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# Review

# Molecular adaptations to cold in psychrophilic enzymes

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**Abstract.** Psychrophiles or cold-loving organisms successfully colonize cold environments of the Earth's biosphere. To cope with the reduction of chemical reaction rates induced by low temperatures, these organisms synthesize enzymes characterized by a high catalytic activity at low temperatures associated, however, with low thermal stability. Thanks to recent advances provided by X-ray crystallography, protein engineering and biophysical studies, we are beginning to understand the molecular adaptations responsible for these properties which appear to be relatively diverse. The emerging picture suggests that psychrophilic enzymes utilize an improved flexibility of the structures involved in the catalytic cycle, whereas other protein regions if not implicated in catalysis may or may not be subjected to genetic drift.

**Key words.** Psychrophile; extremophile; enzyme kinetics; crystal structure; folding; mutagenesis; biophysics.

## The current context

Since our last review in this journal [1], significant advances have been made in the study of proteins and enzymes from cold-adapted organisms, the so-called psychrophiles, demonstrating an intense research activity in the field. Such interest is certainly motivated by the fact that life at extremely low temperatures is no longer regarded as a biological curiosity. Indeed, the cold-active and heat-labile psychrophilic enzymes possess interesting biotechnological potential [2–6] and have been shown to be useful models for protein folding studies [7] and for the analysis of the adaptability of the structure and function of enzymes [8]. In addition, a low-temperature origin of life is still a plausible hypothesis [9]. More exotic reasons for this interest should also be mentioned. For example, living remnants of ancestral microorganisms, such as those expected to survive below 3500 m of ice in the Antarctic subglacial Lake Vostok [10], should display psychrophilic characters, as should the possible living forms on the planet Mars [11]. Psychrophiles were first referred to as cold-adapted bacteria [12]. However, if a psychrophile is defined as an organism living permanently at temperatures close to the freezing point of water in thermal equilibrium with the medium, such a definition includes de facto a large range of species: yeast [13–16], algae [17–19], marine invertebrates and insects or polar fish, which are the largest psychrophiles [20, 21]. These examples underline that psychrophiles are numerous, taxonomically diverse and have a widespread distribution. One should not forget that the major part of Earth is exposed to low temperatures if one considers the vast extent of permanently cold environments such as the Antarctic, Arctic and mountain regions as well as the deep-sea waters covering three-quarters of the planet surface.

To define the properties of a cold-active enzyme, the effect of temperature on the activity of psychrophilic and mesophilic enzymes is illustrated in figure 1. This graph reveals three basic features which will form the body of the next sections.

1) To compensate for the slow reaction rates at low temperatures, psychrophiles synthesize enzymes with an

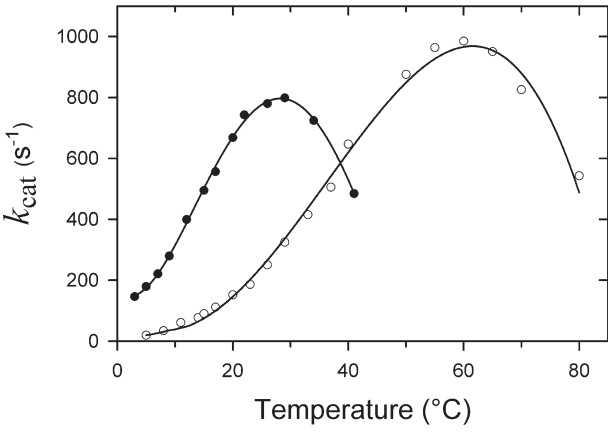


Figure 1. Effect of temperature on the activity of psychrophilic (●) and mesophilic (○) enzymes. The thermodependence of the activity (of α-amylases in this case) illustrates the three main adaptive traits of psychrophilic enzymes.

up to tenfold higher specific activity in this temperature range. This is in fact the main physiological adaptation to cold at the enzyme level.

2) The temperature for apparent maximal activity for cold-active enzymes is shifted towards low temperatures, reflecting the weak stability of these proteins which are prone to unfolding and inactivation at moderate temperatures.
3) Finally, the adaptation to cold is frequently not perfect. Figure 1 illustrates that the specific activity of the psychrophilic enzyme at low temperatures, although very high, remains generally lower than that of the mesophilic enzymes at 37 °C.

Some reviews have been published recently by our group [22–26] but other excellent reviews should also be consulted for a complete coverage of this topic [4, 11, 27–33].

## Recombinant psychrophilic enzymes

A major achievement in the study of psychrophilic enzymes was the availability of recombinant enzymes that have been produced in sufficient amount to determine X-ray structures and for their analysis by sophisticated biophysical methods. This has also opened the way for mutagenesis studies. The first cold-active enzymes were cloned in *Escherichia coli* and selected by the activity of the gene product expressed by the recombinant colonies [34–36]. Later, the high level of sequence identity with mesophilic homologues demonstrated by psychrophilic enzymes prompted the isolation by the polymerase chain reaction of other genes that cannot be detected in expression libraries [37, 38]. Accordingly, the study of rare enzymes was also made possible, such as the elongation factors EF-2 [39–41] and Tu [42] or a DNA ligase [43] which only occurs as 200 molecules per cell, therefore precluding its direct purification in reasonable amounts.

The availability of these genes also allowed an exploration of the overexpression of these psychrophilic enzymes in a mesophilic host. Various *E. coli* strains were used for this purpose, whereas expression in *Bacillus* species [44, 45] or yeast was rarely addressed. The first concern of workers in the field was to establish if an enzyme expressed near 0 °C by the wild-type strain maintains its properties when expressed and folded at higher temperatures that are lethal for the parent microorganisms. Indeed, when expressed at a sufficiently low temperature (15–18 °C), a recombinant psychrophilic enzyme is undistinguishable from its wild-type parent molecule as regards kinetic parameters, folding properties [46] and three-dimensional (3D) structure [47]. By contrast, at 37 °C, recombinant psychrophilic enzymes are frequently not expressed or are quickly inactivated. In addition, when expressed at this temperature, the properties of the recombinant psychrophilic enzyme seem to be altered as a result of misfolding or the occurrence of an inactivated population [46]. Affinity tags added in fusion to a recombinant psychrophilic enzyme for purification purposes are also a concern. A His-tagged phosphoglycerate kinase (PGK) has intermediate kinetic properties between the native psychrophilic PGK and yeast PGK, a difference possibly arising from the stabilization center introduced by the affinity tag [48]. As a practical conclusion, it is therefore advisable to express recombinant psychrophilic enzymes at the lowest possible temperature, to cleave and remove affinity tags, to perform an affinity chromatography step (removing the possible inactive fraction) and to perform active-site titration to calculate the kinetic parameters.

Recently, a recombinant psychrophilic enzyme has been expressed in psychrophilic bacteria [49]. For this purpose, a broad host range expression vector was constructed using a cold-adapted replication element derived from a multicopy cryptic plasmid of the Antarctic Gram-negative bacterium *Pseudoalteromonas haloplanktis*. This study demonstrated that production of recombinant proteins can be performed at low temperatures, revealing a new biotechnological potential for psychrophilic strains.

## Structural factors related to activity at low temperature

To date, four crystal structures of bacterial psychrophilic enzymes have been solved by X-ray crystallography [50–53] as has the structure of three serine proteases from cold-adapted fish [54–56]. Other structures should become available soon as crystals of three other cold-ac-

tive enzymes have been obtained [57–59]. Unfortunately, only four of these X-ray structures have been compared with mesophilic or thermophilic homologues and analyzed in detail with regards to the structural parameters related to cold activity. These include the bacterial $\alpha$-amylase [47], citrate synthase [53], malate dehydrogenase [52] and the fish trypsin [54]. Nevertheless, this limited set of data has already provided valuable insights into the molecular basis of cold adaptation [30, 60–62]. As inferred from their amino acid sequences and homology models, psychrophilic enzymes do not display unusual or exotic conformations. On the contrary, the overall fold is very close to that of their mesophilic homologues. For example, when the 3D structures of mammalian $\alpha$-amylases are superimposed on the psychrophilic $\alpha$-amylase, the root mean square deviation is only about 0.5 Å, reflecting a high similarity of the tertiary structures [47].

As far as the active site of psychrophilic enzymes is concerned, 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 in cold-active enzymes. This aspect is not really surprising as the specific reaction mechanism of enzymes is not prone to drastic variation. However, worth mentioning is that all residues forming the active site can be conserved in a psychrophilic enzyme. This was demonstrated by the X-ray structure solved at high resolution of both the cold-active $\alpha$-amylase and of its closest structural homologue from pig in complex with acarbose, a pseudosaccharide inhibitor mimicking the transition state intermediate [63, 64]. Comparison of residues with direct or water-mediated H bonds to the inhibitor revealed that all 24 residues involved are strictly conserved in the cold-active $\alpha$-amylase. This outstanding example of active-site identity (in terms of side chain identity) demonstrates that the specific properties of psychrophilic enzymes can be reached without any amino acid substitution in the reaction center. As a consequence, changes occurring elsewhere in the molecule are responsible for the optimization of the catalytic parameters of this enzyme, as demonstrated by site-directed mutagenesis [65], probably by modifying the dynamic and conformational properties of the conserved residues.

Another important feature of the active site refers to its accessibility, because in many cases, the catalytic cavity appears to be larger and more accessible to ligands. This larger opening of the catalytic cleft is achieved in various ways, including small deletions in loops bordering the active site or by distinct conformation of these loops, and by replacement of bulky side chains for smaller groups at the entrance. This aspect has been well illustrated for the cold-active citrate synthase [53]. The reasons for such a better accessibility are still not clearly understood but cold-active enzymes are thought to accommodate substrates at 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. This larger size of the active site and the better accessibility have at least two consequences. First, non-specific enzymes accepting various substrates should have a broader specificity, because substrates with slightly distinct conformations or sizes can fit and bind to the site. For example, the observed differences in substrate specificity between Atlantic salmon and mammalian elastases have been interpreted in terms of a somewhat wider and deeper binding pocket for the cold-adapted elastase [28]. The broad specificity of a psychrophilic alcohol dehydrogenase, oxidizing large bulky alcohols, was also assigned to a highly flexible active site [66]. As a second consequence, substrates should bind less firmly in the binding site, if no point mutations have occurred, giving rise to higher $K_m$ values. This is certainly one of the structural explanations for the observation that numerous cold-active enzymes, which are strongly homologous to mesophilic counterparts, have lower binding constants.

Finally, differences in electrostatic potentials in and around the active site of psychrophilic enzymes appear to be a crucial parameter for activity at low temperatures. Interestingly, the cold-active citrate synthase [53], malate dehydrogenase [52] and trypsin [28, 67, 68] are characterized by marked differences in electrostatic potentials near the active-site region when compared with mesophilic or thermophilic counterparts. In the case of malate dehydrogenase for example, the increased positive potential at and around the oxaloacetate-binding site and the significantly decreased negative surface potential at the NADH-binding region may facilitate the interaction of the oppositely charged ligands with the surface of the enzyme. 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. Future work in the field will certainly refine the structural and functional relationships between electrostatic potentials and catalytic efficiency in psychrophilic enzymes.

## Structural factors linked to stability

The numerous homology-based models and especially the recently solved X-ray structures of psychrophilic enzymes have deciphered the molecular origin of the weak stability characterizing these proteins. All structural factors currently known to stabilize the protein molecule can be attenuated in strength and number in the structure of cold-active enzymes [28, 30, 62]. This involves the clus-

tering of glycine residues (providing local mobility), the disappearance of proline residues in loops (providing enhanced chain flexibility between secondary structures), a reduction in arginine residues which are capable of forming multiple salt bridges and H bonds, as well as a lower number of ion pairs, aromatic interactions or H bonds compared to mesophilic enzymes. The size and relative hydrophobicity of non-polar residue clusters forming the protein core are frequently smaller, lowering the compactness of the protein interior. The N and C caps of $\alpha$-helices are also altered (weakening the charge-dipole interaction) and loose or relaxed protein extremities appear to be preferential sites for unzipping. The binding of stabilizing ions, such as calcium, can be extremely weak, 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 (insertion) or the weakening of subunit interactions (deletion).

Calculation of the solvent-accessible area showed that some psychrophilic enzymes expose a higher proportion of non-polar residues to the surrounding medium [47, 53]. This is an entropy-driven destabilizing factor caused by the reorganization of water molecules around exposed hydrophobic side chains. Calculations of the electrostatic potential revealed in some cases an excess of negative charges at the surface of the molecule and, indeed, the pI of cold-active enzymes is frequently more acidic 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 [69]. Besides the balance of charges, the number of salt bridges covering the protein surface is also reduced. 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, thermophiles and hyperthermophiles, the latter showing arginine-mediated multiple ion pairs and interconnected salt bridge networks [70, 71]. Such an altered pattern of electrostatic interactions at the molecular surface is thought to improve the resilience [72] or the 'breathing' of the external shell of cold-active enzymes.

Of course, all these factors are not found in every cold-active enzyme: each enzyme adopts its own strategy by using one or a combination of these altered structural factors to improve the local or global mobility of the protein edifice. An example is given in table 1 which provides a summary of the structural factors possibly involved in the low stability of a psychrophilic $\alpha$-amylase. A recent comparative structural analysis of psychrophilic, mesophilic and thermophilic enzymes reports that each protein family displays a different structural strategy 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 decrease with decreasing optimal temperatures [62].

Table 1. Structural parameters potentially involved in the stability of $\alpha$-amylase conformation as deduced from the X-ray structures [47, 63, 74]

| $\alpha$-amylase | Psychrophile | Mesophile |
|---|---|---|
| Proline content | 13 | 21 |
| Salt bridges | 18 | 26 |
| Arginine content | 13 | 28 |
| Arg-mediated salt bridges | 8 | 17 |
| Arg-mediated amino-aromatic interactions | 2 | 13 |
| Arg-mediated H bonds | 20 | 42 |
| Disulfide bonds | 4 | 5 |
| Core cluster hydrophobicity ($\Sigma H_i$) | 47 | 103 |
| Non-constrained residues at C terminus | 6 | 0 |
| Aromatic-aromatic interactions | 19 | 20 |
| Amino-aromatic interactions | 2 | 13 |
| Oxygen-aromatic interactions | 24 | 19 |
| Sulfur-aromatic interactions | 2 | 5 |
| $\alpha$ helix dipole – charges in N$\alpha$ | 2 | 4 |
| + charges in C$\alpha$ | 3 | 6 |
| Calcium-binding constant ($M^{-1}$) | $5.0 \times 10^7$ | $2.0 \times 10^{11}$ |
| Chloride-binding constant ($M^{-1}$) | $1.6 \times 10^2$ | $3.5 \times 10^3$ |

Data for the psychrophilic $\alpha$-amylase differ slightly from Aghajari et al. [47] as only one crystal form is considered and bond length limits are different.

## Kinetic optimization of cold-active enzymes

As mentioned, improving the turnover number $k_{cat}$ is the main physiological adaptation, because it offsets the inhibitory effect of low temperatures on reaction rates and therefore provides adequate raw metabolic activity to the growing organism. However, both $k_{cat}$ and $K_m$ are fundamental kinetic parameters characterizing an enzymatic reaction and the specificity constant $k_{cat}/K_m$ is generally a better indication of catalytic efficiency than $k_{cat}$ alone. In principle, cold-adapted enzymes can optimize the $k_{cat}/K_m$ ratio by increasing $k_{cat}$, decreasing $K_m$ or by changes in both $k_{cat}$ and $K_m$. Inspection of the available data, although limited [for compilations see refs 4 and 28], reveals two main trends: cold-active 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 $K_m$). As will be discussed in the next sections, both kinetic behaviors seem to be related to cold-active enzymes devoid of adaptive mutations within the active site and to enzymes displaying adaptive mutations within the catalytic center, respectively.

## Improving the turnover number $k_{cat}$: a thermodynamic challenge

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 to enzyme and product. The transition state theory assumes the existence of a stable activated complex $ES^{\#}$ in equilibrium with the ground state ES:

$$E + S \leftrightarrow ES \leftrightarrow ES^{\#} \xrightarrow{k_{cat}} E + P \quad (1)$$

and the temperature dependence of the catalytic rate constant is given by the following relation which is formally equivalent to the Arrhenius law:

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

where $\kappa$ is the transmission coefficient generally close to 1, $k_B$ is the Bolzmann constant ($1.3805 \times 10^{-23}$ J K$^{-1}$), $h$ the Planck constant ($6.6256 \times 10^{-34}$ 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 ES. The transition state theory contains several well-known simplifications and interpretation of the absolute value of the thermodynamic parameters of activation should be taken with caution. In the context of cold adaptation has come the proposal to compare the variation of the thermodynamic parameters [25] between psychrophilic ($_p$) and mesophilic ($_m$) enzymes, namely $\Delta(\Delta G^{\#})_{p-m}$, $\Delta(\Delta H^{\#})_{p-m}$ and $\Delta(\Delta S^{\#})_{p-m}$. Such parameters are compiled in table 2. It should be stressed that commonly found negative values provide useful insights into the way psychrophilic enzymes have evolved.

As inferred from equation 2, the $\Delta(\Delta G^{\#})_{p-m}$ parameter simply reflects the fact that psychrophilic enzymes are more active than their mesophilic homologues. However, the small value of this parameter originates from large differences in the enthalpic and entropic contributions, $\Delta(\Delta H^{\#})_{p-m}$ and $T\Delta(\Delta S^{\#})_{p-m}$, respectively, between cold-adapted and mesophilic enzymes. To appreciate the informative content of $\Delta(\Delta H^{\#})_{p-m}$, the exponential term of equation 2 can be rewritten as:

$$k_{cat} = \frac{k_B T}{h} e^{-(\Delta H^{\#}/RT - \Delta S^{\#}/R)} \quad (3)$$

In this form, one can note that either a decrease of $\Delta H^{\#}$ or an increase of $\Delta S^{\#}$ results in an increase of $k_{cat}$. However, the contribution of the $\Delta H^{\#}/RT$ term to the decrease of $k_{cat}$ is larger when the temperature $T$ is lowered. Interestingly, all psychrophilic enzymes studied so far possess lower

Table 2. Activation parameters for the activity of psychrophilic and mesophilic enzymes [25].

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

$\Delta H^{\#}$ values (table 2) and therefore reduce the temperature dependence of $k_{cat}$ and the inhibitory effect of low temperatures on enzyme reaction rates. Thus, 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 in activation energy is achieved structurally 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 folded protein conformation and, as a corollary, the structural domain of the enzyme bearing the active site should be less stable. This is the first insight suggesting that activity and stability are linked in the process of thermal adaptation. As far as the activation entropy is concerned, the negative value of $\Delta(\Delta S^{\#})_{p-m}$ can also be interpreted assuming a more resilient active site from psychrophilic enzymes [25]. As a consequence of active-site flexibility, the ground state enzyme-substrate complex ES occupies a broader distribution of conformational states translated into increased entropy of this state compared to that of the mesophilic homologues, leading to a negative value of $\Delta(\Delta S^{\#})_{p-m}$.

## Adaptive drift and adaptive optimization of $K_m$

As inferred from the previous section, a broader distribution of the ground state ES should be accompanied by a weaker substrate binding strength if increases in $k_{cat}$ are reached through active-site resilience. In other words, there is an evolutionary pressure on $K_m$ to increase in order to maximize the overall reaction rate [73]. If the substrate is weakly bound, the ground state ES complex will fall in a less deep energy pit and, as a consequence, the activation energy of the reaction ($\Delta G^{\#}$) will be smaller, that is $k_{cat}$ will be increased (eq. 2). This is precisely what is found in psychrophilic enzymes which display identical substrate-binding site and active-site architecture (in terms of side chain identity) when compared with their mesophilic homologues. This fundamental notion was first introduced in the study of fish lactate dehydrogenases ($A_4$-LDHs). The active-site residues are fully conserved in $A_4$-LDHs from South American and Antarctic notothenoid fish, whereas the cold-adapted enzymes are characterized by higher $k_{cat}$ and $K_m$ constants. The conclusion was that temperature-adaptive increases in $k_{cat}$ occur concomitantly with increases in $K_m$ in cold-active LDHs [73]. A second and even more conclusive example is given by the above-mentioned structures of cold-active and porcine $\alpha$-amylases in complex with a large inhibitor [63, 64]. Again, all amino acids forming the active site are strictly conserved, although the cold-active enzyme possesses higher $k_{cat}$ and $K_m$. Worth mentioning is that this aspect seems to be valid for binding constants for cofactors, allosteric effectors and ions [37, 42, 44, 74].

Several enzymes counteract this adaptive drift of $K_m$ to maintain or to improve the substrate-binding affinity by amino acid substitutions within the active site. 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 dependencies. Hydrophobic interactions form endothermically and are weakened by a decrease in temperature. In contrast, interactions of an electrostatic nature (ion pairs, hydrogen bonds, Van der Waals interactions) form exothermically and are stabilized at low temperatures. Therefore, low temperatures not only inhibit the enzyme activity ($k_{cat}$) but can also severely alter the substrate-binding mode and strength according to the type of interaction involved.

The chitobiase from Antarctic bacteria nicely illustrates both aspects as well as the extent of the kinetic optimization that can be reached during cold adaptation of enzymes [75]. As shown in figure 2, the $k_{cat}$ of the cold-active chitobiase is 8 times higher than that of a mesophilic chitobiase at 5 °C. However, the $K_m$ for the substrate is 25 times lower at this temperature and as a result, the $k_{cat}/K_m$ for the cold-active enzyme is nearly 200 times greater at low temperature. In addition, the cross-shaped plot of $K_m$ shows that the $K_m$ of each enzyme tends to minimal and optimal values in the range of the corresponding environmental temperatures, reflecting the fine tuning of this parameter reached in the course of thermal adaptation. In the case of the mesophilic chitobiase, the 3D structure indicates that two tryptophan residues are the main substrate-binding ligands and perform hydrophobic interactions with the substrate. This can be related to the decrease of $K_m$ with temperature, according to the above-mentioned thermal dependence of hydrophobic interactions. Interestingly, the two tryptophan residues are not found in the cold-active chitobiase but are replaced by polar residues that are able to perform stronger interactions as the temperature decreases. Such replacements may explain the cross-shaped plot, revealing a subtle adaptive strategy that takes advantage of the thermodynamic properties of the bonds involved in substrate binding at low temperatures.

## A working hypothesis

The previous sections give the impression that two main types of adaptation occur in psychrophilic enzymes. On the one hand, enzymes that possess strictly identical residues in the active site as their mesophilic homo-

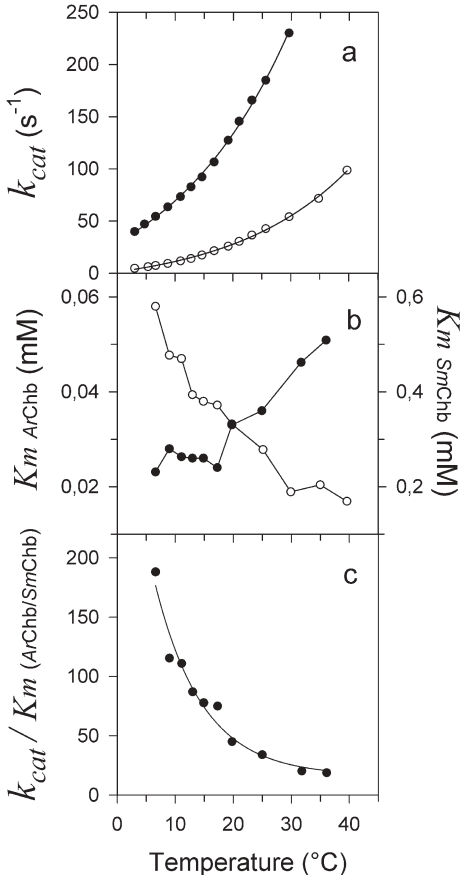


Figure 2. Temperature dependence of the kinetic parameters for psychrophilic (●) and mesophilic (○) chitobiases (from *Arthrobacter* sp. and *Serratia marcescens*, respectively). Data for the catalytic rate constant $k_{cat}$ (*a*), the Michaelis parameter $K_m$ (*b*), note the different scales used and the relative catalytic efficiency $k_{cat}/K_m$ (psychrophile/mesophile) (*c*). The cold-adapted chitobiase is characterized by a higher activity, an optimal $K_m$ value at low temperatures and a 200-fold higher catalytic efficiency at 7 °C [75].

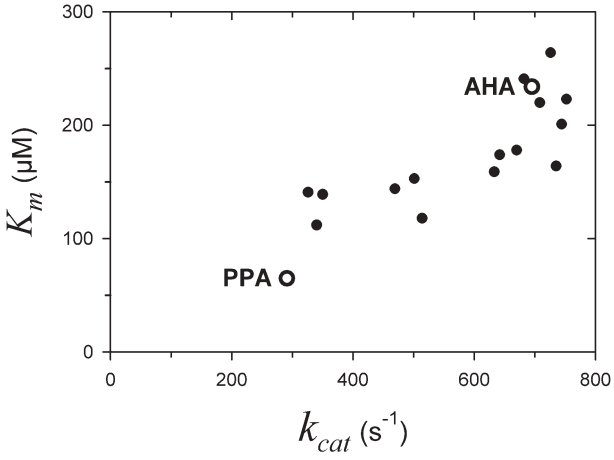


Figure 3. Correlation of the kinetic parameters in a psychrophilic enzyme (AHA), its stabilized mutants (closed circles) and a mesophilic homologue (PPA). The general trend displayed by the stabilized mutants of AHA is to decrease both $k_{cat}$ and $K_m$ values [65].

logues, possibly because the constraints imposed by the structure and the enzyme function do not allow any adjustments at this level. On the other hand, some enzymes possess amino acid replacements in the active site that can contribute to cold activity. According to the above-mentioned energetic aspects of enzyme-catalyzed reactions at low temperature, a working hypothesis can be formulated. The high catalytic activity of psychrophilic enzymes corresponds to a decrease of $\Delta G^{\#}$ of the catalyzed reaction (eq. 2). For all cold-active enzymes, this decrease of $\Delta G^{\#}$ is achieved by a decrease of $\Delta H^{\#}$ (table 2) that renders the catalyzed reaction less temperature sensitive, but requires a more flexible and therefore heat-labile active site. As a result of this active site resilience, the entropic contribution becomes larger and substrate binding is weakened for enzymes devoid of adaptive mutations in the active site. This weak binding also contributes to the decrease in $\Delta G^{\#}$ as the ES complex falls in a less deep energy pit. However, specific mutations in the active site can counteract the adaptive drift of $K_m$ to meet other physiological requirements.

## Mutational analysis of cold-activity determinants

An experimental demonstration of the relationships between $k_{cat}$ and $K_m$ was recently provided using psychrophilic and pig pancreatic $\alpha$-amylases as models [65]. Stabilizing weak interactions found in the porcine $\alpha$-amylase, but absent in the cold-active $\alpha$-amylase, were engineered by site-directed mutagenesis in the psychrophilic enzyme at positions far from the catalytic center. As shown in figure 3, stabilizing and rigidifying the cold-active $\alpha$-amylase tends to decrease the $k_{cat}$ values and to decrease concomitantly the $K_m$ values of the mutant enzymes, revealing the high correlation between these two kinetic parameters. This is also another strong indication that structural flexibility is an essential feature related to catalysis at low temperatures in psychrophilic enzymes. One should note that other mutational studies of cold-active enzymes have been less conclusive. Citrate synthase [76], subtilisin [44], alkaline phosphatase [77, 78], ribonuclease [79] and chitinase (unpublished results) have been engineered to check the involvement of specific adaptive mutations within or close to the active site. These studies have revealed a complex pattern of effects on kinetic parameters, activation energy and stability that cannot be interpreted in simple terms. Double mutants have also demonstrated an unsuspected synergy between individual mutations sometimes giving rise to non-additive or opposite effects. These interesting studies mainly highlight our current inability to predict the effect of single side chain replacement based on rational design of the

active site, not only in psychrophilic catalysts but in enzymes in general. However, a single mutation in the calcium-binding site of subtilisin [44] and in the phosphate-binding helix of triose phosphate isomerase [51] were shown to increase drastically the thermostability of the psychrophilic mutants. These results underline the strong involvement of bound cofactors in the modulation of stability and activity.

Table 3. Thermodynamic parameters for the irreversible inactivation at 52 °C of Antarctic fish and bovine L-glutamate dehydrogenases [80].

| Enzyme | $k_i$ ($min^{-1}$) | $E_a$ (kJ/mol) | $\Delta G^{\#}$ (kJ/mol) | $\Delta H^{\#}$ (kJ/mol) | $T\Delta S^{\#}$ (kJ/mol) |
|---|---|---|---|---|---|
| *C. aceratus* | 0.230 | 659 | 94.9 | 657 | 562 |
| Ox | 0.043 | 493 | 99.4 | 490 | 391 |

## Heat-labile activity and weak structural stability

The fast inactivation of cold-active enzymes by temperature has been recognized for decades. As a rule, the half-life of activity for most psychrophilic enzymes at 50–60 °C is about 10 min, and a few hours at 37 °C. However, some cold-active enzymes already denature at 20–25 °C. As it counteracts the increase of activity with temperature, this marked heat lability is responsible for the shift of the apparent optimal temperature of activity shown in figure 1. The irreversible denaturation between the native state N and the denatured inactive state I can be represented by a simple model characterized by a first-order rate constant $k_i$.

$$N \xrightarrow{k_i} I \qquad (4)$$

Surprisingly, systematic analyses of this well-recognized property are scarce and generally fragmental. Such an analysis has been performed [80] for L-glutamate dehydrogenase (GDH) from the Antarctic fish *Chaenocephalus aceratus* (the white-blooded icefish) and for the bovine enzyme (table 3). As anticipated, the activation energy $\Delta G^{\#}$ is lower for the fish enzyme, because it undergoes a faster denaturation. Because the cold-active GDH is inactivated in a narrow temperature range, the slope of the Arrhenius plot is steep, leading to very high values of both $E_a$ and $\Delta H^{\#}$ and, by difference, to a high value of $T\Delta S^{\#}$. Therefore, the activity loss is entropically driven (the low $\Delta G^{\#}$ value arises from the unfavorable entropy contribution). At a given temperature, the activated state of the psychrophilic enzyme is reached through a larger entropy variation, probably reflecting an increased local disorder of the active site, which is the main driving force of heat inactivation. On the other hand, the larger enthalpy variation accompanying this process possibly reflects the higher cooperativity of heat inactivation in the psychrophilic enzyme, originating from the lower number of interactions required to disrupt the active conformation.

The origin of the heat lability is obviously found in the low stability of the structural elements forming the active site (localized flexibility) or in the weak conformational stability of cold-active enzymes (global flexibility). Global and localized stability of enzymes may have evolved independently [81]. Extrinsic stabilization by 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 cosolvents. This effect has been attributed to a flexible active site in cold-adapted enzymes that can be more compacted by preferential exclusion of solutes. Direct evidence of differences in stability between the active site and the whole structure has been provided by microcalorimetric studies of multidomain proteins, as shown in figure 4. The cold-adapted chitobiase from the psychrophile *Arthrobacter* TAD 20 has a modular architecture consisting of an active-site domain, a galactose-binding domain and an immunoglobulin-like domain, the latter two probably being involved in the binding onto the chitinous exoskeleton of crustaceans. Differential scanning calorimetry (DSC) thermograms of this cold-active chitobiase display both a heat-labile and a heat-stable domain corresponding to the catalytic domain and to the binding domains, respectively, as demonstrated by saccharide-binding experiments [75]. The precursor of the psychrophilic $\alpha$-amylase consists of the native enzyme and an extra domain involved in the bacterial secretion [82]. The unfolding profiles of the precursor (fig. 4b) also display a heat-labile and a heat-stable domain corresponding to the native enzyme and to the secretion domain, respectively. These unfolding patterns clearly demonstrate that heat lability only affects the calorimetric domain containing the active site while leaving stability of the non-catalytic domains less affected or unchanged. Thus heat lability is not simply the result of the lack of selective pressure for stable protein in a cold environment (as demonstrated by the stable non-catalytic domains) but seems to be the consequence of the improved plasticity required around the active site for efficient activity at low temperatures. Another stability pattern detected in a cold-active enzyme is also worth mentioning. The psychrophilic PGK from *Pseudomonas* TACII 18 is a globular enzyme composed of two domains connected by a hinge region that bends during catalysis forming the active site at the interface of the two domains. Surprisingly, one domain is heat labile whereas the second domain has the same stability as mesophilic PGKs (fig. 4c). The heat-labile domain may provide the re-

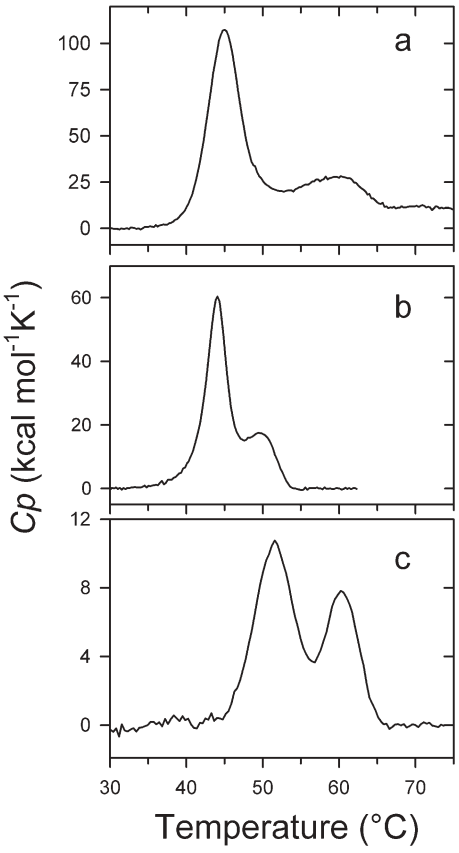


Figure 4. Three examples of psychrophilic enzymes exhibiting local flexibility. In chitobiase (*a*) and the $\alpha$-amylase precursor (*b*), the active site is embedded in the heat-labile domain, whereas the stability of the non-catalytic domains is unaffected. In phosphoglycerate kinase (*c*), the active site is formed at the interface of both heat-labile and heat-stable domains by a hinge-bending mechanism [48, 75].

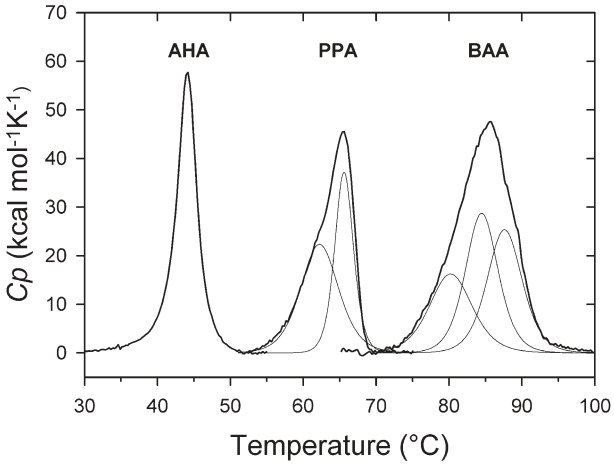


Figure 5. Thermal unfolding of $\alpha$-amylases recorded by DSC. When compared with the psychrophilic AHA (from *P. haloplanktis*), the heat-stable enzymes PPA (from pig pancreas) and BAA (from *Bacillus amyloliquefaciens*) are characterized by higher $T_m$ (top of the transition) and $\Delta H_{cal}$ (area under the transition) values, by a flattening of the transition and by the occurrence of calorimetric domains indicated by deconvolutions in thin lines [65].

quired flexibility around the active site and favor the reaction rate, whereas the heat-stable domain could improve substrate binding (as indicated by low $K_m$ values) as a result of its rigidity [48].

## Conformational stability of psychrophilic enzymes

As a result of their weak stability, psychrophilic enzymes are valuable model compounds for folding studies and are consequently increasingly investigated by more sophisticated techniques. Among these, DSC offers a definitive advantage because several thermodynamic parameters related to protein stability are recorded directly and not extrapolated from indirect data. Figure 5 displays the calorimetric records, or thermograms, of heat-induced unfolding for psychrophilic, mesophilic and heat-stable $\alpha$-amylases. Several interesting observations can already be made from these records.

1) The unfolding of the cold-adapted enzyme 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 are given by the top of the transition.
2) Protein unfolding is an endothermic process. The area under the curves (fig. 5) provides the total amount of heat absorbed during unfolding and is given by the calorimetric enthalpy, $\Delta H_{cal}$. This parameter corresponds to all enthalpic contributions involved in maintaining the compact structure and is markedly lower for the psychrophilic enzyme. In addition, there is a clear trend for increasing $\Delta H_{cal}$ values in the order psychrophile < mesophile < thermophile.
3) The transition for the psychrophilic enzyme is sharp and symmetric whereas other enzymes are characterized by a flattening of the thermograms. This is indicative of a pronounced cooperativity during unfolding of the psychrophilic enzyme: its structure is stabilized by fewer weak interactions and disruption of some of these interactions strongly influences the entire molecular edifice and promotes its unfolding.
4) The psychrophilic enzyme unfolds according to an all-or-none process, revealing a uniformly low stability of its architecture. By contrast, all other homologous enzymes display two to three transitions (either observable or indicated by deconvolution of the heat capacity function in fig. 5). Therefore, the conformation of these mesophilic and thermophilic enzymes contains structural blocks or units of distinct stability that unfold independently.

5) Although not illustrated by figure 5, the unfolding of the psychrophilic enzyme is freely reversible whereas all other homologous enzymes are irreversibly unfolded after a DSC experiment. The weak hydrophobicity of the core clusters in the cold-adapted enzyme and the low melting temperature, at which hydrophobic interactions are restrained, certainly account for this reversible character because, unlike mesophilic $\alpha$-amylases, aggregation does not occur.

From this microcalorimetric study, one can conclude that the psychrophilic enzyme investigated possesses a fragile molecular edifice that is uniformly unstable and stabilized by fewer weak interactions than homologous mesophilic or thermophilic proteins [65, 69]. The contribution of individual weak interaction to the above-mentioned behavior was analyzed by site-directed mutagenesis of the psychrophilic $\alpha$-amylase [65]. Fourteen mutants of this enzyme were constructed, each bearing an engineered residue forming a weak interaction found in mesophilic $\alpha$-amylases but absent in the cold-active $\alpha$-amylase. Single amino acid side chain substitutions were found to significantly modify the melting point $T_m$, the calorimetric enthalpy $\Delta H_{cal}$, the cooperativity and reversibility of unfolding but also the thermal inactivation rate constant $k_i$ and the kinetic parameters $k_{cat}$ and $K_m$. Thus, a fascinating aspect of this study was the ability to dissect the contribution of individual amino acid side chains to the structural properties of heat-labile and heat-stable proteins.

## Thermodynamic stability of psychrophilic enzymes

The reversible unfolding pathway of some proteins between the native state N and the unfolded state U is characterized by an equilibrium constant $K$.

$$N \overset{K}{\longleftrightarrow} U \tag{4}$$

The free energy of conformation or free energy of unfolding is therefore:

$$\begin{aligned} \Delta G_{N-U} &= -RT \ln K \\ &= \Delta H_{N-U} - T\Delta S_{N-U} \end{aligned} \tag{6}$$

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

$$\begin{aligned} \Delta G_{N-U}(T) = \Delta H_{cal}(1-T/T_m) + \Delta Cp(T-T_m) \\ - T\Delta Cp \ln (T/T_m) \end{aligned} \tag{7}$$

where $\Delta Cp$ 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 [83].

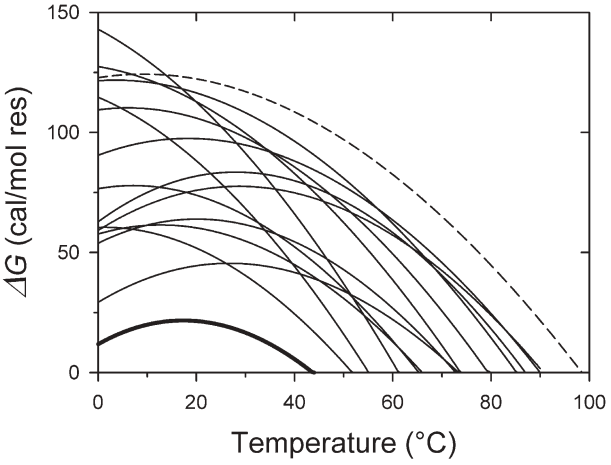


Figure 6. Gibbs free energy of unfolding or conformational stability. Stability curves for a psychrophilic $\alpha$-amylase (heavy line), several mesophilic proteins (continuous lines) and a thermophilic protein (dashed line). To compare proteins of various sizes, the free energy is expressed in specific units per mole of residue [69].

The stability curves, i.e. the Gibbs free energy of unfolding as a function of temperature, for psychrophilic, mesophilic and thermophilic proteins are drawn in figure 6. These curves correspond to the energy required to disrupt the protein structure at any given temperature. This is a parabolic function limited by the temperature of cold denaturation $T_{m'}$ (below 0 °C, not shown) and by the high temperature melting point $T_m$, at which by definition $\Delta G_{N-U} = 0$ [84]. Again, these curves provide valuable insights into the molecular adaptations of psychrophilic enzymes.

1) In principle, the low stability of a protein can be reached via three ways (or their combination): by shifting the stability curve toward low temperatures (with a similar maximal value of $\Delta G_{N-U}$ as mesophiles but at much lower temperatures), by increasing the steepness of the stability curve (with a similar maximal value of $\Delta G_{N-U}$ as mesophiles, at the same temperature but with a lower $T_m$) or by a global collapse of the stability curve. Each of these alternatives corresponds to a combination of alterations in the $\Delta H_{cal}$, $T_m$ or $\Delta Cp$ values in equation 7. Obviously, the stability curve of the cold-active $\alpha$-amylase follows the third alternative: the strikingly weak conformational energy ($\Delta G_{N-U}max$ = 22 cal/mol of residue or 92 J/mol of residue for 453 amino acids) over the entire temperature range where the native state prevails, i.e. between both melting points, arises from low values of both $\Delta H_{cal}$ and $T_m$, whereas $\Delta Cp$ is not significantly affected.
2) The temperature for maximal protein stability does not usually correspond to the environmental temperature of psychrophiles, mesophiles or thermophiles but is, rather, close to room temperature [7]. The temperature experienced by mesophiles and thermophiles lies on the right side of the bell-shaped stability curve, pro-

viding evidence that their enzymes reach the necessary balance between optimal stability (leading to a compact molecule) and flexibility (required for the catalytic function) by using the thermal dissipative force, responsible for unfolding at higher temperatures. In contrast, the environmental temperatures, around 0 °C, that are encountered by psychrophiles are found far on the left side of the stability curve. Mention needs to be made at this point that the decrease in stability within this region of the curve shown in figure 6, and ultimately leading to cold denaturation, arises from the hydration of protein groups which destabilizes the protein at decreasing temperature [85]. It follows that the origin of flexibility of psychrophilic enzymes at low temperatures involves mainly group hydration, and is therefore drastically different from mesophilic and thermophilic proteins, the latter taking advantage of the conformational entropy rise with temperature to gain mobility. The improved interactions with the solvent suggested by the crystal structure of some cold-active enzymes reinforce the idea that group hydration plays an essential role in the acquisition of flexibility in psychrophilic proteins.

3) A surprising consequence of the free energy function for the psychrophilic $\alpha$-amylase shown in figure 6 is its weak stability at low temperatures when compared with mesophilic and thermophilic proteins, whereas intuitively, cold-active proteins were also expected to 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 $\alpha$-amylase should unfold at –10 °C. Incidentally, this temperature closely corresponds to the probable lower limit of bacterial growth and, therefore, cold denaturation of some key enzymes in psychrophiles is an additional, though unsuspected factor fixing the lower limit of life at low temperatures.
4) The psychrophilic $\alpha$-amylase has reached a state close to the lowest possible stability of the native state [65, 69]. This fact indicates that the cold-active $\alpha$-amylase could not be any less stable than it is. If psychrophilic enzymes have indeed gained in flexibility at the expense of stability in the course of evolution, this implies that the actual native state precludes further adaptation toward a more mobile structure. This aspect accounts for the imperfect adaptation of the catalytic function in psychrophilic enzymes, mentioned at the beginning of this review and illustrated in figure 1.

## Natural versus directed evolution

Directed evolution has contributed significantly to explore the role of multiple and dispersed substitutions that act synergistically to modify enzyme properties and functions [86, 87]. Basically, random mutations are introduced in a gene and the resulting library is screened for the acquisition or improvement of a specific property. This approach reveals mutations responsible for immediate and drastic adaptation in response to a strong selection imposed in the laboratory and selected by a physicochemical property. In contrast, natural evolution is a very slow process driven by complex environmental and metabolic constraints, and selected by the viability or performance of a living organism. What can we learn from the results of laboratory evolution and from the general traits of psychrophilic enzymes?

Laboratory evolution has demonstrated that enzyme activity and stability are not physically linked in proteins as previously thought, giving rise to the proposal that the low stability of cold-active enzymes results from genetic drift originating from the lack of selective pressure for stable proteins [8]. However, mutations that simultaneously increase activity and stability are very rare [88] and proved to be extremely complex to obtain [89]. In fact, in nature, these environmental constraints do not exist except for thermophilic enzymes that catalyze the conversion of labile metabolic intermediates. As a matter of fact, phosphoribosyl anthranilate isomerase from *Thermotoga maritima* was found to be both extremely stable and active [90]. When random mutants are only screened for high activity at low temperature during directed evolution, the selected enzymes invariably display the canonical properties of psychrophilic enzymes [91–96]. This can be understood from the above-mentioned results, as improvement of activity at low temperatures associated with lost of stability appears to be the most frequent and accessible strategy at the level of the protein molecule. An elegant demonstration of this aspect was given using ornithine carbamoyltransferase from *Pyrococcus furiosus* because random mutagenesis was associated with a functional screening procedure involving the survival of the host strain [94]. Consequently, the low stability of cold-active enzymes may be the simplest way to acquire activity in the absence of selection for stable proteins. Thus, the occurrence of heat-stable psychrophilic enzymes should not be ruled out.

## Conclusions

The most widely accepted hypothesis accounting for the dominant adaptive traits of cold-active enzymes, i.e. the high activity and the weak stability, suggests that there is a correlation between the activity, the flexibility and the stability of the enzyme molecule. The flexible structure of psychrophilic enzymes can provide enhanced ability to undergo discrete and fast conformational changes at low temperatures imposed by the catalytic events. But the price to

pay for such plasticity is, of course, the low stability of the native enzyme structure [1]. This link between activity, flexibility and stability, although intuitively satisfying, remains to be proven experimentally in cold-active enzymes. Nonetheless, these relationships have found strong experimental support from the comparison of thermophilic and mesophilic enzymes [97–99]. In contrasts, very few attempts have been made to quantify and compare the expected flexibility of cold-adapted proteins. The rate of hydrogen/deuterium exchange was recorded by Fourier transform infrared spectroscopy for two cold-active enzymes [32, 100] but no significant exchange rate was found between psychrophilic enzymes compared with the mesophilic homologues. The nature of the protein motion involved in temperature adaptation is complex and remains largely hypothetical [101]. For example, amide hydrogen exchange experiments using rubredoxin from *P. furiosus*, the most thermostable protein presently known, indicate that conformational fluctuations for solvent access do not differ from those of mesophilic homologues at the millisecond time scale [102], therefore questioning the involvement of conformational rigidity in the extreme heat stability of this protein. Incidentally, this unexpected result also raises some doubts about the connection between exchange rates and conformational flexibility. Directed evolution suggests that low stability results from genetic drift but, in contrast, the biophysical studies mentioned in this review indicate that flexibility is an integral component of the adaptive strategy. At this stage, the emerging picture suggests that psychrophilic enzymes utilize an improved flexibility of the structures involved in the catalytic cycle (as indicated by the common heat-labile activity), whereas other protein regions, if not implicated in catalysis, may or may not be subjected to genetic drift, as nicely illustrated by the cold-active chitobiase and the $\alpha$-amylase precursor (fig. 4). A better understanding of enzyme evolution and protein dynamics is obviously the next challenge for biochemical studies of extremophiles.

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