whereas the bottom for an unstable and flexible psychrophilic protein is rugged and depicts a large population of conformers with low energy barriers to flip between them. Rigidity of the native state is therefore a direct function of the energy barrier heights [79, 80] and is drawn here according to the results of fluorescence quenching (figure 6) and neutron scattering experiments [32]. In this context, the activity–stability relationships in these extremophilic enzymes depend on the bottom properties. Indeed, it has been argued that, upon substrate binding to the association-competent sub-population, the equilibrium between all conformers is shifted towards this sub-population, leading to the active conformational ensemble [79–82]. In the case of the rugged bottom of psychrophilic enzymes, this equilibrium shift only requires a modest free energy change (low energy barriers), a low enthalpy change for interconversion of the conformations but is accompanied by a large entropy change for fluctuations between the wide conformer ensemble. The converse picture holds for thermophilic enzymes, in agreement with the activation parameters shown in table 6 and with the proposed macroscopic interpretation. Such energy landscapes integrate nearly all biochemical and biophysical data currently available for extremophilic enzymes but they will certainly be refined by future investigations of other series of homologous proteins from psychrophiles, mesophiles and thermophiles. This model has nevertheless received support from independent studies [83, 84].

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