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Chapter II

Enzyme Function at Low Temperatures in Psychrophiles

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Abstract

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. This concept is based on the observation that thermal stabilities of protein homologues are almost invariably correlated positively with environmental temperature. Current views of protein molecular dynamics suggest that the enzyme structure must achieve a balance between stability (allowing the retention of a specific conformation at the physiological temperature) and flexibility (allowing the enzyme to perform its catalytic function). This hypothesis is known as the adaptive strategy of "compromise" between activity and stability, leading to "corresponding states" of enzymes adapted to different thermal environments. The main functional and structural adaptive properties of cold-active enzymes are presented in this chapter. A fascinating aspect of the current knowledge on psychrophilic enzymes is the great diversity in the strategies used to maintain the appropriate level of catalysis at low temperatures.

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Introduction

Most of the biotopes on Earth are exposed to low temperatures if one considers the vast extent of permanently cold environments such as the Antarctic continent, the Arctic ice floe and the oceans surrounding them, the permafrost, the mountain and glacier regions and last but not least, the deep-sea waters and sediments covering three-quarters of the planet surface. 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 (without entering the debate on classification), this definition includes *de facto* a large range of species from bacteria, archaea, yeasts, algae, marine invertebrates or polar fish. These examples underline that psychrophiles are numerous, taxonomically diverse and have a widespread distribution.

Most psychrophiles do not simply endure such extreme conditions but instead colonize, grow well and reproduce successfully in these environments. Some psychrophilic bacteria grown in a rich medium at 4°C have doubling times close to that of *Escherichia coli* at 37°C, in some instance yielding huge cell density. In addition, they frequently possess a character of “irreversible adaptation” in the sense that low temperatures are not only the optimal conditions for growth but are also mandatory for sustained cell metabolism. Such deep adaptation of course requires a vast array of metabolic and structural adjustments at nearly all organization levels of the cell, that begin to be understood thanks to the availability of genome sequences [1-4] and of proteomic approaches [5-8]. Overviews on these various aspects have been recently published [9-11].

This chapter focuses on protein structure and mainly on enzyme function at low temperatures. The α-amylase from an Antarctic bacterium (*Pseudoalteromonas haloplanktis*) will be used here as a study model as this is the best investigated psychrophilic enzyme, displaying general features of cold-adapted proteins and usefully illustrating similarities and differences with other psychrophilic enzymes. As a general picture, psychrophilic enzymes are all faced to a main constraint, to be active at low temperatures, but the ways to reach this goal are quite diverse. Previous reviews can also be consulted for a complete coverage of this topic [12-14].

The Thermodynamic Challenge: Enzyme Activity in the Cold

The activity of enzymes is strongly dependent on the surrounding temperature. The catalytic constant k_{cat} corresponds to the maximum number of substrate molecules converted to product per active site per unit of time, and the temperature dependence of the catalytic rate constant is given by the following relation:

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

In this equation, κ is the transmission coefficient generally close to 1, k_B is the Bolzmann constant (1.38×10^{-23} J K^{-1}), h the Planck constant (6.63×10^{-34} J s), R the universal gas constant (8.31 J K^{-1} $mole^{-1}$) and $\Delta G^{\#}$ the free energy of activation or the variation of the Gibbs energy between the activated enzyme-substrate complex ES^* and the ground state ES, according to the transition state theory (see Figure 9). Accordingly, the activity k_{cat} is exponentially dependent on the temperature. As a rule of thumb, for a biochemical reaction catalyzed by an enzyme from a mesophile (a bacterium or a warm-blooded vertebrate), a drop in temperature from 37°C to 0°C results in a 20 to 80 times lower activity. This is the main factor preventing the growth of non-adapted organisms, even the simplest microbial forms, at low temperatures.

The effect of temperature on the activity of psychrophilic and mesophilic α-amylases is illustrated in Figure 1. It should be noted that equation (1) is only valid for the exponential rise of activity with temperature on the left limb of the curves. Deviation from this exponential rise occurs when thermally-induced alterations of the enzyme catalyst itself compete with thermal activation of the reaction, giving rise to an apparent temperature optimum. This is followed by the activity decay at high temperatures, when enzyme inactivation dominates as a result of unfolding or aggregation. Models have been proposed to simulate the effects of these parameters on activity [15, 16] and to take the viscosity of the medium into account [14]. The comparison of the effect of temperature on the activity of psychrophilic and mesophilic enzymes in Figure 1 reveals at least three basic features of cold-adaptation. (*i*) In order to compensate for the slow reaction rates at low temperatures, psychrophiles synthesize enzymes having an up to tenfold higher specific activity in this temperature range. This is in fact the main physiological adaptation to cold at the enzyme level. (*ii*) The temperature for apparent maximal activity for cold-active enzymes is shifted towards low temperatures, reflecting the weak stability of these proteins and their unfolding and inactivation at moderate temperatures. (*iii*) Finally, the adaptation to cold is not perfect. It can be seen in Figure 1 that the specific activity of the psychrophilic enzymes at low temperatures, although very high, remains generally lower than that of the mesophilic enzymes at 37°C.

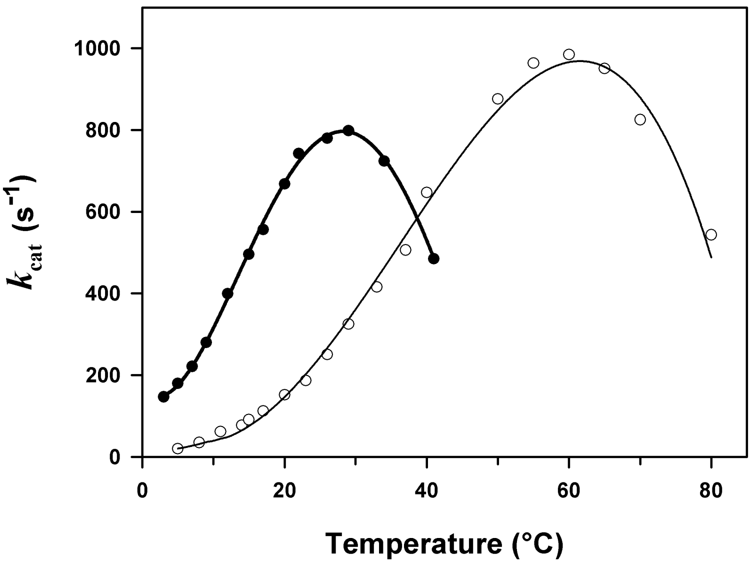

Figure 1: Temperature dependence of the activity. The activity of psychrophilic (filled symbols, heavy line) and mesophilic (open symbols) α-amylases recorded at various temperatures illustrates the main properties of cold-active enzymes (see text for details).

"Flexibility" and "Corresponding States" hypotheses

Similar observations as in Figure 1 on various psychrophilic enzymes have suggested relationships between the activity of the enzyme, the flexibility of the protein and its stability. Indeed, the high activity at low temperatures seems to arise from an increased flexibility of the protein structure, especially at temperatures that strongly slow down molecular motions, but the consequence of this improved mobility of the protein structure is of course a weak stability. In this "flexibility" hypothesis, the high activity and the low stability can be easily demonstrated experimentally, whereas determination of molecular flexibility is much more complex as it requires the definition of the types and amplitudes of atomic motions as well as a timescale for these motions. In this respect, fluorescence quenching of extremophilic enzymes was used as this method averages most of these parameters into a single signal (Figure 2). It was found that the structure of psychrophilic proteins has an improved propensity to be penetrated by a small quencher molecule, when compared to mesophilic and thermophilic proteins, and therefore revealing a less compact conformation undergoing frequent micro-unfolding events [17-19]. The "flexibility" hypothesis has received further support by the quantification of macromolecular dynamics in the whole protein content of psychrophilic, mesophilic, thermophilic and hyperthermophilic bacteria by neutron scattering [20]. This unique tool to study thermal atomic motions has indeed revealed that the resilience (equivalent to macromolecular rigidity in term of a force constant) increases with physiological temperatures. Furthermore, it was also shown that the atomic fluctuation amplitudes (equivalent to macromolecular flexibility) were similar for each microorganism at its physiological temperature. This is in full agreement with Somero's "corresponding state" concept [21] postulating that enzyme homologues exhibit comparable flexibilities to perform catalysis at their physiologically relevant temperatures.

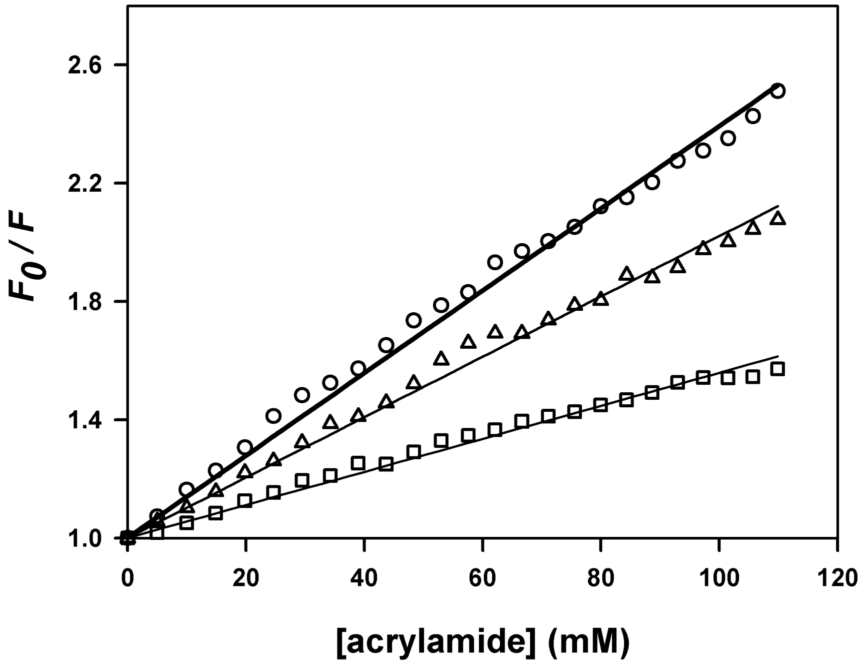

Figure 2: Permeability of the protein structure. Fluorescence quenching experiments on psychrophilic (circles, heavy line) mesophilic (triangles) and heat-stable (squares) α-amylases. The steep slope recorded for the psychrophilic enzyme indicates that its structure is easily penetrated by a small quencher molecule (acrylamide), resulting in a larger attenuation of the intrinsic fluorescence (F_0/F). This graph shows a clear correlation between this permeability index and the stability of the proteins. Adapted from [17].

However, the "flexibility" hypothesis has been challenged from an evolutionary point of view. As a matter of fact, directed evolution [22] and protein engineering [23] of enzymes have demonstrated that activity and stability are not physically linked in protein as previously thought. This is also illustrated by the discovery of a thermophilic enzyme that is about 35-fold more active at 80°C when compared to its mesophilic counterpart at 37°C. Its high activity and stability is thought to allow the enzyme to effectively compete with the spontaneous hydrolysis of its heat-labile substrate at the physiological temperature [24, 25]. Accordingly, it has been proposed that the low stability of cold-active enzymes is the result of a genetic drift related to the lack of selective pressure for stable proteins [22]. Although this seems to be obvious, several lines of evidences indicate that the situation is more subtle. For instance, in multi-domain psychrophilic enzymes containing a catalytic and a non-catalytic domain, the catalytic domain is always heat-labile (Figure 3) whereas the non-catalytic domain can be as stable as mesophilic proteins [26-28]. It is therefore unlikely that a genetic drift only affects the catalytic domain without modifying other regions of the protein. Furthermore, several directed evolution experiments have shown that when libraries of randomly mutated enzymes are only screened for improved activity at low temperatures without any other constraints, the best candidates invariably display the canonical properties of psychrophilic enzymes (see [29] for discussion). Examination of the activity versus stability plots for hundreds of mutants shows that random mutations improving both activity and stability are rare [30, 31]. It follows that improvement of activity at low temperatures associated with loss of stability appears to be the most frequent and accessible event. In conclusion, the current view suggests that the strong evolutionary pressure on psychrophilic enzymes to increase their activity at low temperatures can be accommodated for by the lack of selection for stability and represents the simplest adaptive strategy for enzyme catalysis in the cold.

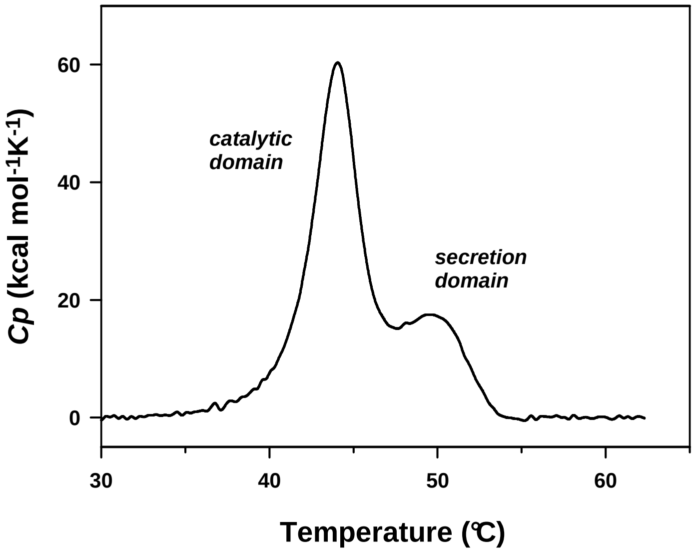

Figure 3: Stability of domains in the α-amylase precursor. The precursor is composed by a catalytic domain and a secretion helper (that is cleaved after membrane translocation to generate the native enzyme). In this thermogram, the top of the transitions corresponds to the melting points and the area under the transitions is roughly proportional to the domain sizes. Assuming that the low stability of psychrophilic enzymes is simply the result of a genetic drift (lack of selection for stable proteins), it is surprising that the more stable non-catalytic domain has not been subjected to the same extent to this drift. In fact, the stability pattern of multidomain psychrophilic enzymes suggests that the reduced stability of the catalytic structures contributes to the activity at low temperatures. Adapted from [27].

Flexibility and Structural Adaptations at the Active Site

Psychrophilic enzymes are extremely diverse in terms of source, cellular location, structure and fold, function or catalytic properties but they all share at least one property: a heat-labile activity, irrespective of the protein structural stability. Furthermore, the active site appears to be the most heat-labile structural element of these proteins [17-19]. Figure 4 illustrates this significant difference between the stability of the activity and the stability of the structure. The lower panel shows the stability of the structure as recorded by fluorescence. When the temperature is raised, the proteins undergo a transition between the native state and the unfolded state (the mid-point of the transition is defined as the melting temperature). As expected, the structure of the cold-active enzyme is less stable than the mesophilic one. In the upper panel, the activity is recorded under the same experimental conditions and it can be seen that the mesophilic enzyme is inactivated when the protein unfolds. By contrast, the situation is drastically different for the cold-active enzyme: the activity is lost well before the protein unfolds. This means that the active site is even more heat-labile than the whole protein structure. Using a combination of mutagenesis and ligand binding, it was also shown that the active site of the psychrophilic α-amylase is the first structural element that unfolds in transverse urea gradient gel electrophoresis [32]. All these aspects point to a very unstable and flexible active site and illustrate a central concept in cold adaptation: localized increases in flexibility at the active site are responsible for the high but heat-labile activity [33], whereas other regions of the enzyme might or might not be characterized by low stability when not involved in catalysis.

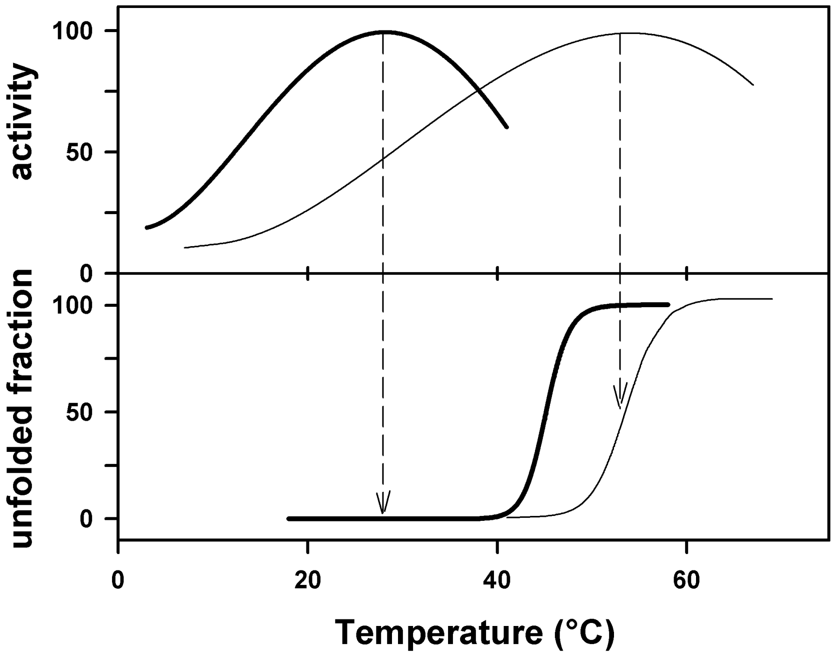

Figure 4: Inactivation and unfolding of psychrophilic enzymes. The activity of psychrophilic enzymes (upper panel, heavy line) is inactivated by temperature before unfolding of the protein structure (lower panel, heavy line) illustrating the pronounced heat-lability of the active site. By contrast, inactivation of mesophilic (thin lines) or thermophilic enzymes closely corresponds to the loss of the protein conformation. Adapted from [17].

Crystal structures of psychrophilic enzymes were of course of prime importance to investigate the properties of these heat-labile and cold-active catalytic centers. The first basic observation is that all reactive side chains as well as most side chains pointing towards the catalytic cavity are strictly conserved. This means that the overall catalytic mechanism and reaction pathway are not modified in cold-active enzymes. This aspect is not really surprising as the specific reaction mechanism of enzymes is not prone to drastic variation. However, the pattern provided by the first structure of a cold-active enzyme, the psychrophilic α-amylase [34, 35], was quite astonishing. Indeed, the X-ray structure of both the cold-active α-amylase and of its closest structural homologue from pig have been solved at high resolution in complex with acarbose, a pseudosaccharide inhibitor mimicking the transition state intermediate [36, 37]. Comparison of residues having direct or water-mediated H-bonds to the inhibitor revealed that all 24 residues involved and forming the catalytic cleft are strictly conserved in the cold-active α-amylase (Figure 5). This outstanding example of active site identity demonstrates that the specific properties of psychrophilic enzymes can be reached without any amino acid substitution in the reaction center. As a consequence, changes occurring elsewhere in the molecule are responsible for the optimization of the catalytic parameters (see *Activity-stability relationships: experimental insights*).

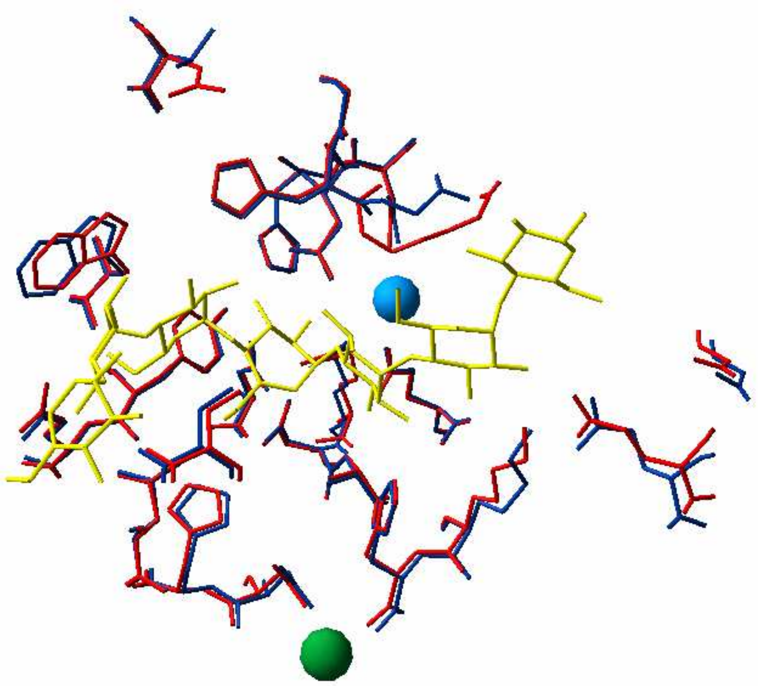

Figure 5: Structure of the active site. Superimposition of the active site residues in psychrophilic (blue) and mesophilic (red) α-amylases. The chloride and calcium ions are shown as blue and green spheres, respectively. The 24 residues performing direct or water-mediated interactions with the substrate analog derived from acarbose (yellow) are identical and superimpose perfectly within the resolution of the structures, demonstrating a structural identity in these psychrophilic and mesophilic enzymes.

Nevertheless, significant structural adjustments at the active site of psychrophilic enzymes have been frequently reported. In many cases, the catalytic cavity appears to be larger and more accessible to ligands. This larger opening of the catalytic cleft is achieved by various ways, including small deletions in loops bordering the active site, as well illustrated by a cold active citrate synthase [38] or by distinct conformation of these loops, and by replacement of bulky side chains for smaller groups at the entrance. In the case of a Ca^{2+}, Zn^{2+}-protease from a psychrophilic *Pseudomonas* species, an additional bound Ca^{2+} ion pull the backbone forming the entrance of the site (Figure 6) and markedly increases its accessibility when compared with the mesophilic homologue [39]. This large conformational change in the proteolytic domain is accompanied by deletions in the surrounding loops and by an increased local flexibility, as deduced from the crystallographic temperature factors. Furthermore, this active site conformation in the cold active protease, without bound substrate, is close to the substrate-bound conformation of the mesophilic homologue: this suggests that the psychrophilic protease already adopts a catalytically competent conformation in its substrate-free structure. As a result of such a better accessibility, cold-active enzymes can 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. The crystal structure of a psychrophilic xylanase in complex with substrate and product [40] provides support to these latter aspects. It was shown that an opening of the active site takes place upon binding of substrate or product in the cold-active enzyme whereas similar large scale movements are not observed in mesophilic or thermophilic structural homologues. This can be tentatively related to higher active site mobility in the psychrophilic enzyme. The xylanase also presents a rather smooth binding profile or gliding surface that could enable directing of the leaving product.

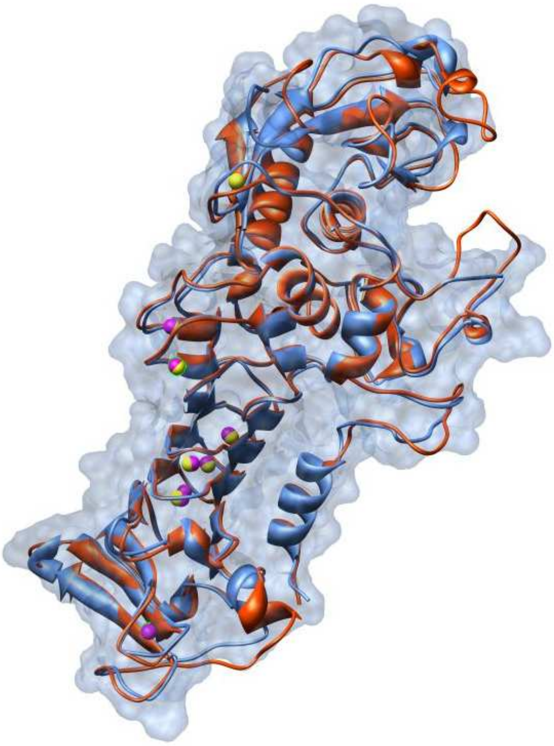

Figure 6: Active site accessibility in a psychrophilic enzyme. The molecular surface (light blue) and main chain (blue) of a cold-active Ca^{2+}, Zn^{2+}-protease is superimposed with the main chain of its mesophilic homologue (red). The active site is formed by the cleft at the upper right of the figure. The larger opening of the catalytic cavity in the psychrophilic protease results from deletions in surrounding loops and mainly from the binding of an extra calcium ion (yellow sphere) triggering large conformational changes in the proteolytic domain [39]. (Courtesy of Dr Nushin Aghajari, CNRS, Lyon, France)

In addition, differences in electrostatic potentials in and around the active site of psychrophilic enzymes appear to be a crucial parameter for activity at low temperatures. Electrostatic surface potentials generated by charged and polar groups are an essential component of the catalytic mechanism at various stages: as the potential extends out into the medium, a substrate can be oriented and attracted before any contact between enzyme and substrate occurs. Interestingly, the cold-active citrate synthase [38], malate dehydrogenase [41], uracyl-DNA glycosylase [42] and trypsin [12, 43, 44] are characterized by marked differences in electrostatic potentials near the active site region compared to their 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.

Finally, two last examples illustrate the unsuspected diversity of strategies used to improve the activity in psychrophilic enzymes. With few exceptions, β-galactosidases are homotetrameric enzymes bearing four active sites. However, the crystal structure of a cold-active β-galactosidase revealed that it is a homohexamer, therefore possessing six active sites [45]. This is the first known β-galactosidase structure in the form of compact hexamers. This unusual quaternary structure, containing two additional active sites, certainly contributes to improve the activity at low temperatures. Cellulases are microbial enzymes displaying a modular organization made of a globular catalytic domain connected by a linker to a cellulose binding domain. Psychrophilic cellulases were found to possess unusually long linkers of more than 100 amino acid residues, *i.e.* about five times longer than in mesophilic cellulases [46, 47]. The molecular dimensions of a psychrophilic cellulase in solution have been determined by small angle X-ray scattering [47]. It was shown that the long linker adopts a large number of conformations between the fully extended and bended conformations, in caterpillar-like motions. Considering the cellulose-binding domain anchored to the cellulose fibers and a rotation of the extended molecule around this axis, it was calculated that the catalytic domain has a 40-fold higher accessible surface area of substrate when compared with a mesophilic cellulase possessing a much shorter linker. Here also, increasing the available surface of the insoluble substrate to the catalytic domain should improve the activity of this enzyme at low temperatures.

Active Site Dynamics

The heat-labile activity of psychrophilic enzymes suggests that the dynamics of the functional side chains at the active site is improved in order to contribute to cold-activity and the above mentioned structural adaptations seem to favor a better accessibility to the substrate and release of the product. This view is strongly supported by the enzymological properties of cold-active enzymes. Non-specific psychrophilic enzymes accept various substrates and have a broader specificity than the mesophilic homologues, because substrates with slightly distinct conformations or sizes can fit and bind to the site. For instance, the observed differences in substrate specificity between Atlantic salmon and mammalian elastases have been interpreted to be based on a somewhat wider and deeper binding pocket for the cold-adapted elastase [12]. The broad specificity of a psychrophilic alcohol dehydrogenase that oxidizes large bulky alcohols was also assigned to a highly flexible active site [48].

The active site flexibility of cold-active enzymes in solution is also well demonstrated by the psychrophilic α-amylase [49]. In this specific case, the above mentioned structural identity of the catalytic cleft with its mesophilic homologue from pig precludes the involvement of adaptive mutations within the active site in the analysis of the results. As shown in Table 1, both the psychrophilic and mesophilic α-amylases degrade large macromolecular polysaccharides made of glucose units linked by α-1,4 bonds. These substrates have a complex structure and are generally branched. Taking the natural substrate, starch, as the reference, it can be seen that the psychrophilic enzyme is more active on all these large substrates. Being more flexible, the active site can accommodate easily these macromolecular polysaccharides. Considering the small substrates, the reverse situation is observed. Both enzymes are active on short oligosaccharides of at least four glucose units but in this case, the psychrophilic α-amylase is less active on all these small substrates. Apparently, the flexible active site accommodates less efficiently these short oligosaccharides.

Table 1. Relative activity of the psychrophilic (AHA) and the mesophilic (PPA) α-amylases on macromolecular polysaccharides and on maltooligasaccharides. Adapted from [49].

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

The inhibition patterns provide additional insights into the specific properties of psychrophilic active sites (Figure 7). Both the mesophilic and the psychrophilic α-amylases are inhibited by maltose, the end product of starch hydrolysis. In the case of the mesophilic enzyme, the enzyme can bind either the substrate (in a productive mode) or the inhibitor, but not both. By contrast, the cold-active enzyme can also bind either the substrate or the inhibitor but also both, forming the ternary complex ESI. Thus, the same active site, formed by the same residues, is able to accommodate a larger complex and this of course suggests a more accessible and flexible active site.

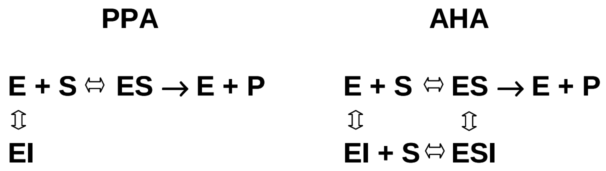

Figure 7. Inhibition models of α-amylases. Reaction pathways for the competitive inhibition of starch hydrolysis by maltose for the mesophilic α-amylase PPA and of the mixed type inhibition for the psychrophilic α-amylase AHA. Under identical experimental conditions, the cold-active enzyme forms the ternary complex ESI [49].

In addition, the psychrophilic enzyme has been crystallized in complex with two competitive inhibitors of α-amylases [36]. Although several α-amylases, including the mesophilic homologue, have been crystallized in the same conditions, only the cold-active enzyme has formed such ternary complex and this seems to be a unique example of the simultaneous occurrence of two competitive inhibitors within the crystal structure of an enzyme active site [36]. These kinetic and crystallographic results are also compatible with a more mobile and flexible active site in psychrophilic enzymes.

Adaptive Drift and Adaptive Optimization of Substrate Affinity

As a consequence of the improved active site dynamics in cold-active enzymes, substrates bind less firmly in the binding site (if no point mutations have occurred) giving rise to higher K_m values. This is the main structural explanation for the observation that most cold-active enzymes, which are strongly homologous to mesophilic counterparts at the active site, have lower binding constants. An example is given in Table 2 showing that the psychrophilic α-amylase is more active on its macromolecular substrates whereas the K_m values are up to 30-fold larger, *i.e.* the affinity for the substrates is up to 30-fold lower. Ideally, a functional adaptation to cold would mean optimizing both k_{cat} and K_m. However, a survey of the available data on psychrophilic enzymes [50] showed that optimization of the k_{cat} / K_m ratio is far from a general rule but on the contrary that the majority of cold-active enzymes improve the k_{cat} value at the expense of K_m, therefore leading to suboptimal values of the k_{cat} / K_m ratio, as also shown in Table 2. There is in fact an evolutionary pressure on K_m to increase in order to maximize the overall reaction rate. This aspect will be developed in the next section. Such adaptive drift of K_m has been well illustrated by the lactate dehydrogenases from Antarctic fish [33] and by the psychrophilic α-amylase [51] because both enzymes display rigorously identical substrate binding site and active site architecture when compared with their mesophilic homologues. The latter condition is essential as such identity ensures that discrete amino acid substitutions are not responsible for the differences in kinetic parameters and hence that the conformation or the dynamic properties of the sites are the relevant parameters that have to be taken into consideration. In both cases, temperature-adaptive increases in k_{cat} occur concomitantly with increases in K_m in cold-active enzymes. As already mentioned, such identity of the sites also implies that adjustments of the kinetic parameters are obtained by structural changes occurring distantly from the reaction center. This aspect has received strong experimental support [52] as discussed latter in this chapter.

Table 2. Kinetic parameters for the hydrolysis of polysaccharides at 25°C by the psychrophilic (AHA) and the mesophilic (PPA) α-amylases. Adapted from [49].

Substrate	AHA			PPA		
	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

Several enzymes, especially in some cold-adapted fish, counteract this adaptive drift of K_m in order to maintain or to improve the substrate binding affinity by amino acid substitutions within the active site [12]. 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. By contrast, interactions of electrostatic nature (ion pairs, hydrogen bounds, Van der Waals interactions) form exothermically and are stabilized at low temperatures. Therefore low temperatures do not only reduce the enzyme activity (k_{cat}), but can also severely alter the substrate binding mode according to the type of interaction involved.

The chitobiase from an Antarctic bacteria nicely illustrates both aspects, as well as the extent of the kinetic optimization that can be reached during cold adaptation of enzymes [26]. Figure 8 shows that 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. Because the cell-bound bacterial chitobiase has to access its substrate in the extracellular medium, the physiological advantage of a high affinity for the substrate is clear. 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 is decreased. 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. In another case, it was also possible to correlate the kinetic optimization with the stability pattern in a psychrophilic enzyme. The phosphoglycerate kinase from an Antarctic bacterium optimizes both k_{cat} and K_m values for its substrates [53]. The stability pattern of this enzyme is unusual as it displays a large unstable domain and a smaller stable domain as shown by microcalorimetry [54]. It was found that the latter domain is formed by the residues at and around the nucleotide binding site. This has led to the proposal that the large heat-labile domain contributes to improve the activity at low temperatures whereas the heat-stable domain provides a compact structure improving the binding affinity for the nucleotide, therefore increasing the catalytic efficiency k_{cat}/K_m.

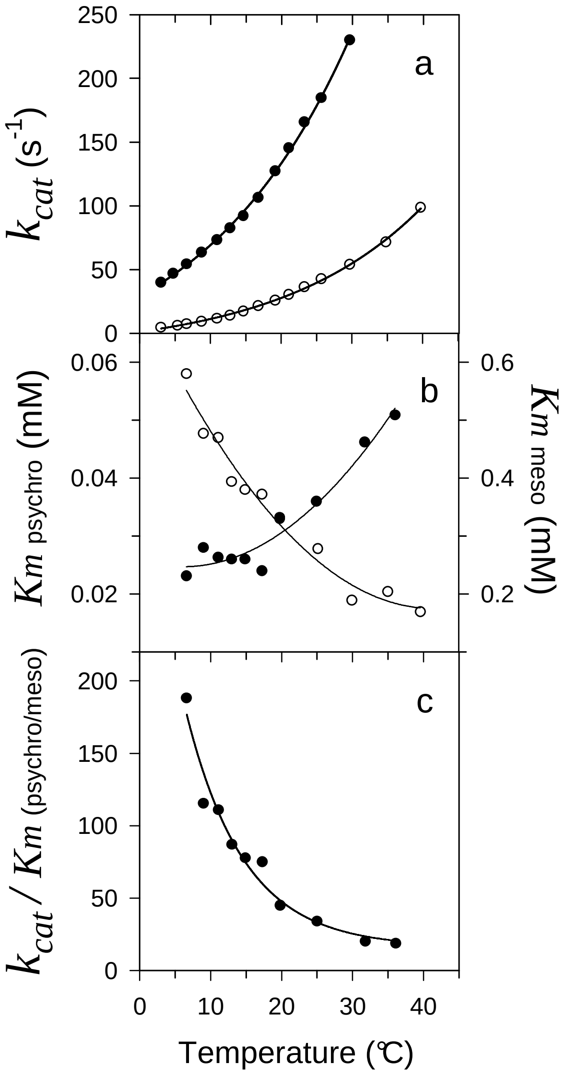

Figure 8: Kinetic optimization in a cold-active chitobiase. Temperature dependence of the kinetic parameters for psychrophilic (closed symbols) and mesophilic (open symbols) chitobiases. Data for (a) the catalytic rate constant k_{cat} , (b) the Michaelis parameter K_m , note the different scales used and (c) the relative catalytic efficiency k_{cat}/K_m (psychrophile/mesophile). The cold-adapted chitobiase is characterized by a higher activity, an optimal K_m value at low temperatures and a 200-times higher catalytic efficiency at 7°C. Adapted from [26].

Energetics of Activity at Low Temperatures

Referring to equation (1), the high activity of cold-adapted enzymes corresponds to a decrease of the free energy of activation $\Delta G^{\#}$. Two strategies have been highlighted to reduce the height of this energy barrier: the first only applies to psychrophilic enzymes displaying a drift of the K_m value, *i.e.* a low affinity for the substrate, whereas the second strategy seems to be more general.

Figure 9 illustrates the first strategy where an evolutionary pressure increases K_m in order to maximize the reaction rate. According to the transition state theory, when the enzyme encounters its substrate, the enzyme-substrate complex ES falls into an energy pit. For the reaction to proceed, an activated state $ES^{\#}$ has to be reached, that eventually breaks down into the enzyme and the product. The height of the energy barrier between the ground state ES and the transition state $ES^{\#}$ is defined as the free energy of activation $\Delta G^{\#}$: the lower this barrier, the higher the activity as reflected in equation (1). In the case of cold active enzymes displaying a weak affinity for the substrate, the energy pit for the ES complex is less deep (dashed in Figure 9). It follows that the magnitude of the energy barrier is reduced and therefore the activity is increased. This thermodynamic link between affinity and activity is valid for most enzymes (extremophilic or not) under saturating substrate concentrations and this link appears to be involved in the improvement of activity at low temperatures in numerous cold-active enzymes [33, 50]. Two examples of crystal structures of psychrophilic enzymes can be cited to support this mechanism. In the structure of a cold-active xylanase in complex with its substrate, analysis of the bound carbohydrate conformation indicates that the psychrophilic enzyme is not optimized for stabilizing the ground state conformation of its substrate [40]. Aspartate carbamoyltransferase is an allosteric enzyme and the structure of a cold-active representative has been solved [55]. It was shown that its active site is more open and hence would significantly alter interactions with bound substrate. Furthermore, the unliganded structure of this allosteric enzyme appears to be in an extreme T state of low affinity (in contrast with the high affinity R state).

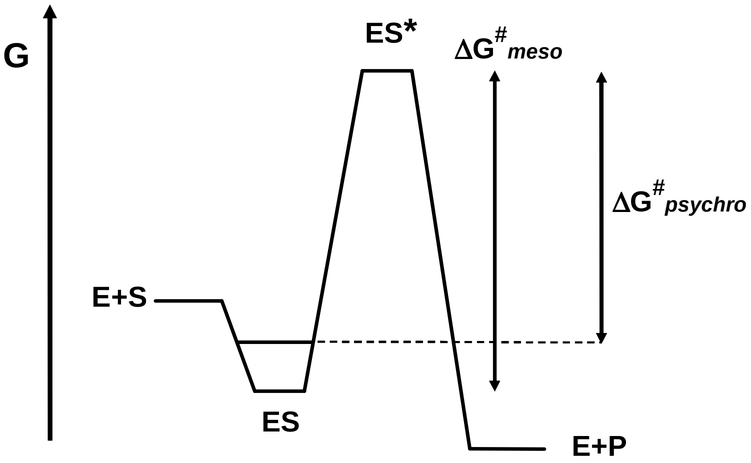

Figure 9: Optimization of activity by decreasing substrate affinity in psychrophilic enzymes. Reaction profile for an enzyme-catalyzed reaction with Gibbs energy changes under saturating substrate concentration. Weak substrate binding (dashed line) decreases the activation energy ($\Delta G^{\#}psychro$) and thereby increases the reaction rate (see text for details).

The second and much more general strategy involves the temperature-dependence of the reaction catalyzed by cold-active enzymes. Table 3 reports the enthalpic and entropic contributions to the free energy of activation in extremophilic α-amylases. The free energy of activation $\Delta G^{\#}$ is calculated from equation (1) using the k_{cat} value at a given temperature and the enthalpy of activation $\Delta H^{\#}$ is obtained by recording the temperature dependence of the activity [56]. Finally, the entropic contribution $T\Delta S^{\#}$ is deduced from the classical Gibbs-Helmholtz equation

$$\Delta G^{\#} = \Delta H^{\#} - T\Delta S^{\#} \tag{2}$$

The enthalpy of activation $\Delta H^{\#}$ basically depicts the temperature dependence of the activity: the lower this value, the lower the variation of activity with temperature. The low value found for almost all psychrophilic enzymes demonstrates that, by keeping a low temperature dependence of the activity, their reaction rate is less reduced than for other enzymes when the temperature is lowered. Accordingly, the decrease of the activation enthalpy in the enzymatic reaction of psychrophilic enzymes can be considered as the main adaptive character to low temperatures. This decrease in activation energy is structurally achieved by a decrease in the number of enthalpy-driven interactions that have to be broken during the activation steps. These interactions also contribute to the stability of the protein folded conformation and, as a corollary, the structural domain of the enzyme bearing the active site should be more flexible. It is interesting to note that such a macroscopic interpretation of the low activation enthalpy in cold-active enzymes fits with the experimental observation of a markedly heat-labile activity illustrated in Figure 4. Table 3 also shows that the entropic contribution $T\Delta S^{\#}$ for the cold-active enzyme is larger and negative. This has been interpreted as a large reduction of the apparent disorder between the ground state with its presumably relatively loose conformation and the well organized and compact transition state [56]. Again, the heat-labile activity of cold-active enzymes suggests a macroscopic interpretation for this thermodynamic parameter. As a consequence of active site flexibility, the enzyme-substrate complex ES (i.e. the ground state) occupies a broader distribution of conformational states translated into increased entropy of this state, compared to that of the mesophilic or thermophilic homologues. It should be noted that this assumption has received strong experimental support by using microcalorimetry to compare the stabilities of free extremophilic enzymes with the same enzymes trapped in the transition state conformation by a non-hydrolysable substrate analog [17]. The larger increase in stability for the psychrophilic enzyme in the transition state conformation demonstrated larger conformational changes between the free and bound states when compared to mesophilic and thermophilic homologues. Furthermore, a broader distribution of the ground state ES should be accompanied by a weaker substrate binding strength, as indeed observed for numerous psychrophilic enzymes. In contrast to the example given in Table 3, the reaction catalyzed by some cold-active enzymes proceed with a positive entropic contribution, for instance when bound water molecules are released from the active site during substrate binding. A general methodology has been proposed to analyze these data by comparing the variation of the thermodynamic parameters between psychrophilic and mesophilic enzymes [56] that also points to a broader distribution of the ground state.

Table 3: Activation parameters of the hydrolytic reaction of α-amylases at 10°C. Adapted from [17].

	k_{cat} s^{-1}	$\Delta G^{\#}$ kcal mol^{-1}	$\Delta H^{\#}$ kcal mol^{-1}	$T\Delta S^{\#}$ kcal mol^{-1}
Psychrophile	294	13.8	8.3	-5.5
Mesophile	97	14.0	11.1	-2.9
Thermophile	14	15.0	16.8	1.8

Conformational Stability of Extremophilic Proteins

Considering the numerous insights for strong relationships between activity and stability in psychrophilic enzymes, the conformational stability of these proteins has been intensively investigated in comparison with mesophilic and thermophilic counterparts. Beside this functional aspect, psychrophilic proteins were found to be valuable molecules for biophysical studies of protein folding and stability in general. The main points are summarized below.

Figure 10 displays the calorimetric records, or thermograms, of heat-induced unfolding for psychrophilic, mesophilic and thermophilic proteins. These enzymes clearly show distinct stability patterns that evolve from a simple profile in the unstable psychrophilic proteins to a more complex profile in very stable thermophilic counterparts. The unfolding of the cold-adapted enzymes occurs at lower temperatures as indicated by the T_m values which correspond to the temperature of half-denaturation (for a two-state process) and is given by the top of the transition. This property is known for decades and has been highlighted by various techniques. By contrast, the energetics of structure stability was essentially revealed by microcalorimetry [18, 19, 51]. The area under the curves in Figure 10 corresponds to the total amount of heat absorbed during unfolding and is given by the calorimetric enthalpy, ΔH_{cal}. This parameter reflects the enthalpy of disruption of bonds involved in maintaining the compact structure and is markedly lower for the psychrophilic enzymes. In addition, there is a clear trend for increasing ΔH_{cal} values in the order psychrophile < mesophile < thermophile. The transition for the psychrophilic enzymes 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 enzymes: the structure is stabilized by fewer weak interactions and disruption of some of these interactions strongly influences the whole molecular edifice and promotes its unfolding. The psychrophilic enzymes unfold according to an all-or-none process, revealing a uniformly low stability of the architecture. By contrast, all other homologous enzymes display two to three transitions (either observable or indicated by deconvolution of the heat capacity function in Figure 10). Therefore, the conformation of these mesophilic and thermophilic enzymes contains structural blocks or units of distinct stability that unfold independently. Finally, the unfolding of the psychrophilic proteins is frequently more reversible than that of other homologous enzymes that are irreversibly unfolded after heating. The weak hydrophobicity of the core clusters in cold-adapted enzymes and the low melting temperature, at which hydrophobic interactions are restrained, certainly account for this reversible character because, unlike mesophilic proteins, aggregation does not occur or occurs to a lower extent. From these observations, it can be concluded that psychrophilic enzymes possess a fragile molecular edifice that is uniformly unstable and stabilized by fewer weak interactions than homologous mesophilic or thermophilic proteins.

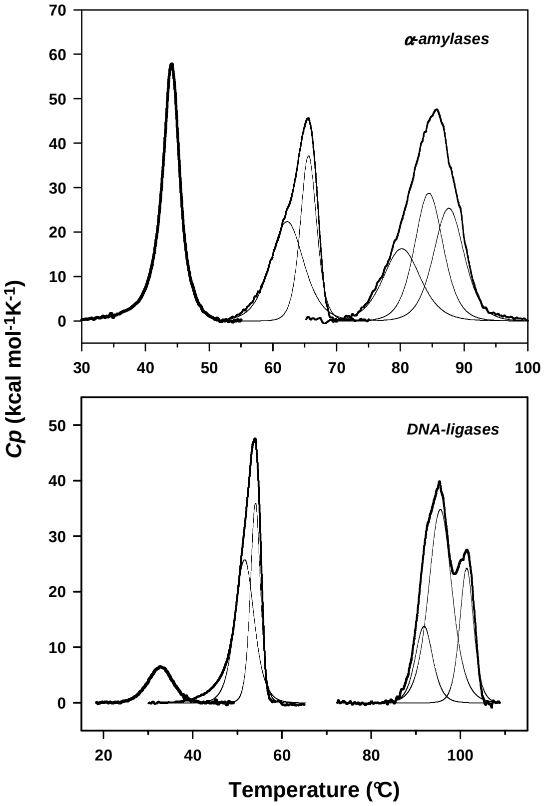

Figure 10: Thermal unfolding of extremophilic enzymes. Thermograms of α-amylases and DNA-ligases recorded by differential scanning microcalorimetry showing, from left to right on each panel, psychrophilic (heavy lines), mesophilic and (hyper)thermophilic proteins. The cold-active enzymes are characterized by a lower T_m (top of the transition) and ΔH_{cal} (area under the transition), by a sharp and cooperative transition and by the lack of stability domains (indicated by thin lines in stable proteins). Adapted from [19, 51].

As a practical and useful consequence of the above-mentioned unfolding reversibility, it has been possible to calculate accurately the conformational stability of the psychrophilic α-amylase over a broad range of temperatures [57]. The comparison of these data with those of other proteins reveals some unsuspected properties of cold-adapted proteins. The thermodynamic stability of a protein that unfolds reversibly according to a two-state mechanism

$$N \rightleftharpoons U \tag{3}$$

is described by its stability curve [58], *i.e.* the free energy of unfolding as a function of temperature (Figure 11). In other words, this is the work required to disrupt the native state at any given temperature [59] and is also referred to as the conformational stability. By definition, this stability is nil at the melting point (equilibrium constant K = [U] / [N] = 1 and $\Delta G = -RT\ln K$). At temperatures below the melting point, the stability increases, as expected, but perhaps surprisingly for the non-specialist, the stability reaches a maximum close to room temperature then it decreases at lower temperatures (Figure 11). In fact, this function predicts a temperature of cold-unfolding, which is generally not observed because it occurs below 0°C. Nevertheless, cold-unfolding has been well demonstrated under specific conditions [60]. Increasing the stability of a protein is essentially obtained by lifting the curve towards higher free energy values, also resulting in an increased melting point [61-64]. As far as extremophiles are concerned, one of the most puzzling observations of the last decade is that most proteins obey this pattern, *i.e.* whatever the microbial source, either from deep-vents or from polar ice, the maximal stability of their proteins is clustered around room temperature (for more details see [64]). Accordingly, the environmental temperatures for mesophiles and (hyper)thermophiles lie on the right limb of the bell-shaped stability curve and obviously, the thermal dissipative force is used to promote molecular motions in these molecules. By contrast, the environmental temperatures for psychrophiles lie on the left limb of the stability curve. It follows that molecular motions in proteins at low temperatures are gained from the factors ultimately leading to cold-unfolding [57], *i.e.* the hydration of polar and non-polar groups [65]. The origin of flexibility in psychrophilic enzymes at low temperatures is therefore drastically different from mesophilic and thermophilic proteins, the latter taking advantage of the conformational entropy rise with temperature to gain in mobility. The improved interactions with the solvent of cold-active enzymes suggested by their crystal structures (see below), reinforces the idea that group hydration plays an essential role in the acquisition of flexibility in psychrophilic proteins.

A surprising consequence of the free energy function for the psychrophilic α-amylase shown in Figure 11 is its weak stability at low temperatures when compared with mesophilic and thermophilic proteins, whereas it was intuitively expected that cold-active proteins should also be cold stable. This protein is in fact both heat and cold labile. Assuming constant properties of the solvent below 0°C (*i.e.* no freezing) and the absence of protective effects from cellular components, this α-amylase should unfold at –10°C. Therefore cold denaturation of some key enzymes in psychrophiles can be an additional, though unsuspected factor fixing the lower limit of life at low temperatures. It has also been shown that the psychrophilic α-amylase has reached a state close to the lowest possible stability of the native state. For instance, site-directed mutagenesis experiments designed to reduce its stability frequently resulted in transiently stable mutant enzymes, which could not be purified [51]. 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 towards a more mobile structure. This aspect can account for the imperfect adaptation of the catalytic function in some psychrophilic enzymes, mentioned at the beginning of this chapter and illustrated in Figure 1.

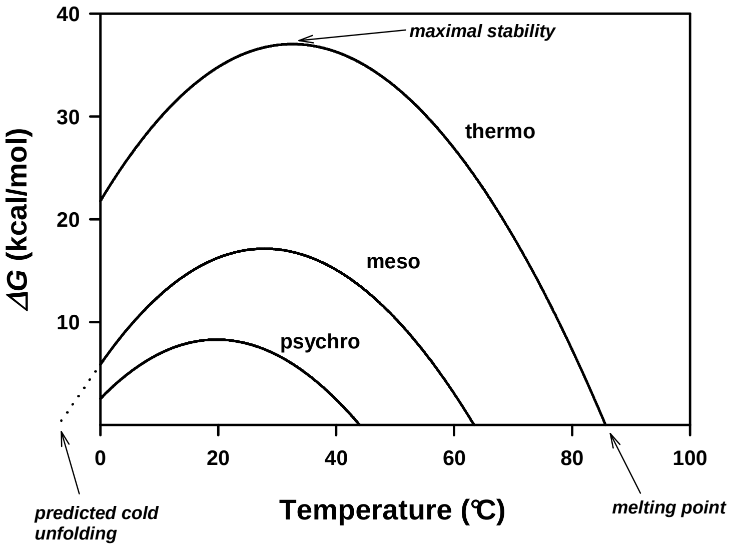

Figure 11: Representative stability curves of homologous extremophilic proteins. The energy required to disrupt the native state (*i.e.* the conformational stability) is plotted as a function of temperature. At the melting point, this energy = 0 and in addition, the curves also predict cold unfolding and a maximal stability close to room temperature. A high stability in thermophiles is reached by lifting the curve towards higher free energy values, whereas the low stability in psychrophiles corresponds to a collapse of the bell-shaped stability curve. Adapted from [17].

Structural Basis of Low Stability

Following the first analyses of homology-based models, the number of X-ray crystal structures from psychrophilic enzymes has increased dramatically, demonstrating the growing interest for these peculiar proteins. However, the interpretation of these structural data is frequently difficult for two main reasons. First, the structural adaptations are extremely discrete, when compared with mesophilic proteins, and can easily escape the analysis. Second, these structural adaptations are very diverse, reflecting the complexity of factors involved in the stability of a macromolecule at the atomic level. For instance, it was found that all structural factors currently known to stabilize the protein molecule could be attenuated in strength and number in the structure of cold-active enzymes [12, 66, 67]. An example is given in Table 4 which provides a summary of the structural factors possibly involved in the low stability of a psychrophilic α-amylase. An exhaustive description of all these factors is beyond the scope of this chapter and only the essential features are summarized below. Two review articles can be consulted for a comprehensive discussion of this topic [12, 14].

The observable parameters related to protein stability include structural factors and mainly weak interactions between atoms of the protein structure. In psychrophilic proteins, this involves the clustering of glycine residues (providing local mobility), 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 by weakening the hydrophobic effect on folding. The N and C-caps of α-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 [35, 38]. 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 instances an excess of negative charges at the surface of the protein 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 [57]. 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, to thermophiles and hyperthermophiles, the latter showing arginine-mediated multiple ion pairs and interconnected salt bridge networks [68, 69]. Such an altered pattern of electrostatic interactions at the molecular surface is thought to improve the dynamics [70] or the “breathing” of the external shell of cold-active enzymes.

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 in order to improve the local or global mobility of the protein edifice. Comparative structural analyses of psychrophilic, mesophilic and thermophilic enzymes indicate that each protein family displays 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 [67, 71-73]. As a result of the great diversity of factors involved in protein stability, the bias in the amino acid composition observed in individual psychrophilic protein (low proline or arginine content, etc…) is not found when analyzing the mean amino acid composition of the whole genome. On the contrary, the available genomic data have produced ambiguous results [1-4] and it is currently difficult to correlate the reported trends in genomic amino acid composition with adaptations to low temperatures or with species-specific differences. By contrast, the comparative genomics of DNA fragments sequenced from Antarctic environmental samples has consistently reproduced the above mentioned trends observed in individual proteins [74]. For instance, this metagenomic approach of psychrophilic protein sequences pointed to a reduction in salt bridge-forming residues, reduced proline content, reduction in stabilizing hydrophobic clusters and longer stretches of disordered residues. This discrepancy between the genomic and metagenomic studies remains to be explained.

Table 4. Structural parameters potentially involved in the stability of α-amylase conformation as deduced from the X-ray structures [35, 37, 75]

α-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 (ΣH_i)		47	103
Non-constrained residues at C terminus		0	6
Aromatic-aromatic interactions		19	20
Amino-aromatic interactions		2	13
Oxygen-aromatic interactions		24	19
Sulfur-aromatic interactions		2	5
α-helix dipole	- charges in Nα	2	4
	+ charges in Cα	3	6
Calcium binding constant (M^{-1})		$5.0\ 10^{7}$	$2.0\ 10^{11}$

Activity-Stability Relationships: Experimental Insights

In order to check the validity of the proposed relationships between the activity and the stability in cold-active enzymes, the psychrophilic α-amylase has been used as a model because, as already mentioned in this chapter, the identical architecture of its active site, when compared with a close mesophilic homologue, indicates that structural adaptations affecting the active site properties occur outside from the catalytic cavity. Accordingly, the crystal structure [34, 35] has been closely inspected to identify structural factors involved in its weak stability, such those described in the previous section and in Table 4. On this basis, 17 mutants of this enzyme were constructed, each of them bearing an engineered residue forming a weak interaction found in mesophilic α-amylases but absent in the cold-active α-amylase, or a combination of up to six stabilizing structural factors [51, 52, 76]. As illustrated in Figure 12, it was found that single amino acid side chain substitutions can significantly modify the melting point T_m and the calorimetric enthalpy ΔH_{cal} but also the cooperativity and reversibility of unfolding as well as the thermal inactivation rate constant. Therefore, these mutants of the psychrophilic α-amylase consistently approximate and reproduce the unfolding patterns of the heat-stable enzymes depicted in Figure 10. Thus, a fascinating aspect of this study was the ability to dissect the contribution of individual amino acid side chain to the structural properties of heat-stable proteins by engineering the psychrophilic enzyme.

However, in the context of catalysis at low temperatures, the most significant observation was that the overall trend of these mutations is to decrease both k_{cat} and K_m. As shown in Figure 13, stabilizing the cold-active α-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 both kinetic parameters (illustrated in Figure 9). In fact, in addition to an engineered mesophilic-like stability, the multiple-mutant bearing six stabilizing structural factors also displays an engineered mesophilic-like activity in terms of alterations in k_{cat} and K_m values and even in thermodynamic parameters of activation [52]. Considering the various available data on the psychrophilic α-amylase, it can be concluded that the improved molecular motions of the side chains forming the active site (motions responsible for the high activity, the low affinity and heat-lability) originate from the lack of structure-stabilizing interactions in the vicinity or even far from the active site. This is also another strong indication that structural flexibility is an essential feature related to catalysis at low temperatures in psychrophilic enzymes.

It should be mentioned that other mutational studies of cold-active enzymes have been less conclusive. Various psychrophilic enzymes (citrate synthase [77], subtilisin [78], alkaline phosphatase [79, 80], ribonuclease [81] or chitinase [82]) have been engineered in order 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 works 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. It should be mentioned however that a single mutation in the calcium-binding site of subtilisin [78] and in the phosphate-binding helix of triose phosphate isomerase [83] were shown to drastically increase the thermostability of the psychrophilic mutants. These results underline the strong involvement of bound cofactors in the modulation of stability and activity.

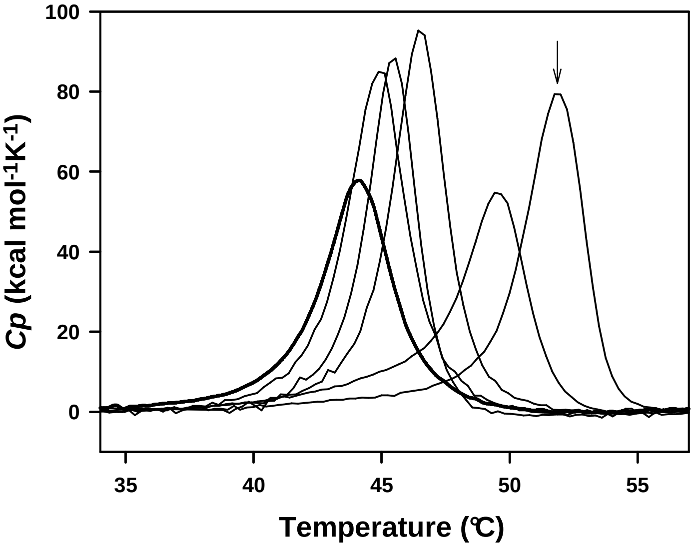

Figure 12: Engineering mesophilic-like stability in mutants of the psychrophilic α-amylase. Structure-stabilizing interactions have been introduced in the heat-labile enzyme (heavy line). As shown by the microcalorimetric thermograms, the resulting mutants (thin lines) display increased melting points (top of the transitions) and calorimetric enthalpies (area below the curves). The most stable mutant (arrow) bears six additional interactions. Adapted from [51, 52].

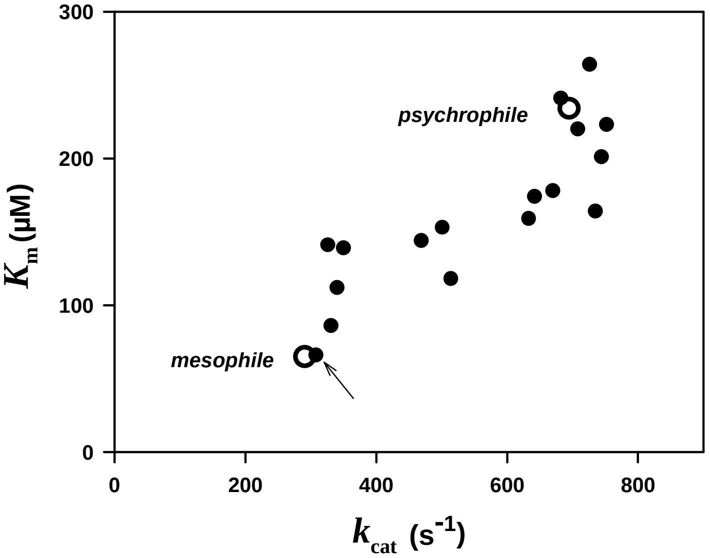

Figure 13: Engineering mesophilic-like activity in mutants of the psychrophilic α-amylase. This plot of the kinetic parameters for the stabilized mutants (filled symbols) shows that the general trend is to decrease the activity and to increase the affinity for the substrate of the wild-type psychrophilic enzyme (open symbol). The most stable mutant bearing six additional interactions (arrow) displays kinetic parameters nearly identical to those of the mesophilic homologue (open symbol). Adapted from [51, 52].

In Vivo Protein Folding in Psychrophiles

Nearly all specific properties of psychrophilic enzymes described in this chapter have been studied *in vitro*. However, cellular factors and regulations directly involved in protein and enzyme functions should not be neglected as they establish an essential link between the macromolecular structure and the cell cycle in the relevant environmental conditions. In this respect, protein folding appears to be finely regulated in psychrophilic bacteria. These mechanisms should be of prime importance for cold-adaptation as they participate to the acquisition of the native and biologically active conformation of psychrophilic proteins.

When emerging from the ribosome, the nascent polypeptide must fold into precise three-dimensional structure. In the crowded cytosol, molecular chaperones promote and catalyze efficient folding by preventing misfolding and aggregation. Amongst these molecular chaperones, the chaperonins GroEL and GroES in the mesophile *E. coli* participate in the folding of over 30% of cellular proteins. The homologues of these chaperonins from the Antarctic bacterium *Oleispira antarctica* have been isolated and characterized [84]. It was shown that these psychrophilic chaperonins have an optimal refolding activity at low temperatures, in sharp contrast with the GroEL and GroES chaperonins from *E. coli* that are nearly inactive at these temperatures. The activity of the psychrophilic chaperonins was further assigned to a transition from a double ring to a single ring conformation of the quaternary structure in the cold [85]. Interestingly, when these cold-active chaperonins are expressed in *E. coli*, the mesophilic bacterium becomes able to grow at much lower temperatures, therefore highlighting that optimizing protein folding in the cold is an essential component of temperature adaptation in psychrophilic bacteria. It should be noted that such strategy may not be a general trait for cold-adapted microorganisms as similar results have not been obtained when using the chaperonins from another Antarctic bacterium, *Pseudoalteromonas haloplanktis* [3, 86].

Another interesting insight into protein folding in the cold has been provided by proteomic studies of psychrophilic bacteria. When comparing the proteomes expressed at high and low temperatures, a peptidyl-prolyl *cis-trans* isomerase was found to be overexpressed by two psychrophilic bacteria growing at low temperature [6, 87]. This enzyme is also directly involved in protein folding as it catalyzes the *cis-trans* isomerization of peptide bonds N-terminal of proline residues. For many proteins, this isomerization is the last and rate-limiting step of their folding before reaching the native and active state. Therefore, overexpression of this enzyme by psychrophilic bacteria obviously contributes to speed up this rate-limiting step and seems to be a key adaptation to growth at low temperatures. The above mentioned studies on chaperonins and isomerases strongly suggest that protein folding is a rate-limiting step for bacterial growth at low temperatures and that adaptive cellular mechanisms are required to improve protein folding in these microorganisms.

Conclusion

The various properties of psychrophilic enzymes that have been presented in this chapter can be integrated in a model based on folding funnels [88-91] to describe the activity-stability relationships in extremophilic enzymes. Figure 14 depicts the energy landscapes of psychrophilic and thermophilic enzymes. The top of the funnel is occupied by the unfolded state, adopting numerous available conformations and having a high free energy (considering the spontaneous folding reaction), whereas the bottom of the funnel is occupied by the stable (low free energy) native state adopting a limited number of catalytically active conformations. The height of the funnel, *i.e.* the free energy of folding, also corresponding to the conformational stability, has been fixed here in a 1 to 5 ratio according to the stability curves shown in Figure 11. The upper edge of the funnels is occupied by the unfolded state in random coil conformations but it should be noted that psychrophilic enzymes tend to have a lower proline content than mesophilic and thermophilic enzymes, a lower number of disulfide bonds and a higher occurrence of glycine clusters [12, 14, 66, 67, 92]. Accordingly, the edge of the funnel for the psychrophilic protein is slightly larger (broader distribution of the unfolded state) and is located at a higher energy level. When the polypeptide is allowed to fold, the free energy level decreases, as well as the conformational ensemble. However, thermophilic proteins pass through intermediate states corresponding to local minima of energy. These minima are responsible for the ruggedness of the funnel slopes and for the reduced cooperativity of the folding-unfolding reaction, as demonstrated by heat-induced unfolding (Figure 10). By contrast, the structural elements of psychrophilic proteins generally unfold cooperatively without intermediates, as a result of fewer stabilizing interactions and stability domains [51, 57] and therefore the funnel slopes are steep and smooth. The bottom of the funnel, which depicts the stability of the native state ensemble, also displays significant differences between both extremophilic enzymes. The bottom for a very stable and rigid thermophilic protein can be depicted as a single global minimum or as having only a few minima with high energy barriers between them [93, 94] 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 [93, 94] and is drawn here according to the results of fluorescence quenching (Figure 2) and neutron scattering experiments [20]. In this context, the activity-stability relationships in these extremophilic enzymes depend on the bottom properties. Indeed, it has been argued that upon substrate binding to the association-competent sub-population, the equilibrium between all conformers is shifted towards this sub-population, leading to the active conformational ensemble [93-95]. In the case of the rugged bottom of psychrophilic enzymes, this equilibrium shift only requires a modest free energy change (low energy barriers), a low enthalpy change for interconversion of the conformations but is accompanied by a large entropy change for fluctuations between the wide conformer ensemble. The converse picture holds for thermophilic enzymes, in agreement with the activation parameters shown in Table 3 and with the proposed macroscopic interpretation. Such energy landscapes integrate nearly all biochemical and biophysical data currently available for extremophilic enzymes but they will certainly be refined by future investigations of other series of homologous proteins from psychrophiles, mesophiles and thermophiles.

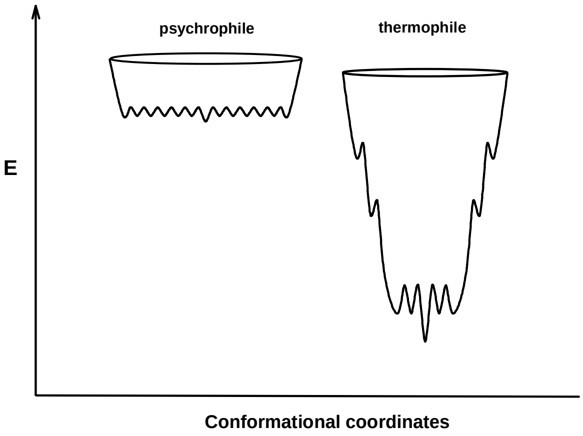

Figure 14: Folding funnel model of enzyme temperature adaptation. In these schematic energy landscapes for extremophilic enzymes, the free energy of folding (E) is depicted as a function of the conformational diversity. The height of the funnels is deduced from the determination of the conformational stabilities. The top of the funnels is occupied by the unfolded states in the numerous random coil conformations, whereas the bottom of the funnels corresponds to native and catalytically active conformations. The ruggedness of the bottom depicts the energy barriers for interconversion, or structural fluctuations of the native state [17].

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