Some like it cold: biocatalysis at low temperatures

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Abstract

In the last few years, increased attention has been focused on a class of organisms called psychrophiles. These organisms, hosts of permanently cold habitats, often display metabolic fluxes more or less comparable to those exhibited by mesophilic organisms at moderate temperatures. Psychrophiles have evolved by producing, among other peculiarities, "cold-adapted" enzymes which have the properties to cope with the reduction of chemical reaction rates induced by low temperatures. Thermal compensation in these enzymes is reached, in most cases, through a high catalytic efficiency associated, however, with a low thermal stability. Thanks to recent advances provided by X-ray crystallography, structure modelling, protein engineering and biophysical studies, the adaptation strategies are beginning to be understood. The emerging picture suggests that psychrophilic enzymes are characterized by an improved flexibility of the structural components involved in the catalytic cycle, whereas other protein regions, if not implicated in catalysis, may be even more rigid than their mesophilic counterparts. Due to their attractive properties, i.e., a high specific activity and a low thermal stability, these enzymes constitute a tremendous potential for fundamental research and biotechnological applications.
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Keywords: Cold-adaptation; Psychrophiles; Extremophiles; Enzyme kinetics; Enzyme activity; Flexibility concept

Contents

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1. Introduction

When one considers the vast extent of permanently cold habitats, most parts of our planet are exposed to low temperatures, often below 0 °C. These environments comprise, among others, the Antarctic, Arctic and mountain regions, as well as the deep-sea waters that cover three-quarters of the planet surface. In 1887, Forster was the first to call attention to the growth and reproduction of bacteria at low temperatures by reporting that microorganisms isolated from fish could grow well at 0 °C [1]. Since then, numerous organisms, prokaryotic but also eukaryotic, have been found to have successfully colonized low-temperatures habitats (for review, see [2]). Evolution has allowed these cold-adapted organisms, called psychrophiles, not to merely survive, but to breed and grow successfully in the restrictive conditions of cold habitats. Psychrophiles display metabolic fluxes at low temperatures that are more or less comparable to those exhibited by closely related mesophiles living at moderate temperatures [3–8], clearly showing that mechanisms of temperature adaptations are involved. Such mechanisms include a vast array of structural and physiological adjustments in order to cope with the reduction of chemical reaction rates induced by low temperatures.

2. Life in the cold

Temperature is one of the most important environmental factors for life, as it influences most biochemical reactions. A reduction in temperature slows down most physiological processes, changes protein–protein interactions, reduces membrane fluidity and provokes an increased viscosity of water. Moreover, a decrease in temperature also induces a reduction in salt solubility and an increase in gas solubility. Decreases in the pH of biological buffers are also noticed with decreasing temperature, affecting both the protein solubility and the charge of amino acids, particularly histidine residues [9,10]. Enzymes are also subject to cold denaturation, leading to the loss of enzyme activity at low temperatures [11]. This phenomenon is thought to occur through the hydration of polar and non-polar groups of proteins [12], a process thermodynamically favoured at low temperatures. It is notably the weakening of hydrophobic forces, which are crucial for protein folding and stability, which causes protein unfolding. Cold-denaturation, which is a very general property of proteins, affects, in particular, multimeric enzymes such as ATP-ases and fatty-acid synthetases as well as proteins showing a high degree of hydrophobicity [13]. As shown in Fig. 1, psychrophilic enzymes are surprisingly more prone to cold-denaturation than their mesophilic and thermophilic counterparts since they can unfold at
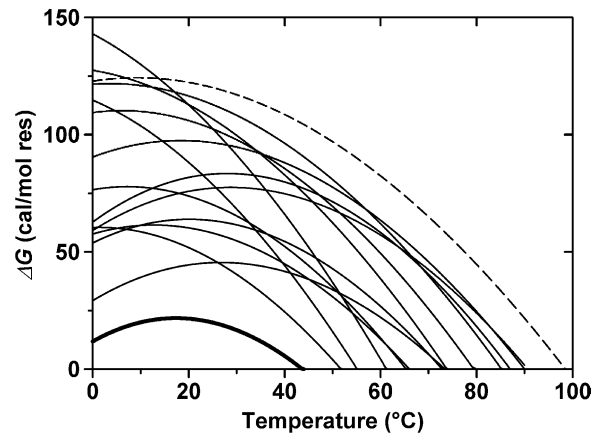

Fig. 1. Gibbs free energy of unfolding or conformational stability. Stability curves for a psychrophilic α-amylase (heavy line), several mesophilic proteins (continuous lines) and a thermophilic protein (dashed line). To compare proteins of various sizes, the free energy is expressed in specific units per mole of residue. Adapted from [14].

temperatures close to −10 °C [14]. On the other hand, biological activities have been recorded in the brine veins of sea-ice at temperatures as low as −20 °C [8]. Therefore, in these types of environments cold denaturation of proteins should be prevented in order to secure life. While in the case of intracellular enzymes, protection towards cold-denaturation can possibly be achieved by compatible solutes such as potassium glutamate, trehalose, etc., in the case of extracellular enzymes, which are essential in providing nutrients to the cell, no specific protectants have been described yet, although exopolymeric substances (EPS) could possibly play such a role [15].

Another consequence of the exposure to low temperatures is a strong inhibition of chemical reaction rates catalysed by enzymes. The temperature dependence of chemical reactions is commonly described by the Arrhenius equation $k = A\,e^{-E_a/RT}$, in which k is the rate constant, A is the pre-exponential factor (related to steric factors and molecular collision frequency), E_a is the activation energy, R is the gas constant (8.314 kJ mol^{-1}) and T is the absolute temperature in Kelvin. Thus, any decrease in temperature will induce an exponential decrease in the reaction rate. Indeed, for most biological systems, a decrease of 10 °C depresses the rate of chemical reactions by a factor ranging from 2 to 3 ($Q_{10} = 2$ to 3), the exact value depending on the activation energy. Accordingly, the activity of a mesophilic enzyme should be 16–80 times lower when the reaction temperature is shifted from 37 to 0 °C.

In order to overcome the detrimental effect of low temperatures, psychrophiles have developed various adaptive strategies from the level of individual types of molecules to that of the whole organism. For instance, cold adaptation has led to the regulation of membrane fluidity, the synthesis of specialized molecules known as cold-shock proteins, cryoprotectors and antifreeze

molecules, the regulation of ion channels permeability, microtubules polymerisation, seasonal dormancy, and importantly, the modification of enzyme kinetics. Some of these crucial adaptations are described below.

Important cellular functions, such as passive and active permeability, nutrient uptake, electron transport, environmental sensing and recognition processes require the maintenance of membrane stability. Psychrophiles achieve this, notably by increasing the proportion of unsaturated fatty acids and the modulation of the activity of the enzymes involved in fatty acid and lipid biosynthesis (for review, see [2]).

Sudden decreases in temperature will also elicit a specific alteration in gene expression known as the cold-shock response [16–20]. The cold-shock response, which is evidently not confined to psychrophilic organisms but constitutes the beginning of cold-adaptation, induces the synthesis of cold-shock proteins named Csp. These proteins act mainly on the regulation of cellular protein synthesis, particularly at the level of transcription and the initiation of translation; and they also act as chaperone by preventing the formation of mRNA secondary structures (mRNA 'folding'). The regulation of cold-shock-protein itself is a complex and multifactorial phenomenon, being controlled at the level of both transcription and translation [20]. A major distinction between psychrophiles and their mesophilic and thermophilic homologues is that, in the former, the synthesis of housekeeping gene products is not inhibited by cold-shock, and the number of cold-shock proteins is usually higher, and proportional to the severity of the cold-shock [19,21,22].

In addition to cold-shock proteins, the production of specialized molecules such as cryoprotectors and anti-freeze molecules has also been reported. Antifreeze proteins (AFPs) are found in some organisms such as fish, insects, plants, fungi and microorganisms that encounter freezing conditions (for review, see [23,24]). By contributing to both freeze resistance and freeze tolerance, AFPs have helped to increase species diversity in some of the harshest and most inhospitable environments. For instance, fish from the polar regions survive at temperatures close to the freezing point of sea water (−1.8 °C) by secreting high concentrations of AFPs molecules into their blood [25,26], preventing the formation of ice crystals which could lead to the destruction of cell membranes and the disruption of osmotic balance. Such molecules act in the blood of these fish by adsorption to the surfaces of microcrystals of ice, thereby preventing the association of additional water molecules and thus, growth of the crystals [25,27–29]. Recent works suggest that the key feature of all anti-freeze molecules could lie in the complementarity between the ice-binding protein surfaces and the ice crystals [30].

Structural proteins of the cell are also challenged by the cold. Microtubules are ubiquitous cytoskeletal structures that are involved in diverse functions including cell movement, vesicle transport within the cell and chromosome segregation during mitosis (for review, see [31]). These structures are formed by the self-assembly of αβ tubulin heterodimers. Around 13 parallel protofilaments, each of which is a linear arrangement of heterodimers, form a hollow cylinder: the microtubule. While microtubules from homeotherms are sensitive to depolymerisation when they experience low temperatures, microtubules from Antarctic fish and algae maintain their structural integrity [32–34]. This phenomenon is due to a small number of amino acid substitutions in the tubulin chains. These alterations seem (i) to stabilize the tubulin monomers in a conformation that favours polymerisation and strengthens protofilament interactions and (ii) to reduce the dynamic instability of microtubules by slowing down the conformational change in the tubulin dimer preceding depolymerisation [34].

3. Evolution of enzymes challenging low temperatures

The catalytic cycle of an enzyme is made up of three main phases: recognition and binding to the substrate; conformational changes induced by the substrate, leading to product formation; and, finally, release of the product. Each of these phases involves weak interactions sensitive to temperature changes. Depending on the type of these weak interactions and on the substrate concentration, the temperature dependence of the enzymatic reaction can be close to that of an uncatalysed reaction, or quite different. Indeed, in the case of Michaelis–Menten kinetics, at high substrate concentrations, the value of K_m, which roughly represents a measure of the affinity of the enzyme for the substrate, becomes inconsequential. Therefore, at a fixed enzyme concentration, the thermodependence of the reaction rate will be dependent only on k_{cat}, the rate constant. However, at subsaturating substrate concentrations, the situation will be quite different, since K_m will also have an influence on reaction rate. Thus the term to be considered is the catalytic efficiency, k_{cat}/K_m.

Efficient binding of substrate by the enzyme is mediated by the nature and strength of weak interactions which are of two types: (i) interactions formed with a negative modification of enthalpy and hence are exothermic (van der Waals interactions, hydrogen bonds, electrostatic interactions) and (ii) interactions formed (at least within the low and moderate temperature ranges) with a positive modification of enthalpy, and thus are endothermic (hydrophobic interactions). The former are destabilized by an increase of temperature, whereas the latter will tend to be stabilized by moderately high temperatures. It is worth mentioning that if hydrophobic interactions are essentially entropically driven at

temperatures up to about 25 °C due to a global positive modification of the enthalpy (dehydration), they become also enthalpically driven through van der Waals interactions at higher temperatures; the entropy change becoming close to zero around 100 °C [12]. Consequently, K_m will be differentially affected by temperature according to the contribution of each type of bond to the enzyme–substrate interaction.

In permanently cold habitats, low temperatures have constrained psychrophiles to develop enzymatic tools allowing metabolic rates compatible to life that are close to those of temperate organisms. Such an objective could be reached by increasing the enzyme concentrations compensating for lowered reaction rates. However, this strategy is energetically expensive and therefore it has been reported only in a very few cases during acclimation of ectotherms [35,36]. Another strategy would be the expression of specific isotypes kinetically adapted to a given environment [37,38]. This kind of adaptation is only feasible when multicopies of genes are available. This process is particularly useful for seasonal reversible adaptation mechanisms, as observed in fish [39] and nematode enzymes [40]. Another alternative in cold-adaptation would be the design of enzymes characterized by temperature-independent reaction rates. In these perfectly evolved enzymes, the reaction rate would be only diffusion-controlled. The analysis of the Arrhenius equation shows that in this case, E_a tends to zero and therefore the exponential term E_a/RT tends to 1, leading to fast and essentially temperature-independent reactions. Such enzymes are relatively rare; some known examples are erythrocytic carbonic anhydrase, catalases from various species or *Vibrio marinus* triosephosphate isomerase. More generally however, most organisms living in cold habitats have adapted their metabolic rates by increasing the catalytic potential of their enzymes.

Many scientists have investigated the adaptation process of psychrophilic enzymes. As described in Fig. 2, three basic key features emerge from the analysis of the effect of temperature on the activity of psychrophilic and mesophilic enzymes. First, psychrophiles produce enzymes having an up to tenfold higher specific activity (k_{cat}) at low temperatures. This is in fact main physiological adaptation to cold at the enzyme level, since it offsets the inhibitory effect of low temperatures on reaction rates and therefore provides adequate raw metabolic activity to the growing organism. Second, the apparent maximal activity is shifted towards low temperatures, reflecting the weak stability of these enzymes that are prone to inactivation and unfolding at moderate temperatures. Third, the adaptation is usually not complete, as the specific activity exhibited by most psychrophilic enzymes around 0 °C, although very high, remains generally lower than that of their mesophilic counterparts at 37 °C.
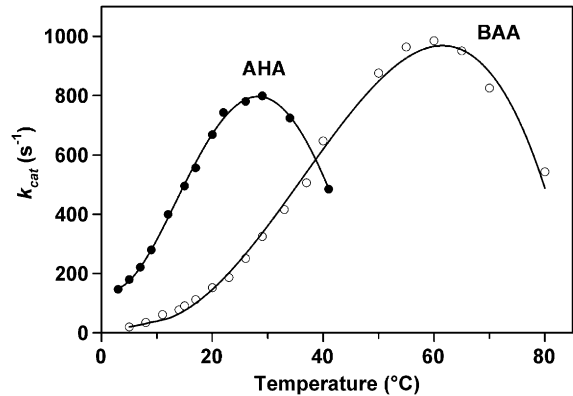

Fig. 2. Effect of temperature on the activity of psychrophilic and mesophilic enzymes. Curves representing the thermal dependence of the specific activity of the psychrophilic α-amylase from *P. haloplanktis* AHA (closed circles) and of its thermostable homologue from *B. amyloliquefaciens* BAA (open circles). Adapted from [124].

In principle, cold-adapted enzymes can optimize the k_{cat}/K_m ratio by increasing k_{cat}, decreasing K_m, or altering both parameters. A survey of the available kinetic parameters reveals two main trends: psychrophilic enzymes that improve k_{cat} at the expense of K_m (both k_{cat} and K_m increase) and those improving the k_{cat}/K_m ratio (increase of k_{cat} and decrease of K_m) (for compilation, see [41,42]). As discussed in the next sections, both kinetic behaviours seem to be related to cold-active enzymes devoid or not of adaptive mutations within the catalytic centre.

The high turnover number (k_{cat}) exhibited by cold-adapted enzymes is mainly correlated with a high efficiency in lowering the activation energy of chemical reactions [43]. 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 Michaelis–Menten equation, the k_{cat} parameter is the first-order rate constant for the chemical conversion of the enzyme–substrate complex (ES) to enzyme and product. The transition state theory assumes the existence of an activated complex ES$^{\#}$ in equilibrium with the ground-state ES

$$\mathrm{E + S \leftrightarrow ES \leftrightarrow ES^{\#} \overset{k_{cat}}{\rightarrow} E + P} \qquad (1)$$
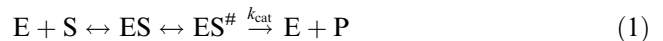
and the temperature dependence of the catalytic rate constant is given by the following equation, equivalent to the Arrhenius law

$$k_{cat} = \kappa \frac{k_B T}{h} e^{-\Delta G^{\#}/RT}, \qquad (2)$$

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^{-1}) and $\Delta G^{\#}$ the free energy of activation or the variation of the Gibbs energy between the activated enzyme–substrate complex ES$^{\#}$ and ground-state ES. The equations used to cal-

culate the thermodynamic parameters of activation leading to $\Delta G^{\#}$, $\Delta H^{\#}$ (activation enthalpy) and $\Delta S^{\#}$ (activation entropy) can be found elsewhere [44]. In order to understand how cold-active enzymes deal with low temperatures, a comparison of the variations of the thermodynamic parameters between psychrophilic (p) and mesophilic (m) enzymes (namely $\Delta(\Delta G^{\#})_{p-m}$, $\Delta(\Delta H^{\#})_{p-m}$ and $\Delta(\Delta S^{\#})_{p-m}$) is particularly striking. Such comparison is compiled in Table 1 and gives access to the understanding of the way followed by cold-enzymes to deal with low temperatures. Due to the relation between k_{cat} and $\Delta G^{\#}$ (see Eq. (2)), the $\Delta(\Delta G^{\#})_{p-m}$ parameter reflects the variation of k_{cat} between the compared enzymes. The negative value of $\Delta(\Delta G^{\#})_{p-m}$ reveals, as expected, that psychrophilic enzymes are more active than their mesophilic homologues. However, the small value originates from large differences in the enthalpic ($\Delta(\Delta H^{\#})_{p-m}$) and entropic contributions ($\Delta(\Delta S^{\#})_{p-m}$) between psychrophilic and mesophilic enzymes studied. Besides, all psychrophilic enzymes studied so far possess lower $\Delta H^{\#}$ values, reducing therefore the temperature dependence of k_{cat} and consequently the deleterious effect of low temperatures on enzyme reaction rates. However, the observed activation energy $\Delta G^{\#}$ is never as low as it would be expected from the decrease in $\Delta H^{\#}$, due to the antagonistic effect of the activation entropy. In effect, numerous analyses carried on cold-adapted enzymes revealed that the $\Delta(\Delta S^{\#})_{p-m}$ was always negative whatever the sign (positive or negative) of the activation entropy. Thus, in an enzymatic reaction catalysed by cold-adapted enzymes, the decrease of the

activation enthalpy can be considered as the main adaptive parameter. The corresponding decrease in activation energy is achieved structurally by a decrease in the number of enthalpy-driven interactions that have to be broken to reach the transition state, thus indicating a lower stability and hence also a greater flexibility at or near the catalytic site of psychrophilic enzymes [45]. This is the first insight suggesting that the activity and stability are linked in the process of thermal adaptation. Recently, this concept of 'localized flexibility' has been confirmed experimentally for a psychrophilic α-amylase [46], a DNA ligase [47] and a xylanase [48] from *Pseudoalteromonas haloplanktis*. As shown in Fig. 3, the maximal activity of the psychrophilic enzymes is reached at temperatures which do not induce any significant conformational changes, pointing out that the active site of cold-adapted enzymes or/and the catalytic intermediates are even more heat-labile than is the general protein structure. In contrast, in the case of the heat-stable homologues, the temperature for maximal activity closely corresponds to the unfolding transition, showing that structural unfolding is a main determinant for the loss of activity at high temperatures. Moreover, the increased resilience of the active site is probably the factor explaining the commonly observed negative $\Delta(\Delta S^{\#})_{p-m}$. Indeed, as a consequence of its high structural flexibility, the ground-state ES occupies a broader distribution of conformational states translated into an increase in entropy of this state compared to that of the mesophilic homologues, leading to the negative value of $\Delta(\Delta S^{\#})_{p-m}$. Thus the negative value of $\Delta(\Delta S^{\#})_{p-m}$ appears to be in-

Table 1
Activation parameters for the activity of psychrophilic and mesophilic enzymes [44]

| Enzyme	Source[a]	T (°C)	k_{cat} (s^{-1})	$\Delta G^{\#}$ (kJ mol^{-1})	$\Delta H^{\#}$ (kJ mol^{-1})	$T\Delta S^{\#}$ (kJ mol^{-1})	$\Delta(\Delta G^{\#})_{p-m}$ (kJ mol^{-1})	$\Delta(\Delta H^{\#})_{p-m}$ (kJ mol^{-1})	$T\Delta(\Delta S^{\#})_{p-m}$ (kJ mol^{-1})
Amylase	Alteromonas$_p$	15	495.2	55.6	73.6	18.0	−4.1	−26.0	−21.9
	Bacillus$_m$		90.5	59.7	99.6	39.9			
Chitinase A	Arthrobacter$_p$	15	1.7	69.2	60.2	−9.0	2.0	−14.1	−16.1
	S. marcescens$_m$		3.9	67.2	74.3	7.1			
Chitobiase	Arthrobacter$_p$	15	98.0	59.5	44.7	−14.8	−4.0	−26.8	−22.8
	S. marcescens$_m$		18.0	63.5	71.5	8.0			
Glucose-6-phosphate dehydrogenase	Antarctic fish$_p$	0	106.5	56.1	36.8	−19.3	−2.1	−15.1	−13.0
	Human$_m$		42.4	58.2	51.9	−6.3			
Glutamate dehydrogenase	Antarctic fish$_p$	5	3.8	64.8	33.4	−31.4	−3.3	−25.6	−22.3
	Bovine$_m$		0.9	68.1	59.0	−9.1			
Subtilisin	Bacillus TA41$_p$	15	25.4	62.7	36	−26.7	−3.7	−10.0	− 6.3
	B. subtillis$_m$		5.4	66.4	46	−20.4			
Trypsin	Greenland cod$_p$	5	82.0	57.7	33.0	−24.5	−2.5	−20.9	−18.3
	Bovine$_m$		27.7	60.2	53.9	−6.2			
Trypsin	Antarctic cod$_p$	15	0.97	70.5	40.6	−29.9	−0.9	−42.0	−41.2
	Bovine$_m$		0.68	71.4	82.6	11.3			
Xylanase	Antarctic yeast$_p$	5	14.8	61.7	45.4	−16.3	−2.6	−4.5	−1.9
	Yeast$_m$		4.9	64.3	49.9	−14.4			

[a] p and m for psychrophile and mesophile, respectively.
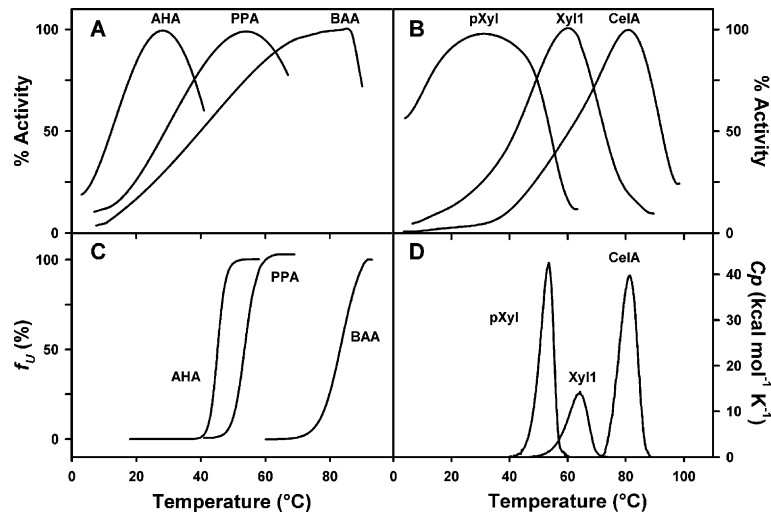
Fig. 3. Temperature dependence of activity (% Activity) (A, B) and unfolding as recorded by fluorescence emission (f_U, C) and DSC (C_p, D) of extremophilic enzymes. AHA, psychrophilic α-amylase; PPA, mesophilic α-amylase; BAA, thermostable α-amylase; pXyl, psychrophilic xylanase; Xyl1, mesophilic xylanase; CelA, thermophilic endoglucanase. Note that the maximal activity of the psychrophilic enzymes AHA and pXyl is reached at temperatures lower than those of any significant conformational event. Adapted from [46,48].

herently associated with the negative value of $\Delta(\Delta H^{\#})_{p-m}$, and to be an unavoidable counter-effect for the acquirement of a high specific activity of psychrophilic enzymes.

The broader distribution of the ground-state ES is predicted to be accompanied by a weaker substrate binding strength (i.e., increased K_m) if increases in k_{cat} are reached through active-site resilience. Such phenomenon is catalytically advantageous: the ground-state ES falls in a less deep energy pit therefore reducing the energy barrier $\Delta G^{\#}$ (and increasing k_{cat}) of the reaction [46]. This has indeed been reported for numerous psychrophilic enzymes displaying identical substrate binding site and active site architecture, when compared with their mesophilic homologues. Recently, the experimental demonstration of the inverse relationship between k_{cat} and K_m was provided by comparing the A4-lactate dehydrogenases from South American and Antarctic notothenoid fishes [45], and the chloride-dependent α-amylases from the Antarctic bacterium *P. haloplanktis* and pig pancreas [49]. In both cases, at low temperatures, the increased k_{cat} noticed in the psychrophilic enzymes was correlated with an increased K_m. For the psychrophilic α-amylase, the correlation between both kinetic parameters was demonstrated by reintroducing in the protein those stabilising weak interactions that initially were missing. This led to rigidification of the enzymes and to stabilisation of the mutants with the consequence that genetically-engineered enzymes displayed decreased k_{cat} and K_m (Fig. 4) [49].

However, several enzymes counteract the adaptive drift of K_m in order to maintain or even improve the substrate-binding affinity. Such strategy is notably crucial for enzymes typically operating at subsaturating
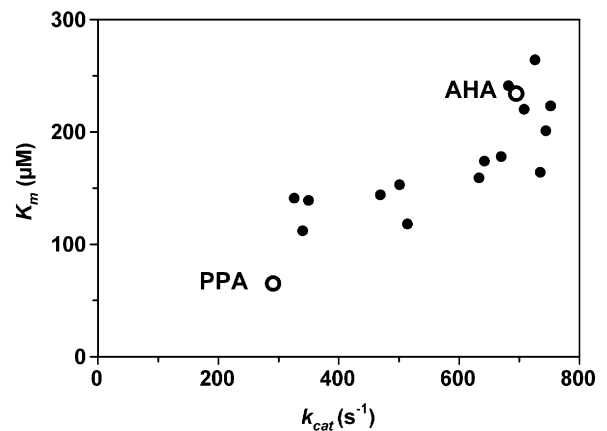

Fig. 4. Correlation of the kinetic parameters in a psychrophilic α-amylase (AHA), its stabilized mutants (closed circles) and a mesophilic homologue (PPA). The general trend displayed by the stabilized mutants of AHA is to decrease both k_{cat} and K_m values. Adapted from [49].

substrate concentrations (such as intracellular enzymes), where substrate affinity represents the critical parameter for reaction rate. Moreover, as previously mentioned substrate binding is very temperature sensitive and depends not only on the active site geometry but also on the type of bonds involved in the substrate interaction. Therefore, low temperatures not only inhibit the enzyme activity (k_{cat}) but can also severely alter the substrate-binding mode and strength. Evolution through an increase of k_{cat} and decrease of K_m has been found in some cold-adapted enzymes such as a phosphoglycerate kinase from *Pseudomonas* sp. TACII18 [50], β-galactosidase from *P. haloplanktis* [51] and psychrophilic chitobiase from *Arthrobacter* sp. TAD20 [52]. Compar-
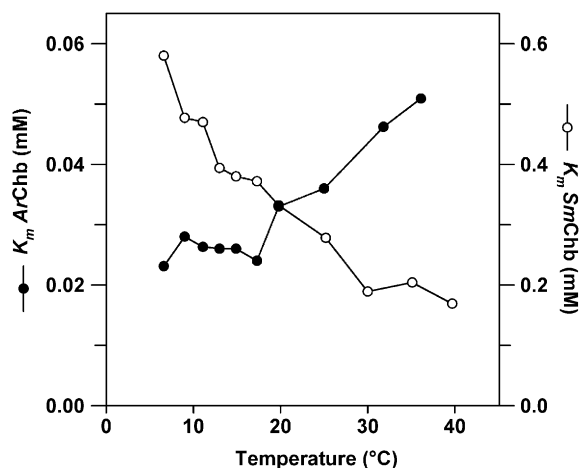
Fig. 5. Temperature dependence of the Michaelis parameter K_m for psychrophilic (closed circles) and mesophilic (open circles) chitobiases. Note the different scales used. Adapted from [52].

ison of the K_m of the latter with that of the mesophilic counterpart (Fig. 5) shows that the kinetic parameter is optimized in accordance with the physiological temperature of the organisms. Here, it has been suggested that the subtle replacement of two tryptophan residues, involved in substrate binding in the mesophilic chitobiase, by polar residues in the psychrophile homologue leads to the observed reduction of the K_m at low temperatures [52].

4. The activity–stability–flexibility trilogy

4.1. The flexibility concept

Enzyme catalysis generally involves the "breathing" of all or of a particular region of the enzyme, enabling the accommodation of the substrate. The ease of such molecular movement may be one of the determinants of catalytic efficiency. Therefore optimising a function of an enzyme at a given temperature requires a proper balance between two often opposing factors: structural rigidity, allowing the retention of a specific 3D conformation at the physiological temperature, and flexibility, allowing the protein to perform its catalytic function [53,54].

At room temperature, a thermophilic enzyme would therefore be stable but poorly active. This is certainly due to an increase in the molecular edifice rigidity induced by the low thermal energy in the surroundings, thus preventing essential movement of residues. In order to secure the appropriate stability at high temperatures, thermophilic enzymes appear to have in general a very rigid and compact structure at moderate temperatures, which, in most cases, is characterized by a

tightly packed hydrophobic core and a maximal exposure of surface ion-pairs organized in networks (for review, see [55–57]). Hydrophobic interactions have been initially proposed as the major stabilising force in proteins [58,59]. However, in 1995, Ragone and Colonna [60] suggested that hydrophobic forces would not significantly stabilize proteins having melting temperatures of about 87 °C and above (ΔS close to zero). So, other forces, such as salt bridges and hydrogen bonds, would be expected to play a major role in the extra thermostabilisation of such proteins (for review, see [57]). Furthermore, this hypothesis is corroborated by studies suggesting that hydrogen bonding and the hydrophobic effect make comparable contributions to the stability of globular proteins [61,62]. The presumed increased rigidity (and thus reduced flexibility) of thermophilic enzymes is indeed supported by a growing body of experimental data including frequency-domain fluorometry and anisotropy decay [63], hydrogen–deuterium exchange [64–66], and tryptophan phosphorescence [67] experiments. One has to note, however, that protein stability can also be achieved in some cases through an increase in the entropy of the native state [68]. Therefore, no universal rules can be proposed to fully explain the high thermostability of thermophilic enzymes, since in addition a few hyperthermophilic enzymes appear to be more active than their mesophilic counterparts, even at 37 °C [69–71]. Their high catalytic activity at such temperatures suggests that as in the case of the β-glucosidase from *Sulfolobus solfataricus* [68], they could combine local flexibility of their active site (which is responsible for their activity at "low" temperatures), and high overall stability. The existence of such enzymes also suggests that thermostability is not incompatible with high activity at moderate temperatures.

Since thermophily is in general correlated with the rigidity of a protein, psychrophily, at the opposite end of the temperature scale, should be characterized by an increase of the plasticity or flexibility of appropriate parts of the molecular structure in order to compensate for the lower thermal energy provided by the low temperature habitat [37]. This plasticity would enable a good complementarity with the substrate at a low energy cost, thus explaining the high specific activity of psychrophilic enzymes. In return, this flexibility would be responsible for the weak thermal stability of psychrophilic enzymes. While the decreased stability of psychrophilic enzymes does support this hypothesis, there is, however, no direct experimental evidence of an increase in flexibility. Indeed, the flexibility of a protein, especially that related to activity and/or stability, remains a difficult parameter to determine experimentally, as the increase in flexibility can be limited only to a small but crucial part of the protein. In the context of temperature adaptation, the

notion of flexibility has to be defined as a parameter related directly to the specific activity of the enzyme. In other words, which type of flexibility is needed for the improvement of the activity? If flexibility can be described in terms of a dynamic motion that is related to a specific time-scale, it can also be considered as a concept related to the amplitude of the deformation of the protein conformation at a given temperature [72]. These uncertainties certainly explain why some thermophilic enzymes were found by hydrogen–deuterium exchange to display a similar [73] or an increased flexibility when compared to their mesophilic homologues [74,75]. The same kind of controversial result has been obtained when monitoring the rate of hydrogen–deuterium exchange of psychrophilic, mesophilic and thermophilic 3-isopropylmalate dehydrogenases [76]. While the psychrophilic and mesophilic enzymes were found to be more flexible than the thermophilic counterpart, the psychrophilic enzyme was, using this technique, more rigid than the mesophilic one. Nevertheless, in this case, the technique suffered from the disadvantage of being a measure of the accessibility of deeply buried residues and thus does not detect local flexibility, in particular that associated with the active site which is generally quite accessible [76]. Therefore, one can address the question: are amide-proton exchange rates always in agreement with the type of flexibility related to the catalytic efficiency at low temperatures? Additional measurements over a wide range of timescales are obviously needed to distinguish the flexibility of psychrophilic, mesophilic and thermophilic enzymes. In turn, NMR experiments on small psychrophilic enzymes should prove to be an attractive alternative in further investigation of the expected stability–flexibility relationship. So far, the conformational flexibility of psychrophilic enzymes was strongly supported by some dynamic fluorescence quenching experiments, using acrylamide as quencher [46–48,77]. In this technique, 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 consequently be viewed as an index of protein permeability [78]. Recently, fluorescence quenching performed on the psychrophilic α-amylase from *P. haloplanktis* (AHA) and its pig pancreatic mesophilic (PPA) and *Bacillus amyloliquefaciens* thermostable homologues (BAA) revealed that the variation of fluorescence quenching between 10 and 37 °C decreases in the order psychrophile→mesophile→thermophile (Fig. 6) [46]. Thus, the increase in protein permeability in a temperature range where the native state prevails is much larger for AHA, intermediate for PPA, and low for BAA, indicating a strong correlation between stability and conformational flexibility.
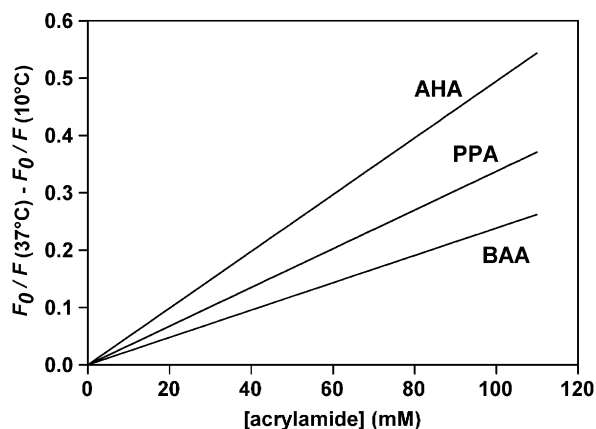

Fig. 6. Conformational flexibility of psychrophilic, mesophilic and thermophilic enzymes. Curves representing the acrylamide quenching of the tryptophan fluorescence of psychrophilic (AHA), mesophilic (PPA) and thermostable (BBA) α-amylases. In order to abolish the artifactual effect of additional tryptophan present in the mesophilic and thermophilic enzymes, the differences of the fluorescence ratios F_0/F at 37 and 10 °C were plotted as a function of acrylamide concentration (mM). Adapted from [46].

4.2. Psychrophiles and instability

A common characteristic of nearly all psychrophilic enzymes studied so far is their low stability in comparison with their mesophilic homologues. This feature has been demonstrated by the drastic shift of their apparent optimal temperature of activity, the low resistance of the protein to denaturing agents and the high propensity of the structure to unfold at moderate temperatures. The decreased stability of psychrophilic enzymes, in addition to their increased low temperature activity, suggests that there is a direct link between activity and stability i.e., maintenance of activity at low temperatures requires the weakening of intramolecular forces which results in reduced stability. On the other hand, it has been suggested that this lower stability may be due to random genetic drift as a consequence of the lack of evolutionary pressure for stable enzymes in the low temperature environment [79].

The stability of most mesophilic globular proteins is only marginal at the environmental temperature of the organism, typically 30–65 kJ mol^{-1} – the equivalent of a small number of weak intramolecular interactions [53], whereas that of psychrophilic proteins is generally one-third to one-quarter of this [14]. The lower stability of psychrophilic enzymes, brought about by a weakening of intramolecular forces contributing to the cohesion of the native protein molecule, may either arise from a general reduction in strength of intramolecular forces (global flexibility), or from weakened interactions in one or a few important regions of the structure (localized flexibility).

Decrease of the global stability has been observed in a number of psychrophilic proteins, in particular those

operating on large-size substrates. In this context, it has been shown that the psychrophilic α-amylase from *P. haloplanktis* (AHA) has reached the lowest possible stability of its native state. This has notably been demonstrated by differential scanning calorimetry (DSC), which is a powerful tool to investigate the thermal unfolding of proteins. Unlike other methods, several thermodynamic parameters related to protein stability are directly accessible. Calorimetric records, or thermograms, of heat-induced unfolding of psychrophilic, mesophilic and heat-stable α-amylases are shown in Fig. 7 [14], from which a number of observations can be drawn. First, the unfolding of the psychrophilic enzyme occurs at a lower temperature, as indicated by the T_m values (peak of the curve), corresponding to the temperature of half-denaturation. Second, protein unfolding is an endothermic process; the unfolding enthalpy (ΔH_{cal}) (area under the curves) corresponds to the heat absorbed during the unfolding process. This parameter reflects the enthalpy of disruption of bonds involved in maintaining the compact structure and is markedly lower for the psychrophilic enzyme. In addition, there is a clear trend for increasing ΔH_{cal} values in the order psychrophile < mesophile < thermophile. Finally, the psychrophilic α-amylase unfolds reversibly and without any stable intermediate according to a highly cooperative process. In contrast, more stable α-amylases unfold irreversibly and display more than one denaturation peak indicative of distinct thermodynamic units or domains with different stability. The weak hydrophobicity of the core clusters in the cold-adapted enzyme [80] and the low melting temperature, at which hydrophobic interactions are restrained, certainly account for this re-

versible character because, unlike mesophilic α-amylases, aggregation does not occur.

From this study, it was thus inferred that the psychrophilic enzyme possesses a fragile molecular edifice that is uniformly unstable and stabilized by fewer weak interactions than homologous mesophilic or thermophilic proteins. The contribution of individual weak interactions in the behaviour of the psychrophilic α-amylase from *P. haloplanktis* was analysed by site-directed mutagenesis [49]. Fourteen mutants of this enzyme were constructed, each of them bearing an engineered residue re-forming a weak interaction found in mesophilic α-amylases but absent in the cold-active α-amylase. It was found that single amino acid side chain substitutions can significantly modify not only the melting point T_m, the calorimetric enthalpy ΔH_{cal}, the cooperativity and reversibility of unfolding, but also the thermal inactivation rate constant k_i and the kinetic parameters k_{cat} and K_m (Fig. 4). From the analysis of the data, it is clear that the adaptation to cold of the psychrophilic α-amylase consists of a weakening of intramolecular interactions leading to an overall decrease of the thermostability of the protein. This in turn provides the appropriate plasticity around the catalytic residues necessary to adapt, more or less successfully, the catalytic efficiency of the enzyme to the low temperature of the environment. It is also clear that in the case of the α-amylase, the limit of stability of the protein has been reached, as demonstrated by the inability of producing destabilized mutants by site-directed mutagenesis, as well as by the disappearance of ion-mediated extra-stability and the simultaneous unfolding of all structural elements [14]. The lowest possible stability of the enzyme therefore prevents any further decrease in stability and thus any further increase in activity via the flexibility strategy [14]. In such enzyme, even in this case, the specific activity of the cold-adapted enzyme at the environmental temperature (0 °C) is still much lower than that of the mesophilic counterpart at 37 °C [81], illustrating the limit of the adaptation.

Nevertheless, the reduced stability related to the low stability of the enzyme may not necessarily arise from a general reduction in strength of intramolecular forces, but from weakened interactions in one or a few important regions of the structure (localized flexibility). Some recent works have corroborated this statement. For instance, in the case of citrate synthase [82], a calculation of crystallographic temperature (*B*) factors, which, in certain conditions, reflects the flexibility or disorder of the crystal structure in a given region, revealed an unexpected higher flexibility for the thermophilic enzyme from *Pyrococcus furiosus* when considering the overall protein structure. However, in the psychrophilic homologue from *Arthrobacter* strain DS2-3R, the small domain showed a higher degree of flexibility than that observed in the large domain. This inequality could
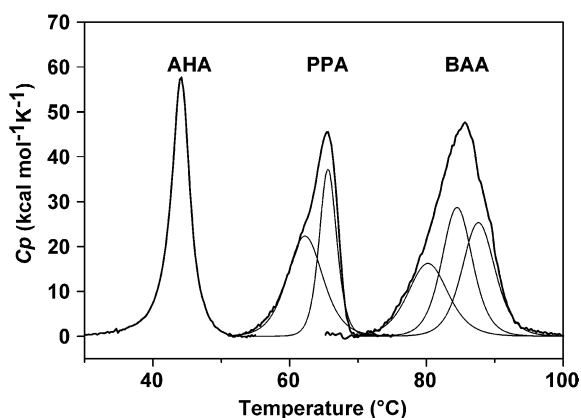

Fig. 7. Thermal unfolding of three α-amylases recorded by differential scanning calorimetry (DSC). When compared with the psychrophilic AHA (from *P. haloplanktis*), the heat-stable enzymes PPA (from pig pancreas) and BAA (from *B. amyloliquefaciens*) are characterized by higher T_m (top of the transition) and ΔH_{cal} (area under the transition) values, by a flattening of the transition and by the occurrence of calorimetric domains indicated by deconvolutions in thin lines. Adapted from [49].

promote activity at low temperatures, since a precise positioning of the small domain following substrate binding is necessary for efficient catalysis. As mentioned by Fields and Somero [45], notothenioid fish A4-lactate dehydrogenase (A4-LDH) has adapted to cold by increasing flexibility of small areas of the molecule that affect the mobility of adjacent active-site structures. The increased flexibility may reduce the energy barrier of the rate-governing shifts in conformation and thereby, increase k_{cat}. In addition, the same authors pointed out that global stability and localized flexibility of enzymes may have evolved independently [83]. Indeed, extrinsic stabilisation by compatible solutes of lactate dehydrogenase from warm- and cold-adapted fish indicates that the stability of the enzyme molecules is affected to the same extent whereas the kinetic parameters of cold-active lactate dehydrogenases is more affected by the co-solvents. This effect has been attributed to the flexibility of the active site in cold A4-LDH that can be more compacted by the preferential exclusion of solutes. Besides these examples, a direct evidence of differences in stability between some domains and the whole structure has been provided by DSC studies of multi-domain proteins such as phospsphoglycerate kinase (PGK) from *Pseudomonas* sp. TACII18 [50] and psychrophilic chitobiase from *Arthrobacter* sp. TAD20 [52], as shown in Fig. 8. Both enzymes are indeed composed of a heat labile and a heat stable domain, the latter domain displaying a higher stability than that of the mesophilic counterpart. Regarding the cold-adapted PGK, Bentahir and his colleagues [50] have proposed that the PGK heat labile domain acts as a destabilising domain providing the required flexibility around the active site and favouring the reaction rate by reducing the energy cost of induced-fit mechanisms. On the other hand, the heat-stable domain could improve the binding of substrate (as indicated by low K_m values, see Section 3) as a result of its rigidity. In the case of the psychrophilic chitobiase, the heat-labile domain corresponds to the catalytic domain whereas the heat-stable domain (which plays no part in the chitobiase activity) corresponds to the binding domain, as demonstrated by saccharide-binding experiments [52].

Thus cold-adapted proteins have evolved, either by displaying a reduced stability of all calorimetric units giving rise to native states of the lowest possible stability [14], or by being constituted of different elements, some controlling protein stability and affinity for the substrate and others conferring the required flexibility for efficient catalysis at the habitat temperature.

Some significant advances in the understanding of the activity–stability relationship have also been drawn from the analysis of heat inactivation of activity and irreversible unfolding of some psychrophilic enzymes such as a psychrophilic α-amylase AHA [46], DNA ligase *Phlig* [47], and xylanase pXyl [48] from *P. halo-*
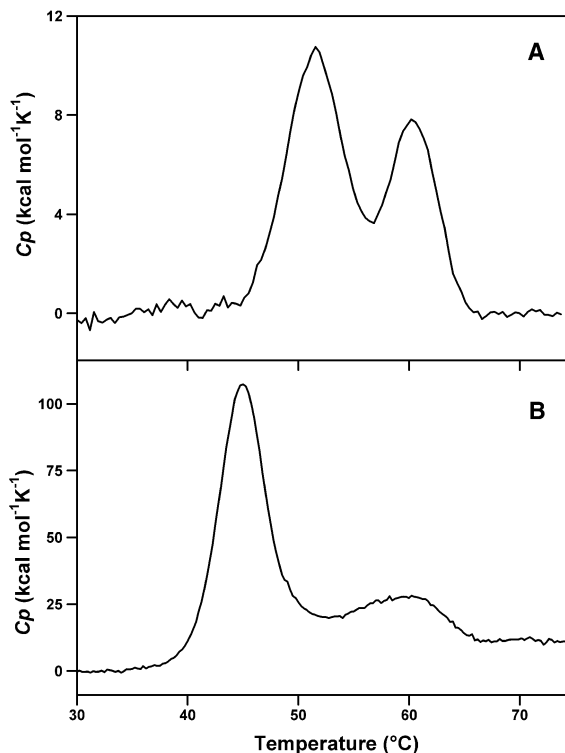

Fig. 8. Thermal unfolding of psychrophilic enzymes with local flexibility. In phosphoglycerate kinase (A) the active site is formed at the interface of both heat-labile and heat-stable domains by a hinge-bending mechanism. In chitobiase (B), the active site is embedded in the heat-labile domain whereas the stability of the non-catalytic domain is unaffected. Adapted from [50,52].

planktis, compared to some of their mesophilic and thermophilic counterparts. Table 2 displays the corresponding activation parameters, from which it can be seen that both heat inactivation of activity and irreversible unfolding of the structures display the same trends. Indeed, the increase in free energies of activation from psychrophilic enzymes to the heat-stable counterparts reflects that the same denaturation rate constant k is reached at increasing temperatures. The small differences in ΔG^* values however arise from large differences in the enthalpic and entropic contributions. Indeed, the lowest ΔG^* values for the psychrophilic enzymes corresponds to the largest ΔH^* and $T\Delta S^*$ contributions, and conversely for the heat-stable homologues. The decrease of ΔH^* as enzyme stability increases mainly reflects the decrease in cooperativity of inactivation and of unfolding: indeed, the heat-labile enzymes denature over a shorter temperature range, leading to a steep slope of Arrhenius plots and subsequently to high activation energy E_a and ΔH^*. Such high cooperativity probably originates from the lower number of interactions required to disrupt the active conformation. In this formalism, $\Delta S^* > 0$ suggests that the randomness of the activated transition state increases before irreversible inactivation or unfolding. Accordingly, the transition

Table 2
Thermodynamic parameters for the irreversible heat inactivation of activity and for irreversible unfolding

	Inactivation				Unfolding			
	ΔG^* (kcal mol^{-1})	ΔH^* (kcal mol^{-1})	$T\Delta S^*$ (kcal mol^{-1})	ΔS^* (kcal mol^{-1})	ΔG^* (kcal mol^{-1})	ΔH^* (kcal mol^{-1})	$T\Delta S^*$ (kcal mol^{-1})	ΔS^* (kcal mol^{-1})
α-Amylase[a]								
AHA	20.5	172.3	151.8	479	20.2	109.7	89.2	286
PPA	21.5	153.0	131.5	395	21.5	84.7	63.2	190
BAA	22.9	58.6	35.7	101	22.9	74.2	51.3	145
Xylanase[b]								
pXyl	19.6	109.4	89.8	265.6	19.6	98.9	79.3	234.5
CelA	27.5	72.9	45.4	134.2	27	82.4	55.4	163.9

[a] As the three α-amylases (psychrophilic AHA, mesophilic PPA and thermophilic BAA) are denatured in different temperature ranges, activation data are reported for an identical rate constant, $k = 0.05$ s^{-1}. Adapted from [46].

[b] Parameters recorded at 65 °C, corresponding to a $k_{inactivation} = 1.4$ s^{-1} and 1.2×10^{-5} s^{-1} for psychrophilic pXyl and thermophilic CelA, respectively, and a $k_{unfolding} = 1.7$ s^{-1} and 1.9×10^{-5} s^{-1} for pXyl and CelA, respectively. Adapted from [48].

state of the psychrophilic enzymes is reached through a larger entropy variation than that of heat-stable homologues, reflecting an increased disorder (of the active site or of the structure), which is the main driving force of heat denaturation. In addition, the thermophilic enzymes seem to resist denaturation before the concomitant irreversible loss of activity and conformation (low ΔS^*). A thermophilic enzyme can then be regarded as an intrinsically stable protein that counteracts heat denaturation by a weak cooperativity of unfolding and inactivation. The entropy loss associated with hydration of non-polar groups upon denaturation can also contribute to the differences in the entropic contributions reported in Table 2.

4.3. A working hypothesis

Recently, D'Amico and colleagues [46] have proposed to integrate the biochemical and biophysical data available on enzymes adapted to extreme temperatures in a model based on the "new view" using the folding funnel model [84–87] to describe the folding–unfolding reactions. The energy landscape of psychrophilic and thermophilic enzymes is described in Fig. 9. The height of the funnel, i.e., the free energy of folding, corresponds to the conformational stability and the upper edge of the funnel is occupied by the unfolded state in random coil conformation. It should be noted that psychrophilic enzymes tend to have lower proline content than mesophilic and thermophilic enzymes, a lower number of disulfide bonds and a higher occurrence of glycine clusters (see Section 5). Accordingly, the edge of the funnel for the psychrophilic protein is slightly larger (broader distribution of the unfolded state). When the folding of the polypeptide occurs, 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
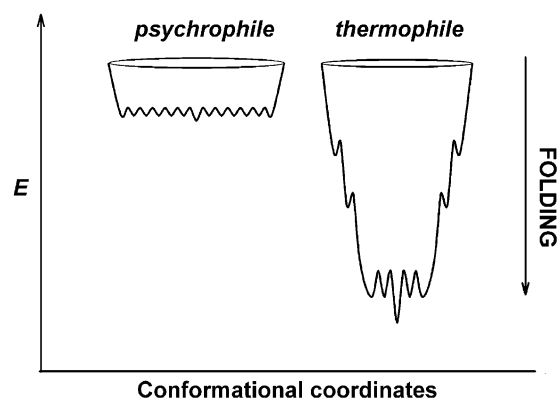

Fig. 9. Proposed model of folding funnels for psychrophilic and thermophilic enzymes. In these schematic energy landscapes, the conformational stability (E) is represented as a function of the conformational diversity. Adapted from [46].

funnel slopes and for the reduced cooperativity of the folding-unfolding reaction. By contrast, the structural elements of psychrophilic proteins generally unfold cooperatively without intermediates, as a result of fewer stabilising interactions and stability domains (see Section 4.2) 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 extremozymes. 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 [88,89] 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 height [88,89]. In this context, the activity–stability relationships in these extremozymes 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 [88–90]. In the case of the rugged bottom of psychrophilic enzymes, this equilibrium shift only requires a modest free energy change (i.e., low energy barriers) and a low enthalpy change for interconversion of the different conformations, but is nonetheless accompanied by a large entropy change resulting from the shift in conformation of the numerous conformational isomers existing in the wide conformer ensemble, whereas the converse picture holds for thermophilic enzymes.

4.4. Stability and low temperature activity

The discussion of the previous section indicates that all psychrophilic enzymes have the common trait of a low conformational stability that could be required to secure an appropriate structural flexibility for low temperature activity. Some authors consider that the instability of psychrophilic enzymes is due to random genetic drift. However, while nature always produces cold-active enzymes with decreased stability, indicating an inverse relationship between stability and activity, a number of studies have indicated that these are not strictly related.

Recently, random mutagenesis techniques using essentially error-prone polymerase chain (PCR) amplification, DNA shuffling or in a few cases, mutating strains [91,92] have been used to study the thermal adaptation of different enzymes, to understand the relationship, if there is any, between stability, activity and flexibility. Basically, random mutations are introduced in a gene and the resulting library is screened for the acquisition or improvement of a specific property such as thermostability, or improved activity. This approach detects mutations responsible for immediate and drastic adaptation in response to a strong selection imposed in the laboratory and based on a physico-chemical property. In contrast, natural evolution is a very slow process driven by complex environmental and metabolic constraints, and enabling the viability or the performance of a living organism in a specific environment.

To our knowledge, only one psychrophilic enzyme, subtilisin S41 [93], has been submitted to directed evolution [79,94]. Following several generations of mutagenesis/recombination and screening, a seven mutant variant with 13 mutations was found to exhibit an increased thermostability without sacrificing low-temperature activity, at least towards a synthetic substrate. Even if this seems to be in contradiction with the current hypothesis of thermal adaptation, the mutated enzyme, however, is also characterized by a decrease in the K_m value, an increased affinity for calcium, additional salt bridges and an apparently decreased conformational flexibility. All these results fit perfectly with the model of activity–flexibility–stability relationships discussed above (see Sections 4.1 and 4.2).

On the other hand, random mutagenesis experiments have been carried out on mesophilic and thermophilic enzymes to either improve the catalytic activity at low temperatures, or, in the same context, to obtain a higher thermostability. When random mutants were only screened for high activity at low temperatures during directed evolution [92,95–99], the selected enzymes invariably displayed a decreased stability as well as a higher (supposed) flexibility, i.e., the canonical properties of psychrophilic enzymes. By contrast, when the selection pressure was thermal stability [67,100–106], mutants with higher stability and unchanged or even enhanced catalytic activity were found in contradiction with the hypothesis that a high stability induces a low catalytic efficiency. However, a number of important points must be taken into account when interpreting such results. In most cases, synthetic substrates were used and may give results differing to those of natural substrates, in particular when those are macromolecular substrates. For some enzymes [101,103], early generations of stabilized mutants exhibited a decreased activity, as observed in nature, while several generations of mutants were required to obtain both a high thermostability and high activity. It is also important to note that an increased thermostability is not necessarily indicative of an increased thermoactivity (as determined from the temperature optimum for activity). Indeed, mutational studies of a cold-active citrate synthase from *Arthrobacter* strain DS2-3R produced a variant with an increased thermostability and a decreased thermoactivity [107]. This suggests that an increased flexibility and hence a decreased stability of the active site could be masked by stabilising mutations elsewhere in the molecule when mutants are selected on the basis of their thermostability. This underlines the importance of analysing all parameters when engineering proteins.

If it appears possible, at least in the laboratory, to uncouple activity and stability, the fact is, however, that enzymes displaying a high stability together with a high flexibility and activity do not exist in nature, except for thermophilic enzymes that catalyse the conversion of labile metabolic intermediates [69]. In addition, if it has been proposed that the low stability of cold-adapted enzymes results from a genetic drift originating from the lack of selective pressure for stable proteins [108], the microcalorimetric studies described in Section 4.2 have indicated that the active site is always in the less stable region of the protein, other domains remaining even more stable than their mesophilic homologues, leading to the question: "Why would the genetic drift only affect one part of the protein?". Moreover, in psychrophilic organisms a selective pressure towards a low stability of proteins does exist; it is exerted by the low environment

temperature which has to select molecular structures flexible enough to secure efficient catalysis. This is achieved in proteins through an unfavourable stabilisation enthalpy driven by hydration of polar and non-polar groups at low temperatures preventing the formation of stabilising weak bonds and securing an appropriate plasticity of the molecular edifice. This cannot be achieved in mesophilic and thermophilic counterparts which are much more resistant to cold denaturation. Therefore, improvement of activity at low temperatures associated with a loss of stability appears to be the most frequent and accessible strategy at the level of psychrophilic proteins. In consequence, the low stability of cold-adapted enzymes may be the simplest way to acquire activity in the absence of selection for stable proteins but the occurrence of heat-stable psychrophilic enzymes should not necessarily be ruled out.

5. Structural adaptations of enzymes

The thermostability of thermophilic enzymes has been extensively investigated and several possible determinants of this stability have been proposed (for review, see [57]). In contrast, identifying the structural determinants of adaptation in cold-adapted enzymes has proven to be difficult due to the low number of available sequences, and more importantly, of three dimensional structures. A few crystallographic structures of cold-adapted enzymes have been elucidated: five from bacteria (α-amylase [109] and xylanase [112] from *P. haloplanktis*, citrate synthase from *Arthrobacter* strain DS2-3R [82], malate dehydrogenase from *Aquaspirillium arcticum* [110] and alkaline Ca^{2+}–Zn^{2+} protease from *Pseudomonas* sp. TACII18 [111]), one from the Arctic shrimp *Pandalus borealis* (alkaline phophatase [113]), and four from cold-adapted fish (two anionic trypsin from the North Atlantic salmon *Salmo salar* and from the chum salmon *Oncorhynchus keta* [114,115], elastase from North Atlantic salmon [116] and pepsin from the Atlantic cod *Gadus morhua* [117]). Other structures should become available soon since crystals of two other psychrophilic enzymes have been obtained [118,119]. With the help of homology-modelled structures, these studies have revealed that only subtle modifications of the enzyme conformation account for the low stability.

The analysis of 3D structures often reveals a better accessibility of the catalytic cavity to ligands. This aspect has notably been described for two salmon trypsin from *S. salar* and *O. keta* [114,115], α-amylase from *P. haloplanktis* (D'Amico S, personal communication), citrate synthase from *Arthrobacter* strain DS2-3R [82] and alkaline Ca^{2+}–Zn^{2+} protease from *Pseudomonas* sp. TACII18 [111]. The larger opening of the catalytic cleft is achieved in various ways, including small deletions in loops bordering the active site or by distinct confor-

mations of these loops (Fig. 10), and by replacement of bulky side chains by smaller groups at the entrance. Such strategy is likely to reduce the energy required for substrate accommodation and/or product release, steps that are accompanied by conformational changes especially in the case of macromolecular substrates. The typical configuration of the active site suggests two reflections. First, these enzymes could display a broader specificity, due to the fact that diverse substrates having slightly distinct conformations or sizes can fit and bind to the active site. This property was indeed notably reported for cold-active subtilisin from *Bacillus* sp. TA41 [93] and alcohol dehydrogenase from *Moraxella* sp. TAE123 [120]. As a second consequence, substrate(s) should bind less firmly in the binding site, giving rise to higher K_m values as reported for cold active enzymes devoid of adaptive mutations within the catalytic centre (see also Section 3). Moreover, although the main residues implicated in catalysis are conserved, differences in
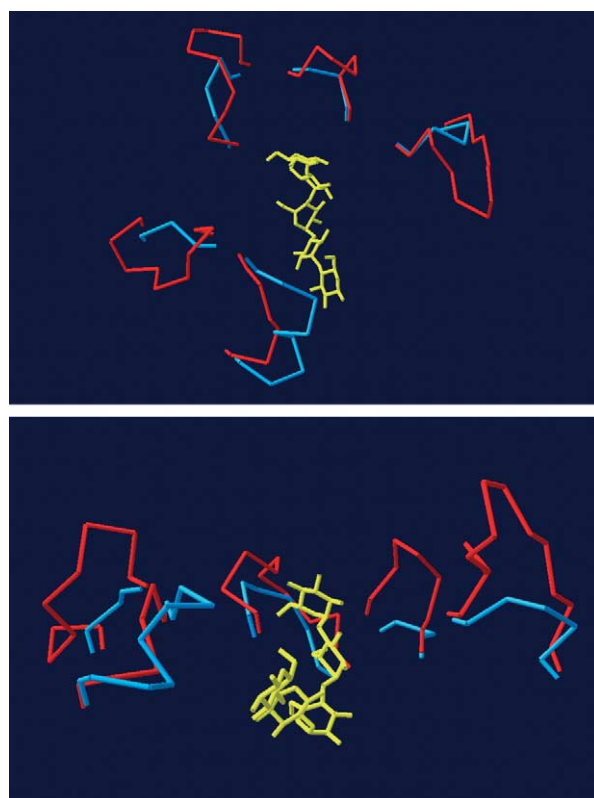

Fig. 10. Active site accessibility. Upper panel: superimposition of the variable loops bordering the active site of the psychrophilic α-amylase from *P. haloplanktis* (backbone in blue) and of pig pancreatic α-amylase (backbone in red). The carbohydrate inhibitor acarbose bound to the active site is also shown (centre of the figure, in yellow). These variable loops are markedly shorter in the cold-active enzyme. Lower panel: tangential view of the molecular surfaces facing the external medium (upper side). The loops around the catalytic cleft of the cold-active enzyme are less protruding and favour active site accessibility. Picture generated by Swiss-PDBViewer [125] using data from [109,126].

electrostatic potentials in and around the active site of psychrophilic enzymes have also been noted and could be responsible for a better substrate binding, by facilitating the interaction of oppositely charged ligands with the surface of the enzyme [82,110,113–115].

As previously mentioned, cold-adapted enzymes are characterized by a decreased conformational stability and increased flexibility of the molecular structure. An inventory of the structural factors possibly involved in cold adaptation has been obtained from comparative studies between psychrophilic enzymes and their heat-stable homologues based on homology models and 3D structures. Compared to mesophilic counterparts, the observed structural alterations include, among others, an increased number and clustering of glycine residues (providing local mobility); a decrease of proline residues in loops (providing enhanced chain flexibility between secondary structures), a reduction in arginine residues, capable of forming multiple salt bridges and hydrogen bonds, as well as a lower number of ion pairs, aromatic interactions or hydrogen bonds, and a weakening of charge–dipole interactions in α-helices. They also involve in some cases a decrease of ion binding strength, of the density of charged surface residues and overall hydrophobicity, and an increased exposure of hydrophobic residues to solvent (entropy-driven destabilising factor). All these factors have been extensively reviewed by Feller et al. [80,121] and Gerday et al. [122]. Obviously, each protein uses some or a few of the above structural adjustments in order to enable catalysis to proceed at the required rate at the environmental temperature, contributing to improve the local or global resilience of the protein edifice. Interestingly, the same structural factors have been implicated in the stability of thermophilic proteins [53,57,123] suggesting that there is a continuum in the strategy of protein adaptation to temperature.

6. Concluding remarks

Over recent years, increased attention has been focused on cold-adapted microorganisms and their catalysts. The future in this field is fascinating and boundless. It has indeed emerged that psychrophilic enzymes represent an extremely powerful tool in both protein folding investigations and for biotechnological purposes.

Naturally evolved psychrophilic enzymes require an improved flexibility of the structural components involved in the catalytic cycle, whereas other protein regions, if not implicated in catalysis, may or may not keep a high rigidity. The price to pay for such increased resilience is the low stability of the native enzyme structure, establishing a direct correlation between the activity, the flexibility and the stability of the enzyme molecule. However, one has to keep in mind that our working hypotheses derive from an oversimplified model that attempts to explain the effect of temperature and of its selective pressure on the conformation of a protein. It is clear that throughout its history, an enzyme may encounter numerous selective pressures that are completely unrelated to temperature adaptation. It is not therefore surprising to note that mutated enzymes obtained by directed evolution, using as the selective parameter a high specific activity at low temperature, display structural adaptations different from those that are observed in a natural environment.

Structure modelling and recent crystallographic data have contributed to better define the structural parameters that could be involved in the increased flexibility of cold-adapted enzymes. It was demonstrated that each psychrophilic enzyme adopts its own adaptive strategy and that there is a continuum in the strategy of protein adaptation to temperature. Additional 3D crystal structures, site-directed and random mutagenesis experiments, as well as biophysical studies should now be undertaken to further investigate the stability–flexibility–activity relationship.

Finally, further advances in the understanding of the mechanisms of cold adaptation at the cellular and molecular levels should emerge from the fulfillment of genome sequencing projects undertaken on psychrophiles.

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