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Review Article

Psychrophilic enzymes: strategies for cold-adaptation

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Psychrophilic organisms thriving at near-zero temperatures synthesize cold-adapted enzymes to sustain cell metabolism. These enzymes have overcome the reduced molecular kinetic energy and increased viscosity inherent to their environment and maintained high catalytic rates by development of a diverse range of structural solutions. Most commonly, they are characterized by a high flexibility coupled with an intrinsic structural instability and reduced substrate affinity. However, this paradigm for cold-adaptation is not universal as some cold-active enzymes with high stability and/or high substrate affinity and/or even an unaltered flexibility have been reported, pointing to alternative adaptation strategies. Indeed, cold-adaptation can involve any of a number of a diverse range of structural modifications, or combinations of modifications, depending on the enzyme involved, its function, structure, stability, and evolutionary history. This paper presents the challenges, properties, and adaptation strategies of these enzymes.

Introduction

Low-temperature environments predominate on Earth and include the ocean depths, the polar, glacier, and alpine regions, deep caves, and permafrost terrain. These permanently low-temperature environments are rich in life, having been successfully colonized by a diverse group of organisms termed the psychrophiles (meaning 'cold-loving'). These organisms have been found to thrive in the most hostile of low-temperature environments [1], even at subzero temperatures in supercooled liquid water such as at −10°C in Arctic permafrost cryopegs [2,3], at −20°C in sea ice brine veins [4,5], and in supercooled cloud droplets [6,7]. Indeed, microbial populations surviving at −60°C in porous rocks in the Antarctic dry valleys have even been described [8,9]. To date, the lowest temperature reported for cell division is −15°C, and for metabolic activity is −25°C [10], this latter possibly representing the lowest temperature for sustained life before dormancy.

Psychrophiles include a remarkably diverse range of organisms; from bacteria, archaea, and yeasts, which are the most common, up to multicellular eukaryotes including plants and ectothermic animals [11]. Importantly, these organisms do not merely survive in the cold, they thrive and indeed require low temperatures. They have adapted their cellular components and processes to overcome the barriers inherent to their extreme environment: from adaptation of their enzymatic repertoire and cell envelop; to the production of chaperones and cryoprotectants such as antifreeze and ice-nucleating compounds; and even metabolic adjustments [12]. Of these adaptations, enzymes, which drive microbial metabolism and the cell cycle, have been the most intensely studied [13,14], and their adaptation is seen as being a key component to life in the cold. The present paper, based on updates of previous publications [15–21], will focus on enzymes produced by psychrophiles, presenting the general features of these while also discussing their less common characteristics and delineating their adaptation strategies.

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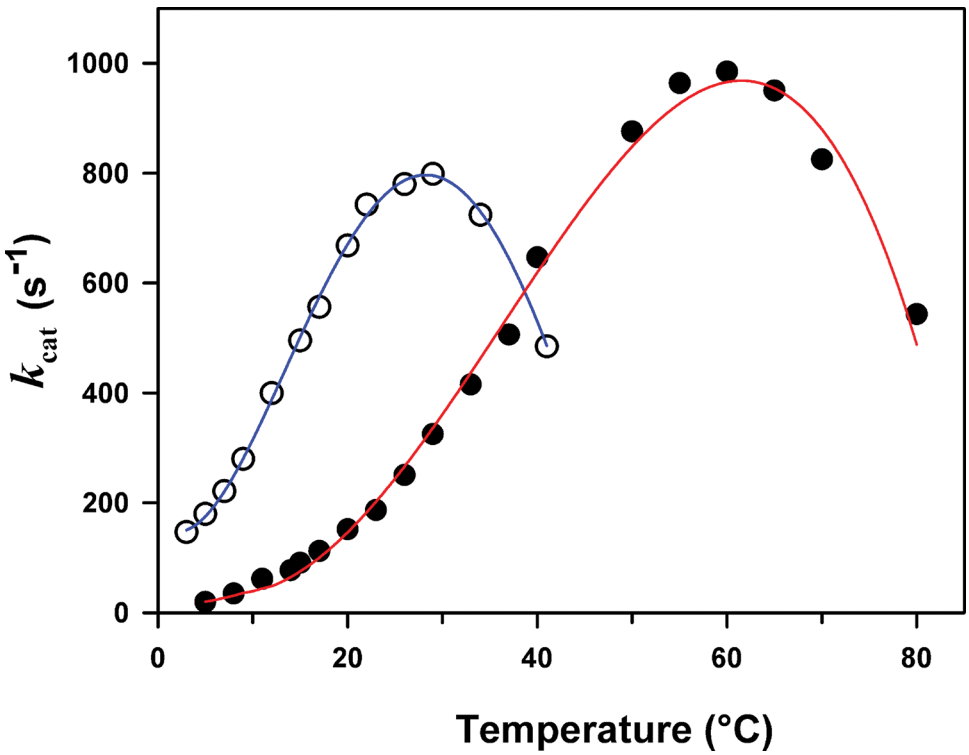

Figure 1. Cold-active and heat-labile psychrophilic enzymes

Comparison of the temperature dependence of activity of a psychrophilic (open symbols, blue line) and mesophilic (closed symbols, red line) α-amylase. It can be seen that the cold-adapted enzyme is characterized by a higher activity at low temperatures and a shift in the apparent optimum temperature toward lower temperatures. Adapted from [80].

Enzyme kinetics and the low-temperature challenge

Enzyme activity is strongly dependent on temperature as described by (eqn 1) from the Eyring equation:

$$k_{\mathrm{cat}} = \kappa \frac{k_B T}{h} \mathrm{e}^{-\triangle \mathrm{G}^{\#}/\mathrm{RT}} \tag{1}$$

In this equation, k_{cat} is the enzyme activity, and the constants, k_{B}, h, and R, are, respectively, the Bolzmann constant (1.38×10^{-23} J K^{-1}), Planck constant (6.63×10^{-34} J s), and the universal gas constant (8.31 J K^{-1} mol^{-1}). κ and $\Delta G^{\#}$ are the equation variables wherein κ is the transmission coefficient, that, among other factors, is inversely related to solvent viscosity; and $\Delta G^{\#}$ is the variation of the Gibbs energy between the activated enzyme–substrate complex ES* and the ground-state ES. From this equation, it can be seen that enzymes face a double challenge at low temperatures: an exponential decrease in reaction rates (k_{cat}) as temperature (T) is decreased, and a further decrease in reaction rates because of the increased solvent viscosity (i.e., reduced κ) brought about by the low temperatures. In agreement with this, a strongly decreased activity at low temperatures is observed for enzymes isolated from moderate- and high-temperature adapted organisms, yet, in contrast, enzymes from psychrophiles maintain high rates [22]. Comparisons of the effects of temperature on the activity of various enzymes adapted to different thermal environments (see Figure 1 for an example) allow for identification of at least three general features of cold-adaptation: (i) psychrophilic enzymes display a high activity at low to moderate temperatures; (ii) the temperature for apparent maximum activity of psychrophilic enzymes is shifted to low temperatures; and (iii) the adaptation to cold is not always perfect as the low-temperature catalytic rates of many psychrophilic enzymes, while being high, are often lower than those of mesophilic enzymes at 37°C. This latter characteristic is in accordance with and probably contributes to the slower doubling times reported for psychrophilic microorganisms [23]. It must also be noted here that not all enzymes of psychrophiles need to be adapted to the cold. Examples include heat-shock proteins, which are required to be active during habitat temperature increases [24,25], and enzymes-catalyzing electron transfer reactions, wherein electron flow is not significantly affected by temperature [26,27].

Table 1 Kinetic parameters for the hydrolysis of various polysaccharides at 25°C by a psychrophilic and mesophilic α-amylase

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

It can be seen that for all substrates examined, the psychrophilic enzyme displays a higher activity (k_{cat}) but also a higher K_m, resulting in a reduced catalytic efficiency (k_{cat}/K_m). Adapted from [57].

In addition to reducing reaction rates (k_{cat}), low temperatures can also affect substrate binding (related to the kinetic parameter K_m) as the weak interactions involved are temperature-dependent. Both positive and negative effects are possible as electrostatic interactions are strengthened when temperature is decreased, whereas in contrast hydrophobic interactions are weakened. Ideally, adaptation to cold should involve optimization of both k_{cat} and K_m, yet, in fact, an increase in K_m, i.e., reduction in substrate affinity, is most commonly observed [19]. This leads to suboptimal values for the k_{cat}/K_m ratio as shown in the example given in Table 1 and the frequency of this occurrence has led to suggestions that there is an evolutionary pressure on K_m to increase so as to enable maximization of k_{cat} [28,29]. Nevertheless, while an increased K_m does in fact appear to be the prevailing observation, a number of reports of cold-adapted enzymes with a reduced K_m have also been published [30–35]. Interestingly, many of these enzymes have been found to be characterized by an optimized electrostatic potential at the active site permitting for the improved substrate affinity. In addition to this, another, less commonly reported strategy for K_m reduction is that displayed by a cold-adapted chitobiase wherein two tryptophan residues involved in hydrophobic substrate binding in mesophilic homologs were replaced with polar residues for improved substrate binding at low temperatures [36]. Interestingly, many of these enzymes are intracellular enzymes in which K_m may be associated with a regulatory function requiring high affinity, and thus there is an evolutionary pressure toward the maintenance of a low K_m.

Global and local protein instability/stability

Figure 2 displays another dominant feature of the majority of cold-adapted enzymes, i.e., a reduced stability as compared with mesophilic homologs. Psychrophilic enzymes generally have a reduced thermal and chemical stability as displayed by low melting temperatures, reduced calorimetric enthalpies for unfolding, reduced stabilization energies ($\Delta G_{N\text{-}U}$), high rates of thermal inactivation, shifts in the apparent optimal temperature for activity towards low temperatures, and sensitivity to chemical denaturants [15,16,18]. In particular, the active site and/or structural elements involved in enzyme function, be they proximal or distal to the active site, are often the most heat-labile structural elements of cold enzymes [37–40], whereas other regions not involved in catalysis may or may not be characterized by low stability. From the example in Figure 2, it can be seen that the activity decrease for the mesophilic enzyme is concomitant with protein unfolding, but for the psychrophile, the rate decline occurs before protein unfolding. Thus, while the activity loss for the mesophile is probably a result of protein unfolding that for the psychrophile appears to be due to a more temperature-sensitive active site and/or enzyme–substrate complex [41].

Structural instability is possibly not an absolute requirement for cold-adaptation and in recent years a growing number of enzymes of psychrophilic origin have been reported to be of similar or higher stability than their mesophilic homologs as displayed by thermal inactivation and unfolding studies [42–45]. It remains to be confirmed however that all these are truly cold-adapted enzymes, and not exceptions that are not required to be adapted.

Global and local protein flexibility

Enzymes are dynamic molecules in a constant state of motion, and these motions, or conformational fluctuations, are essential in conferring to the enzyme the flexibility required for the conformational changes associated with catalysis. While analyses of protein flexibility is a complex matter, a number of studies point to a less compact conformation with increased flexibility for psychrophilic enzymes as compared with mesophilic homologs [37–39,46,47]. From the original comparative studies using hydrogen–deuterium exchange, to neutron spectroscopy, X-ray crystallography relative temperature B-factors, fluorescence spectroscopy, fluorescence quenching (Figure 3), and the more recent

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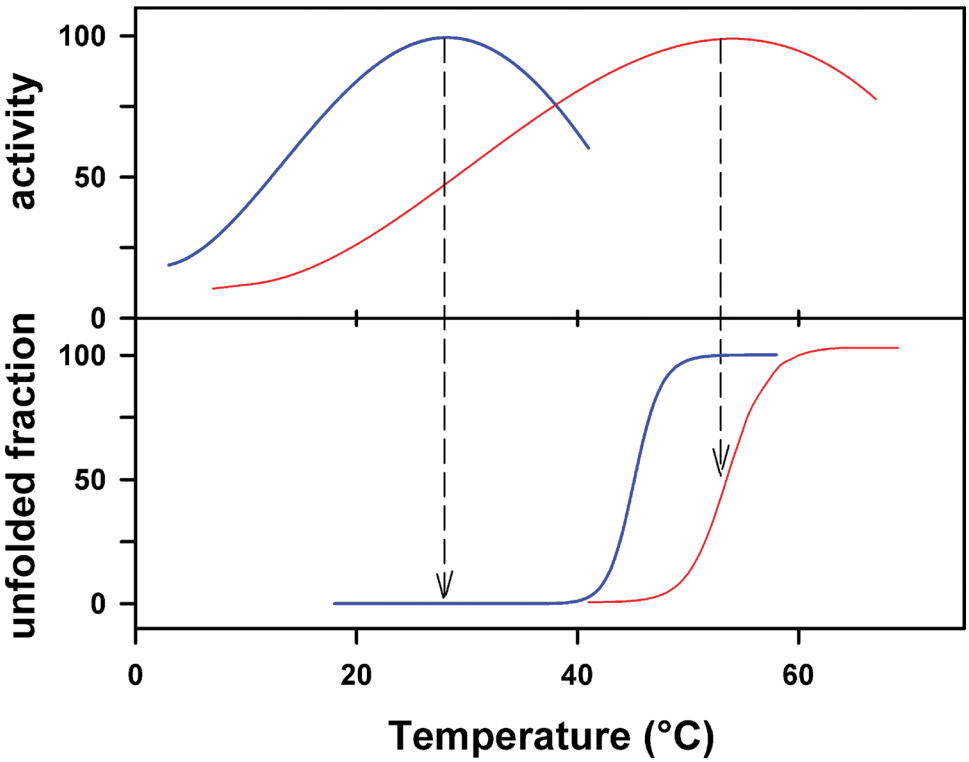

Figure 2. Global and local structural instability of cold-active enzymes

Comparison of the effects of temperature on enzyme activity (upper panel), and thermal stability as measured by fluorescence spectroscopy (lower panel), for a psychrophilic (blue lines) and mesophilic (red lines) α-amylase. The psychrophilic enzyme is characterized by a reduced thermal stability as exhibited by unfolding at lower temperatures, and a decline in enzyme activity before the onset of protein unfolding. In contrast, the decline in enzyme activity for the mesophilic enzyme corresponds with protein unfolding. Adapted from [38].

molecular dynamics simulations approaches, results in many cases point to an increased protein flexibility with decrease in physiological temperature. In particular, molecular dynamics simulations have become popular as they allow for more detailed definitions of flexibility [48] and the types of movements, atomic fluctuations, and vibrations involved [14], while also enabling for analysis of localized regions [49–51] and descriptions of catalytic reaction pathways [41,52–54]. Moreover, and in agreement with an apparently localized heat lability at the active site as discussed above, the active site, associated surface loops, and other regions involved in enzyme function, have also been reported to be the most flexible structural elements of the molecular edifice of many cold-adapted enzymes [41,50]. This is supported by the reduced substrate specificity reported for some cold-active enzymes. It is believed that here, the 'looser', more flexible active site of cold-active enzymes permits accommodation of substrates with slightly different conformations and/or sizes, in contrast to enzymes adapted to higher temperatures where the more rigid structure impedes binding of substrate variants [32,55–57].

Structural origins of cold-adaptation

Numerous studies comparing the structures of homologous psychrophilic, mesophilic, and thermophilic enzymes have been carried out in an attempt to identify the structural determinant of cold-adaptation. It was shown that proteins adapted to different temperatures are highly similar, that no alterations in the reaction mechanism are involved, that all catalytic residues are highly conserved (see Figure 4 for an example), and that the structural changes necessary for cold-adaptation are discrete and enzyme-specific [58]. As compared to enzymes adapted to higher temperatures, cold-adapted enzymes frequently display a reduction in strength and/or number of stabilizing interactions, and/or an introduction of specific protein flexibility-enhancing modifications [32,59–62]. These alterations may be localized, commonly near the active site, or global. Less commonly, active site-specific alterations such as an improved

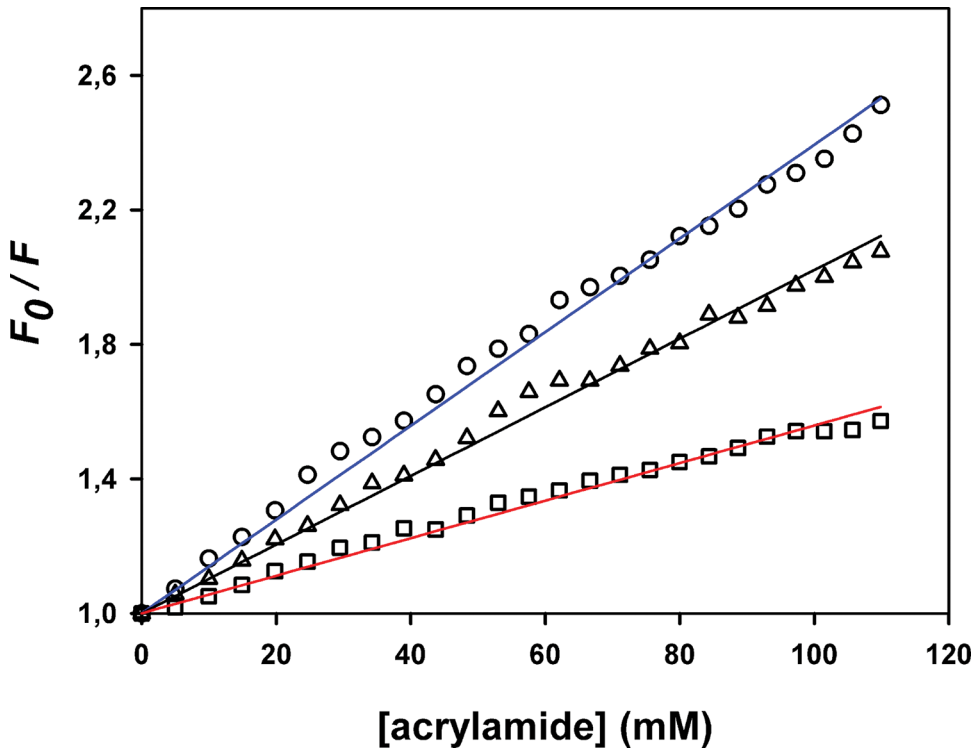

Figure 3. High structural flexibility of the psychrophilic enzyme

Protein flexibility as monitored by fluorescence-quenching experiments with psychrophilic (circles, blue line), mesophilic (triangles, black line), and thermophilic (squares, red line) α-amylases. The psychrophilic enzyme is characterized by an increased flexibility as indicated by an increased permeability to the small quencher molecule acrylamide, resulting in a larger attenuation of the intrinsic fluorescence (F) and hence leading to a steeper slope of the fluorescence ratio (F_0/F). Adapted from [38].

accessibility to substrate and release of product, and/or an active site optimized for substrate binding, have also been reported.

The destabilizing alterations commonly observed include an attenuation of ionic, H-bonding, and/or aromatic interactions and networks, a reduction in the strength and/or compactness of the protein hydrophobic core, and/or an increased hydrophobic surface leading to an entropy-driven destabilization via a reorganization of water molecules around the exposed hydrophobic side chains [18,32]. Further destabilizing alterations observed include weakened charge–dipole interactions in α-helices, a reduced number of binding sites and/or reduced binding affinities for stabilizing ions and, an excess of surface charges potentially leading to destabilization through repulsion. It can be understood that these different structural modifications can reduce protein stability, but would also be expected to allow for a greater freedom of movement of the structural elements, that is to say, a greater protein flexibility. This is in accordance with the reduced stability and increased flexibility observed for many cold-adapted enzymes as discussed above. In addition to these approaches, which enable for a potentially increased protein flexibility, a more direct approach, via specific flexibility-enhancing factors have also been reported. These include the clustering of glycine residues (providing local mobility) [63,64], a decreased content of proline residues in loops (enhancing chain flexibility between secondary structures) [65], and/or longer loop structures. In addition, structural modifications promoting increased protein–solvent interactions and thereby promoting improved protein 'breathing', have also been reported, namely an excess of surface negative charge [66] and/or increased cavity content and size [67]. Recently, enzyme oligomerization, which is normally associated with thermophilic enzymes, has been observed in a number of cold-adapted enzymes [43–45]. It is still not clear how this plays a role in cold-adaptation, but suggestions include the presence of large internal active site cavities that can reduce protein core compactness and enhance structural flexibility while also promoting substrate binding. Interestingly also, oligomerization appears to be common in stable cold-active enzymes, suggesting a role in maintaining stability while also promoting cold activity. Further studies are required to better understand this.

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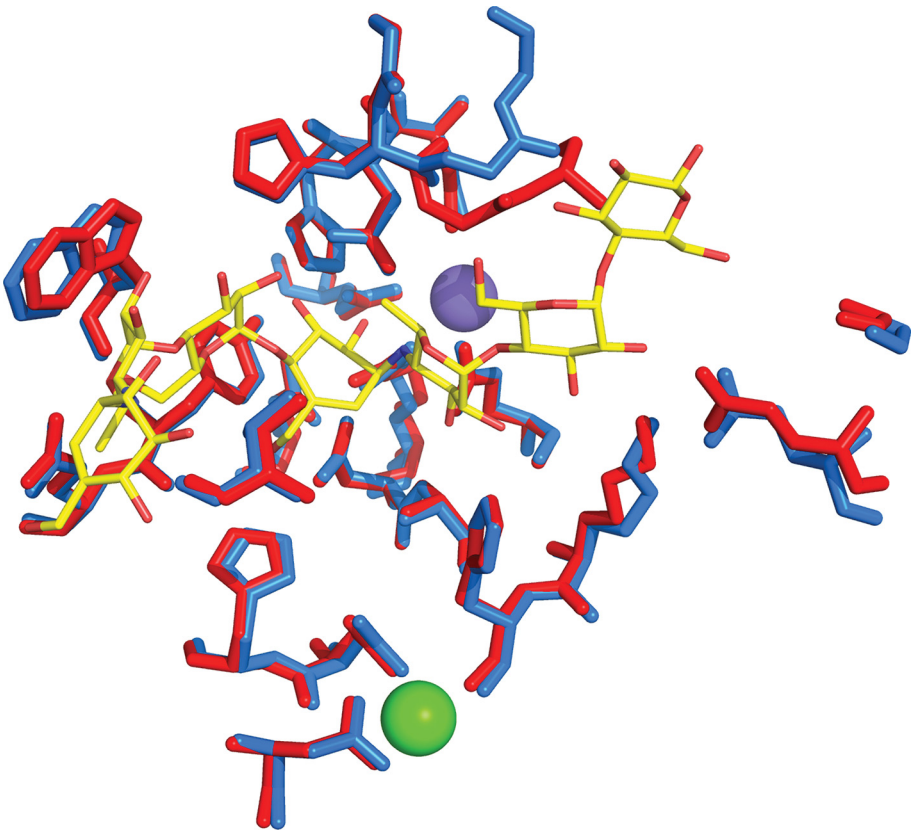

Figure 4. Cold activity can be reached without changes in the active site architecture

Comparison of the catalytic reaction centers of a psychrophilic (blue) and mesophilic (red) α-amylase in complex with the inhibitor acarbose (yellow). The 24 amino acids forming direct or water-mediated interactions with the substrate are identical and superimpose perfectly within the resolution of the structures [81–84]. The blue and green spheres represent, respectively, a chloride and calcium ion known to be essential in enzyme activity.

In relation to adjustments facilitating substrate access and/or product release, less bulky amino acids lining the catalytic cleft, a repositioning of loops bordering the active site and/or loop deletions can lead to a larger opening of the catalytic cleft [30]. Thus, substrates can be accommodated and products released at a lower energy cost due to the reduced conformational changes required, but, on the other hand, looser substrate binding may also occur leading to a higher K_m. In contrast with this, optimization of the electrostatic potential of the active site [30,31,34,68,69] can allow for improved substrate recognition and binding and a reduction in K_m. Here, the electrostatic potential of the active site can extend out into the medium and attract and orient the substrate even before this enters in contact with the enzyme while also enhancing binding. Similarly, substitution of active site hydrophobic residues with polar residues [36], and even optimization of both the hydrophobicity and electrostatics of the active site for improved substrate binding, have also been reported [70,71]. Indeed, when possible within the structural fold and for the reaction catalyzed, these strategies, possibly in conjunction with other activity-enhancing modifications, can enable for optimization of both kinetic parameters, i.e., an increased k_{cat} and decreased K_m.

While various structural alterations have been identified in proteins adapted to different temperatures as listed above, identification of those specific alterations permitting cold-adaptation has been more difficult. Both site-directed and random mutagenesis have been used with varying success in identifying these as the structural modifications observed in the protein may be related to the other environmental conditions of the habitat or may be resultant of an evolutionary drift related to the absence of a selective pressure on protein stability. Notwithstanding these difficulties, a number of studies have nevertheless been successful in pinpointing the structural modifications involved in cold-adaptation [72–74], with some studies even enabling for the engineering of mesophilic-like characteristics into a psychrophilic enzyme (see Figure 5 for an example) and psychrophilic-like characteristics into a mesophilic enzyme. Finally, of the numerous structural adaptations that have been identified, it has become evident

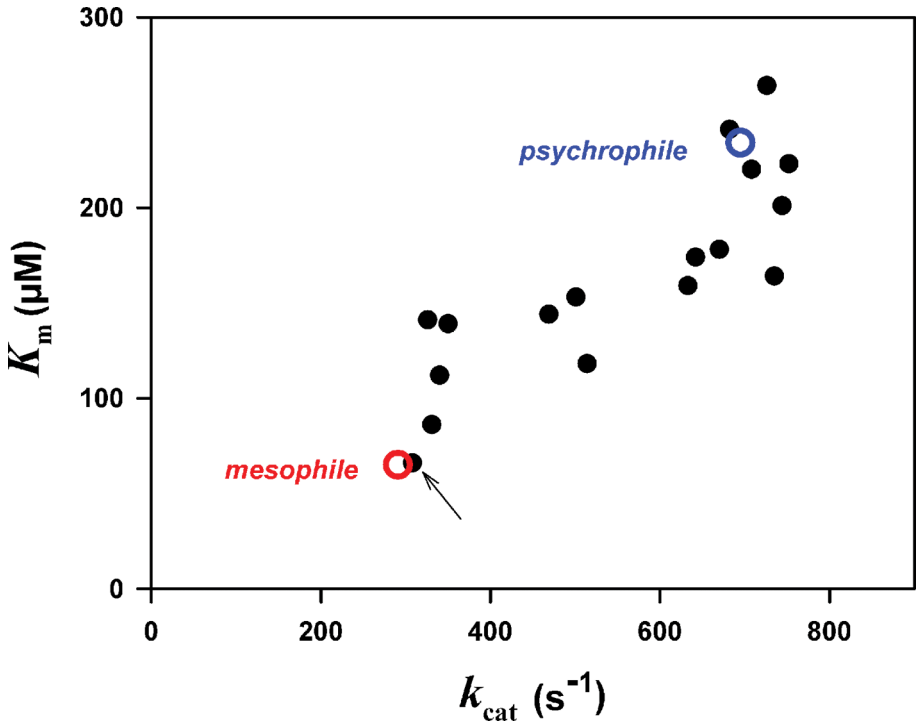

Figure 5. Engineering mesophilic-like activity in a psychrophilic α-amylase

The kinetic parameters k_{cat} and K_m are shown for the psychrophilic (blue circle) and mesophilic (red circle) α-amylases and for stabilized mutants of the psychrophilic enzyme (filled black symbols). It can be seen that the general trend is to decrease both the activity (k_{cat}) and K_m (i.e., increase substrate affinity) when approaching mesophilic-like characteristics. The most stable mutant bearing six additional interactions (arrow) displays kinetic parameters nearly identical to those of the mesophilic enzyme. Data from [29,85–87].

that each enzyme or closely related enzyme family, adapts its own strategy, making use of any one or combination of the structural adjustments identified in accordance with the enzyme structure, function, substrate, and evolutionary background.

Kinetic origin of cold activity

From (eqn 1), it can be seen that maintenance of a high activity at low temperatures can be achieved by decreasing the free energy of activation ($\Delta G^{\#}$) for the reaction. This equation is derived from the transition-state theory (see schematic description in Figure 6) wherein the enzyme (E) interacts with substrate (S) to form the enzyme–substrate complex ES, which, most commonly, is of lower free energy. For the reaction to proceed, the activated-state ES$^{\#}$ has to be reached before then breaking down to the E and the product (P). Herein, the free energy of activation $\Delta G^{\#}$ for the reaction is defined as the height of the energy barrier between the ground-state ES and the transition-state ES$^{\#}$. This energy barrier $\Delta G^{\#}$ is composed of both enthalpic and entropic contributions as described in (eqn 2) and from this, it can be seen that $\Delta G^{\#}$ can be reduced by decreasing the enthalpy of activation ($\Delta H^{\#}$) or/and increasing the entropic contribution ($T\Delta S^{\#}$).

$$\triangle G^{\#} = \triangle H^{\#} - T \triangle S^{\#} \quad (2)$$

Both laboratory studies [38,75] and reaction kinetic simulations [76] demonstrate that catalysis by cold-active enzymes is indeed characterized by a low value for $\Delta H^{\#}$ as compared to mesophilic enzymes. Alternatively, an unfavorable, counteracting, lower and/or more negative $T\Delta S^{\#}$ has also been found (Table 2). The reduced $\Delta H^{\#}$ signifies a reduced temperature dependence of activity for these enzymes, in agreement with the observed decreased reduction in reaction rates as temperature is lowered, and is considered the main adaptive character to low temperatures. At the structural level, this decreased $\Delta H^{\#}$ points to a decrease in the number of enthalpy-driven interactions that are broken during the activation step. These interactions also contribute to protein stability and rigidification, and thus this

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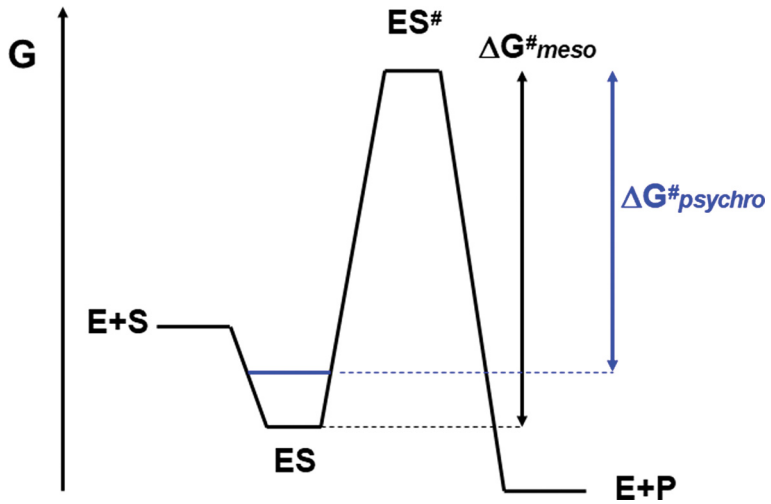

Figure 6. Effects of a reduced substrate affinity on the free energy barrier of the reaction

Schematic description showing the Gibbs free energy profile for an enzyme-catalyzed reaction under saturating substrate concentration and effects of substrate binding strength on the free energy barrier ($\Delta G^{\#}$). E interacts with S to form the ES complex, which has to evolve toward the transition state ($ES^{\#}$) before breaking down to E and P. Weak substrate binding by psychrophilic enzymes would give rise to a higher energy level of the ES complex (solid blue line) and thereby decreases the energy barrier ($\Delta G^{\#}$*psychro*) of the reaction, which leads to an increased reaction rate. Taken from [15].

Table 2 Activation parameters for the enzymatic hydrolysis reactions at 10°C of psychrophilic, mesophilic, and thermophilic α-amylases

	k_{cat} s^{-1}	$\Delta G^{\#}$ kcal mol^{-1}	$\Delta H^{\#}$ kcal mol^{-1}	$T\Delta S^{\#}$ kcal mol^{-1}
Psychrophile	294	13.3	8.3	−5.0
Mesophile	97	14.0	11.1	−2.9
Thermophile	14	15.0	16.8	1.8

The psychrophilic enzyme is characterized by a lower free energy of activation $\Delta G^{\#}$, permitting a higher catalytic rate, resultant of a lower enthalpy of activation ($\Delta H^{\#}$) which is counteracted by a lower, more negative entropic change. Adapted from [38].

observation is in accordance with the reduced stability and stabilizing interactions and the increased flexibility observed for cold-adapted proteins, in particular at their active site. Moreover, the reduced stabilizing interactions and increased flexibility would result in a broader distribution of conformational states for the ES complex. This translates into an increased entropy for the complex that would require a greater reordering to reach the more organized and compact transition-state $ES^{\#}$ [75], and thereby gives rise to the more negative $T\Delta S^{\#}$ values observed. Thus, while the less favorable lower and/or more negative $T\Delta S^{\#}$ values for cold-adapted enzymes do reduce the positive effect of the reduced $\Delta H^{\#}$ in decreasing $\Delta G^{\#}$, they are also in fact a consequence of this reduced $\Delta H^{\#}$. That is to say, there is a thermodynamic link between enzyme stability, flexibility, and activity. This macroscopic interpretation of the thermodynamic data and specifically of a broader distribution of the ground-state ES is supported by the commonly observed weaker substrate binding of cold-adapted enzymes. Indeed, from Figure 6, it can also be seen how weak substrate binding could benefit the reaction rate, whereby weaker binding would lead to an ES complex of higher energy and thereby reduce the free energy barrier $\Delta G^{\#}$. Furthermore, it must also be highlighted that this proposal of a greater reorganization during activation for the psychrophilic enzyme has received strong experimental support from calorimetry studies comparing extremophilic enzymes in the free state (E) and blocked in the transition state ($ES^{\#}$) by a substrate analog [38]. It was shown that the variations in compactness followed both in sign and magnitude the calculated activation entropy values provided in Table 2.

The central concept(s) in cold-adaptation

Currently, the most dominant theory for enzyme cold-adaptation is for a correlation between high activity at low temperatures and high structural flexibility, with these being achieved at the expense of reductions in protein stability, substrate affinity, and sometimes also, substrate specificity. It is believed that cold-adapted enzymes overcome the low-temperature challenge by increasing flexibility of key regions of the structure to allow for the conformational motions required for activity at a lower energy cost. This increased flexibility is most commonly attained via a reduction in stability of the structural edifice and thus there is a limit in the adaptation achievable related to the minimum stability permitted for the protein. Furthermore, resultant of the more dynamic nature of the enzyme, a reduction in substrate affinity and substrate specificity are frequently observed. It must however be noted that, while being the most commonly reported, this is not the only adaptation strategy to deal with low temperatures, and cold-activity is not always associated with low stability and a poor substrate affinity. Other strategies reported include specific flexibility-enhancing alterations that can avoid reductions in stability; increased active site accessibility for enhanced low-temperature activity without impacting flexibility nor stability; and/or optimized enzyme–substrate binding for promotion of high substrate affinity without affecting protein stability nor flexibility. Importantly, any of a large number of structural modification may be employed in achieving these cold-adaptation strategies or combinations of strategies, with the modification(s) and strategy/strategies used being dependant on the enzyme involved and on what is permitted within its structural fold and for the reaction catalyzed. Future developments in advanced computational biology technologies, such as in artificial intelligence systems for accurate, fast, structure prediction and analyses, e.g., AlphaFold, ESMFold [77–79], in conjunction with molecular dynamics simulations for characterization of protein conformational ensembles and dynamics, should enable for a more rapid pinpointing of these strategies for each specific protein in the future. Such developments will enable for the high-throughput, large-scale comparative analysis of proteins adapted to different temperatures and accelerate the identification of novel adaptation strategies.

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Summary

- Psychrophiles thrive in permanently cold environments and even at subzero temperatures in supercooled liquid water.
- These organisms synthesize cold-active enzymes to sustain their metabolic activity and cell cycle.
- Cold-active enzymes display high catalytic rates at low temperatures and are most frequently, but not always, characterized by an increased flexibility and reduced stability and substrate affinity.
- Any of a number of strategies may be employed to maintain high catalytic rates at low temperatures with each enzyme employing its own strategy or combination of strategies dependent on its characteristics and requirements.
- Adaptation strategies include augmenting structural flexibility, increasing active site accessibility, and/or optimizing enzyme–substrate binding.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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Abbreviations

E, enzyme; ES, ground state enzyme–substrate complex; ES#, transition state enzyme-substrate complex; ΔG#, free energy of activation of reaction; P, product; S, substrate.

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