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ORIGINAL PAPER

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Did psychrophilic enzymes really win the challenge?

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Abstract Organisms living in permanently cold environments, which actually represent the greatest proportion of our planet, display at low temperatures metabolic fluxes comparable to those exhibited by mesophilic organisms at moderate temperatures. They produce cold-evolved enzymes partially able to cope with the reduction in chemical reaction rates and the increased viscosity of the medium induced by low temperatures. In most cases, the adaptation is achieved through a reduction in the activation energy, leading to a high catalytic efficiency, which possibly originates from an increased flexibility of either a selected area of or the overall protein structure. This enhanced plasticity seems in return to be responsible for the weak thermal stability of cold enzymes. These particular properties render cold enzymes particularly useful in investigating the possible relationships existing between stability, flexibility, and specific activity and make them potentially unrivaled for numerous biotechnological tasks. In most cases, however, the adaptation appears to be far from being fully achieved.

Key words Psychrophile · Cold-adaptation · Enzyme · Flexibility · Microcalorimetry · Directed evolution · Biotechnological tools

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Life in the cold: an amazing achievement

Life under low-temperature conditions was identified as early as 1887 by Forster, who reported that microorganisms isolated from fish could grow well at 0°C. Since then, numerous organisms, in particular bacteria, yeasts, unicellular algae, and fungi, have been found to have successfully colonized low-temperature environments, in which they contribute to nutrient cycling and primary biomass production (for reviews, see Russell 1992; Gounot and Russell 1999).

Cold-adapted microorganisms are obviously unique because despite an internal temperature close, if not identical, to that of their surroundings, and despite the strong negative effect of low temperatures on biochemical reactions, they not only survive but breed and grow successfully, exhibiting metabolic fluxes more or less comparable to those exhibited by closely related mesophilic species living at moderate temperatures (Clarke 1983; Delille and Bouvy 1990).

Living at temperatures close to 0°C requires a multiplicity of crucial adaptations, including the expression of enzymatic activities at appropriate levels as well as the maintenance of membrane stability and permeability, the latter being essential for many of the major cellular functions such as nutrient uptake, passive and active transport of various compounds, electron and proton transport, photosynthesis, environmental sensing, and recognition processes. These permanent adaptations are fixed in genomic DNA, which through the nucleotide sequence determines the amino acid sequence of proteins and hence their three-dimensional structures. An organism cannot deliberately modify these parameters in order to adapt to unusual thermal conditions, but it can, to some extent, modify the overall pattern of proteins expressed through the thermal regulation of gene expression.

Sudden decreases in temperature will therefore initiate the so-called cold-shock response (Jones and Inouye 1994; Graumann and Marahiel 1996; Lim et al. 2000), which is evidently not confined to psychrophilic (cold-loving) and psychrotrophic (cold-tolerant) microorganisms but constitutes

the beginning of cold adaptation. It involves the induction and synthesis of cold-shock proteins (Csp), the main functions of which are related to the regulation of protein synthesis and mRNA folding. The regulation of the expression of Csp and Csp homologues is a complex phenomenon also involving autoregulation and is controlled at the level of transcription and translation, as well as by the stability of mRNAs and proteins. The Csp number seems to increase with the severity of the cold shock, and analysis of the Csp genes reveals different characteristics. A major distinction between psychrophiles/psychrotrophs and mesophiles/thermophiles is that the synthesis of gene products is not inhibited by cold shock in cold-adapted microorganisms (Gounot and Russell 1999).

In most cases, low temperatures also strongly depress enzyme activity according to the basic equation proposed by Svante Arrhenius in 1889: $k = Ae^{-Ea/RT}$, where k is the rate constant; A the preexponential factor, not really a constant but related to steric factors and the temperature-dependent molecular collision frequency; Ea the activation energy, or rather the difference in heat content between the activated and ground states of the enzyme–substrate complex; R the gas constant (8.31 kJ/mol), and T the absolute temperature in Kelvin. Any decrease in temperature will thus induce an exponential decrease in the reaction rate, the amplitude of which depends on the value of the activation energy. If we also take into account the lower diffusion rate of molecules caused by the increased viscosity of the medium at low temperatures, one can easily understand that cold adaptation is not an easy challenge.

A possible strategy for psychrophilic enzymes to adapt to low temperatures is to decrease their Ea (Arpigny et al. 1997; Feller et al. 1997b; Lonhienne et al. 2000). Indeed, the energy needed to surmount the activation barrier possibly originates from the entropy loss resulting from the binding of substrate to enzyme (Jenks 1975) and from thermal fluctuations, the magnitude of which is reduced at low temperatures. In the extreme, a few so-called perfectly evolved enzymes such as triose phosphate isomerase of *Vibrio marinus* (Alvarez et al. 1998) or catalases from various species (Yumoto et al. 2000) exhibit almost temperature-independent (small Ea) reaction rates and are close to diffusion-controlled catalysts.

In fact, the behavior toward the temperature parameter in the case of enzyme-catalyzed reactions can be similar or quite different from that of a noncatalyzed reaction, depending on the type of weak interactions involved in the formation of the enzyme–substrate complex and also on the substrate concentration. Indeed, if one solely considers the case of enzymes obeying Michaelis-Menten kinetics, it is clear that, for extracellular enzymes, which often operate at high substrate concentrations, the K_m value becomes negligible, so the adaptation will involve increasing k_{cat} (Feller et al. 1994; Narinx et al. 1997; Petrescu et al. 2000).

The situation is quite different at low substrate concentration since the reaction rate will also depend on K_m and globally on the catalytic efficiency, k_{cat}/K_m. Increasing this ratio through a decrease in K_m can constitute a useful strategy for some enzymes in the course of cold adaptation (Feller and Gerday 1997; Kim et al. 1999; Bentahir et al. 2000; Georlette et al. 2000b).

Trademarks of cold enzymes and the flexibility concept

A higher activity at low and moderate temperatures and a lower thermostability

Looking at the effect of temperature on the activity of psychrophilic enzymes (Fig. 1) brings to light three basic features of cold enzymes: (i) an up to tenfold higher specific activity at low temperatures; (ii) an apparent maximal activity temperature shifted towards low temperatures; and (iii) a specific activity around 0°C, which is never as high as that displayed by mesophilic counterparts at their environmental temperature, showing that the adaptation to cold is not complete.

The high activity at low temperatures could be associated with a modification of the active site, but elaborate investigations have revealed that all amino acid residues involved in the reaction mechanism are strictly conserved in psychrophilic and mesophilic enzymes (Qian et al. 1994; Fields and Somero 1998; D'Amico et al. 2000), suggesting that the molecular basis of cold adaptation must be found elsewhere.

The second main feature of naturally evolved psychrophilic enzymes, a higher heat lability, is illustrated in Fig. 2, which shows the residual activity plotted as a function of time for psychrophilic and mesophilic β-lactamases exposed to a temperature of 50°C. One has to be aware, however, that the loss of activity is not necessarily strictly correlated with the unfolding of the enzyme. Indeed, from thermal denaturation experiments followed by spectroscopic techniques such as fluorescence emission or circular dichroism carried out on cold α-amylase, it has been shown (Feller et al. 1994) that enzyme inactivation precedes the modifica-

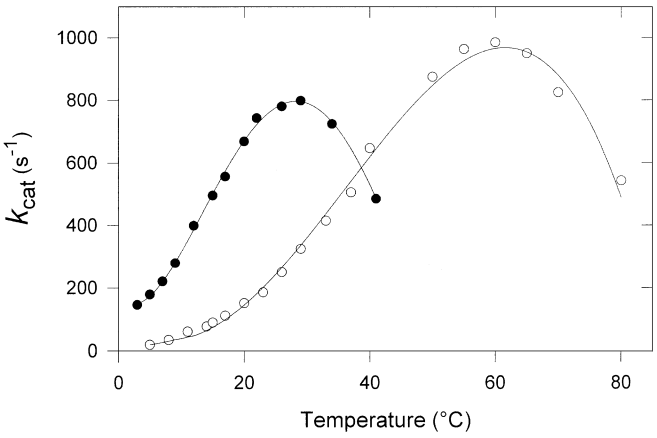

Fig. 1. Effect of temperature on the activity of psychrophilic and mesophilic enzymes. Curves representing the thermal dependence of the specific activity of the psychrophilic α-amylase from *Pseudoalteromonas haloplanktis* (*solid circles*) and of its mesophilic homologue from pig pancreas (*open circles*). Adapted from Feller et al. (1992)

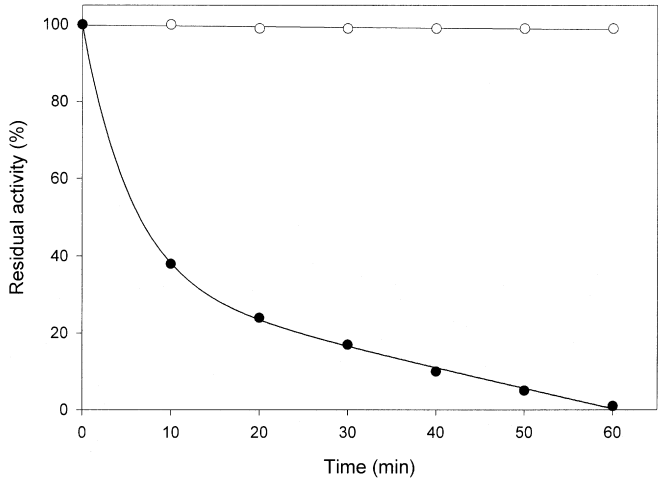

Fig. 2. Thermal stability of psychrophilic and mesophilic enzymes. Curves represent the thermal stability at 50°C, as measured by the residual activity as a function of time using nitrocefin as substrate, of the Antarctic β-lactamase from *Psychrobacter immobilis A5* (*solid circles*) and of the mesophilic β-lactamase from *E. cloacae Q908R* (*open circles*). Adapted from Feller et al. (1997b)

tions the enzyme structure. Moreover, the possible reversibility of the unfolding can considerably alter the data obtained from measurements of the residual activity. All these experiments further indicate that, although the high specific activity of an enzyme is not systematically associated with a low thermostability, the trend does exist in nature, and psychrophilic enzymes generally display high catalytic efficiency and low thermostability. The low thermostability of psychrophilic enzymes has been recently considered as the result of a lack of selective pressure rather than the product of physical or chemical constraints induced by the necessity to improve the activity at low temperature (Miyazaki et al. 2000). Indeed, higher stability variants of the psychrophilic subtilisin S41 protease obtained by directed evolution were found to be even more active than the wild-type enzyme over a large temperature range, suggesting that cold-active enzymes are not necessarily less stable. Several generations were, however, necessary to obtain the stable variant. The activity was measured using the small, synthetic substrate S-AAPF-pNa. It remains, therefore, to be demonstrated that the mutated enzymes have the same high specific activity toward macromolecular substrates. In support of this idea, mutants significantly more active on small substrates than mesophilic BPN′ subtilisin at 10°C do not show any enhancement of the caseinolytic activity (Taguchi et al. 2000), and, in contrast to the statement put forward by Miyazaki et al. (2000), all of the subtilisin BPN′ mutants showing cold adaptation were also found to be much less stable. In another set of experiments designed to improve the low-temperature-specific activity of a mesophilic *Bacillus sphaericus* SS II subtilisin, which is very closely related to psychrophilic subtilisin S41 (Wintrode et al., 2000), three of the four cold-adapted mutants also displayed a reduced stability. Clearly, more experiments are needed to clarify the possible relationship between the cold adaptation of an enzyme and its thermosensitivity.

A higher flexibility

According to the current accepted hypothesis (Fields and Somero 1998; Zavodszky et al. 1998), increased activity at low and moderate temperatures and higher heat lability are tightly correlated with an increase in protein flexibility.

If activity and (thermo)stability are easy terms to define, the flexibility notion appears to be more complex. It must be recalled that a protein in its native state is not in a single conformation but displays a Boltzmann distribution, in which one form often predominates over smaller populations of fluctuating conformations. These fluctuations, often collectively referred to as "flexibility," possibly give rise to an ensemble of conformational isomers and are presumably very important for the catalytic function of globular proteins limiting the energy cost of induced-fit mechanisms of substrate binding and favoring allosteric regulation (Ma et al. 1999). This flexibility can be defined either as a static or as a dynamic property (Tang and Dill 1998) of the protein structure.

Static flexibility refers to the different conformations (number and structural diversity) in the equilibrium ensemble, irrespective of the barriers that must be overcome to interconvert one into the other. It therefore refers to energy minima, pertaining to equilibrium constants. Static flexibility can be measured by experiments that see fast processes averaged over long times, such as X-ray crystallography and hydrogen/deuterium (H/D) exchange. With this latter technique, the ability of an amino acid to exchange a proton is taken to be proportional to the average accessibility of that residue to the solvent. Fluctuations can expose protons that otherwise cannot be exchanged in a fixed native conformation.

In contrast, dynamic flexibility refers to how quickly the protein can interconvert between the conformations and is a measure of the energy barriers between the native conformation and its fluctuation variants. It therefore refers to energy maxima, which pertain to rate constants.

Low temperatures tend to increase the compactness of a protein, disabling the catalytic efficiency of a nonadapted enzyme. Indeed, according to the previous statement for mesophilic enzymes at low temperature, only the low-energy conformations are populated, high-energy conformations are not. This is supported by Ditzel et al. (1996), who have shown that an antibody displaying a broad specificity at 37°C becomes monospecific at 4°C. Therefore an increase in the flexibility of appropriate parts of an enzyme is essential to enable an easier accommodation of substrates. In psychrophilic enzymes, a better accessibility of the active site has been demonstrated in an Antarctic fish elastase (Aittaleb et al. 1997), a cold-active citrate synthase (Russell et al. 1998), and an Antarctic α-amylase (D'Amico, unpublished data). This improved accessibility reduces the energy required to accommodate relatively voluminous substrates and/or facilitates products release. It is, however, worth mentioning that comparison of the overall so-called

temperature or B factors (reflecting the disorder in a crystal lattice) often fails to reveal any significant difference between psychrophilic, mesophilic, and thermophilic homologues. Cold-adapted malate dehydrogenase constitutes an interesting case: a lower average B factor was observed for the psychrophilic enzyme when compared with the thermophilic one, a fact in opposition to the hypothesis of a higher flexibility; however, when the relative size of B factors of atoms involved in cofactor and substrate binding were considered, the values were twofold higher in the case of the psychrophilic enzyme, illustrating the importance of flexibility in the catalytic properties of the enzyme (Kim et al. 1999). It is clear, however, that the crystallographic B factors primarily depend on the crystal packing and are not necessarily related to the flexibility of the molecular edifice.

The presumably enhanced plasticity of cold-adapted enzymes is often considered to be responsible for their weak thermal stability: the greater the flexibility of a protein, the lower the stability. Some experiments based on H/D exchange (Wrba et al. 1990; Jaenicke 1991; Zavodszky et al. 1998) and fluorescence quenching (Varley and Pain 1991) do support this hypothesis. However, amide-proton exchange kinetics have also shown that the rates of slowly exchanging amide-protons in mesophilic and thermophilic α-amylases could be similar. In addition, on a shorter time scale, the thermophilic enzyme displayed a higher flexibility when compared with its mesophilic counterpart (Fitter and Heberle 2000). The hypothesis of an inverse relationship between protein thermostability and flexibility of the molecular edifice as seen by hydrogen experiments has also been questioned in another recent work on rubredoxin (Hernandez et al. 2000). Therefore, one can address the question: are amide-proton exchange rates always in agreement with the type of flexibility required to improve the catalytic efficiency at low temperature? Additional measurements over a wide range of timescales are obviously needed to distinguish the flexibility of mesophilic and psychrophilic enzymes. To our knowledge, probably only one experiment has been successful in demonstrating the higher flexibility of a cold-adapted enzyme: the quenching effect of acrylamide on the fluorescence of tryptophanyl residues identically positioned in the polypeptide chains was clearly much more important in the case of a cold Ca^{2+}–Zn^{2+} protease when compared with its mesophilic counterpart (Fig. 3).

The relationship between the stability and the flexibility of a protein has been described in terms of the energy distribution (Tang and Dill 1998): if the energy level of the nearest nonnative conformation is close to that of the native state, or if there are many nearest nonnative conformations, the native state will readily fluctuate to populate those conformations, and it will not be very stable. This leads to a quite general prediction: stability and static equilibrium flexibility correlate inversely. It follows that thermostability is not a property that can be determined by knowing the native structure alone. A good native structure is necessary for high thermostability, but not sufficient: some proteins having low-energy native states are not particularly stable because of the high number of low-energy nonnative states. This implies that much of the protein stability message may

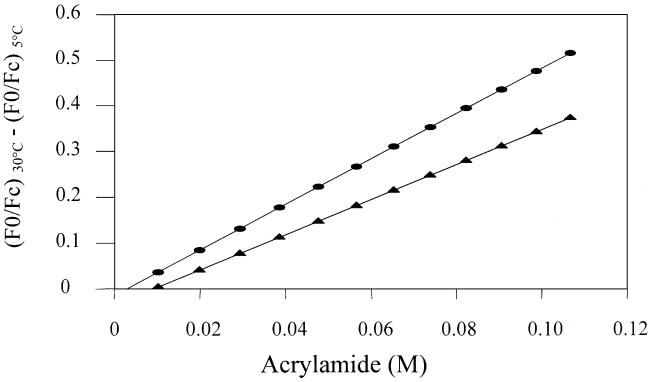

Fig. 3. Flexibility of psychrophilic and mesophilic enzymes. Curves representing the quenching of the tryptophan (Trp) fluorescence of mesophilic (*triangles*) and psychrophilic (*circles*) Ca^{2+}–Zn^{2+} protease by acrylamide. In order to abolish the artifactual effect of the additional Trp present in the mesophilic enzyme, the differences of the fluorescence ratios (*F0/Fc*) at 30°C and 5°C were plotted as a function of acrylamide concentration. Adapted from Gerday et al. (1999)

be in the unobservable states (the fluctuations and excited states), rather than in the observable native state. In the same way, if increasing the hydrophobicity of some surface residues in proteins destabilizes them, the cause could be that such modifications affect the denatured states more strongly than the native one (Shortle et al. 1992; Herrmann et al. 1995).

Thus, activity, stability, and flexibility seem in fact tightly related by the evolutionary route. In this context, recent microcalorimetry (Dsc) data related to the conformational and thermodynamic stability of cold-adapted enzymes shed new light on the above-mentioned relationship. From the limited number of psychrophilic enzymes investigated, two types of adaptations already have emerged (Fig. 4): an evolution toward the lowest possible stability of the native state, exemplified by a cold-adapted α-amylase (Feller et al. 1999), or an evolution toward a clear-cut distinction between the stability of the domains of the protein, one obviously acting as a heat-labile destabilizing domain providing local flexibility, as exemplified in the case of a cold-adapted phosphoglycerate kinase (PGK) (Bentahir et al. 2000). These two strategies are discussed below.

Local flexibility

The psychrophilic PGK exhibits an unexpected calorimetric melting profile because it is composed of a heat-labile and a heat-stable domain (two denaturation peaks), whereas the mesophilic counterpart from yeast displays a cooperative transition at an intermediate temperature value (one peak). The first domain in the cold-adapted PGK is responsible for the low thermostability of the whole edifice and presumably for the higher specific activity due to its high flexibility, whereas the second unit, which is more stable than yeast PGK, counteracts the unfavorable entropic contribution of the first. This has also been encountered (Lonhienne 2000) in a psychrophilic chitobiase also studied by Dsc; this enzyme displays two domains having quite different stabil-

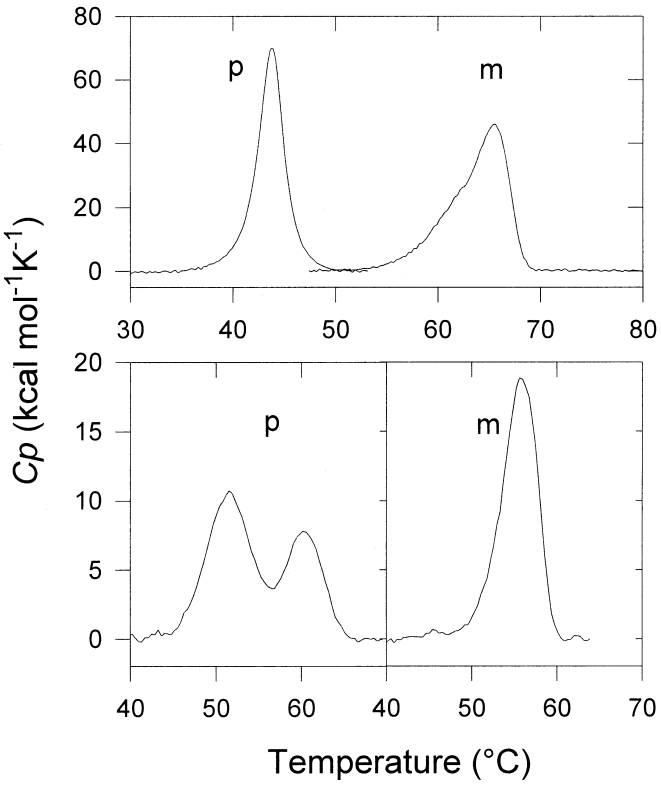

Fig. 4. Conformational stability (*Cp*) in psychrophilic (*p*) and mesophilic (*m*) enzymes. *Upper panel*: all structural elements of a cold-active α-amylase (*p*) unfold into a cooperative unit at low temperature when compared with a mesophilic homologue from pig pancreas (*m*) (Feller et al. 1999). *Lower panel*: the heat-induced unfolding of a cold-active phosphoglycerate kinase (*p*) displays a heat-labile domain and a heat-stable domain when compared with a mesophilic counterpart from yeast (*m*) (Bentahir et al. 2000)

ities, with the less stable domain including the active site. In the case of the cold-adapted PGK, the loss of cooperativity between the two calorimetric units can be due to a decrease in the interdomain hydrophobic (endothermic) interactions because Gast et al. (1995) have shown that cold denaturation of the yeast homologue also exhibits two peaks.

In these cases of proteins interacting with small substrates, increasing the flexibility of the whole protein would probably give rise to high K_m values, thus contributing to a decrease in the efficiency at low substrate concentrations. Keeping one domain very rigid could lower the entropy of the ground state, limiting the effect created by the disorder of the low-stability domain.

Overall flexibility

The second strategy derives from investigations carried out on a cold-adapted α-amylase (Feller et al. 1999). The unfolding of this enzyme occurs through a single transition from the native to the unfolded state, even though the protein is composed of three domains. More interesting is that the unfolding is fully reversible, allowing the determination of the thermodynamic parameters. Stabilization energy curves as a function of temperature show that not only does this cold-adapted protein display the lowest stability ever recorded for an α-amylase but also that the specific stabilization energy, i.e., the stabilization energy per residue, is the lowest ever recorded for proteins displaying a reversible unfolding. It is worth mentioning that, contrary to the above described cases of psychrophilic PGK and chitobiase, the natural substrate of α-amylase is a high-molecular-weight substrate. The voluminous size of the substrate probably requires an overall plasticity of the molecular edifice to secure good accommodation at low temperatures. It is clear t hat in this case and as indicated by the very low value of stabilization energy, especially at low temperatures, we have reached the limit of stability, precluding any further decrease in stability, and also precluding any further improvement of the catalytic efficiency of the enzyme using the flexibility strategy. As a consequence, the specific activity of the cold-adapted enzyme at its environmental temperature (0°C) remains much lower than that of the reference mesophilic enzyme at 37°C (Feller et al. 1992).

Structural adaptation of psychrophilic enzymes

Structural comparisons of psychrophilic enzymes with their mesophilic and thermophilic counterparts were initially limited to the analysis of homology models and/or sequence alignments of a few enzymes from bacteria, yeast, and fish, such as α-amylase (Feller et al. 1992, 1994), triose phosphate isomerase (Rentier-Delrue et al. 1993), subtilisin (Davail et al. 1994), fish trypsin (Genicot et al. 1996), β-lactamase (Feller et al. 1997b), fish elastase (Aittaleb et al. 1997), 3-isopropylmalate dehydrogenase (Wallon et al. 1997), lipase (Arpigny et al. 1997), elongation factors 2 (Thomas and Cavicchioli 1998) and G (Berchet et al. 2000), yeast xylanase (Petrescu et al. 2000), and phosphoglycerate kinase (Bentahir et al. 2000). A few crystallographic structures are also available: α-amylase (Aghajari et al. 1998a, b), triose phosphate isomerase (Alvarez et al. 1998), citrate synthase (Russell et al. 1998), malate dehydrogenase (Kim et al. 1999), Ca^{2+}–Zn^{2+} protease (Villeret et al. 1997), cod pepsin (Karlsen et al. 1998), and elastase (Berglund et al. 1995) and trypsin from Atlantic salmon (Smalas and Horvick 1993). These structures have provided the basis for the analysis of the molecular adaptation to cold by revealing that only minor modifications are necessary to convert a mesophilic or thermophilic enzyme into a cold-adapted one (Gerday et al. 1997, 1999, 2000; Russell 2000; Georlette et al. 2000a; Smalas et al. 2000). But it is understood that each enzyme has evolved following its own route, displaying a set of structural adjustments selected from quite a large panel of possibilities (Arpigny et al. 1994; Feller et al. 1997a; Feller and Gerday 1997): (i) a decrease in salt bridges, H-bonds and aromatic interactions, ion binding constants, hydrophobic interactions, arginine content, or proline residues in loops; (ii) a decrease in the stabilization of α-helix dipoles; (iii) an increase in the clustering of glycine residues; and/or (iv) insertions or deletions of loops responsible for specific properties. To these factors one can add a trend to

decrease the density of charged residues at the surface of the cold-adapted enzymes, which could form the ion-pair network often found in thermophilic enzymes, and another trend to increase the density of hydrophobic residues exposed to the solvent, which also contributes to lower the stability of the molecular edifice (Bentahir et al. 2000). The comparison of the refined structures does not, however, provide a direct access to the structural parameters involved in the adaptation to low temperatures because of the multiple constraints that in a natural environment contribute to shape the enzyme. Site-directed mutagenesis, oriented toward the detection of amino acid substitutions, which, according to a rational approach, tend to decrease the stability of the molecular structure and in this way to increase the flexibility of appropriate parts of the structure, is an appropriate tool to check the hypotheses put forward.

Very recently, random mutagenesis techniques – error-prone polymerase chain reaction (PCR) amplification, DNA shuffling, and mutator cells (Greener et al. 1997) – were used to submit a protein of interest to the so-called laboratory evolution: mutagenesis and recombination steps provided a multitude of mutants afterwards selected on the basis of the desired altered property (thermostability in the case of cold enzymes, or improved activity). This powerful technique therefore allows the researcher to obtain a protein submitted to a "selected" selective pressure. As already mentioned, this process was applied to psychrophilic subtilisin S41 (Davail et al. 1994; Miyazaki et al. 2000), leading to a seven-amino acid substitution variant exhibiting a higher thermostability without sacrificing low-temperature activity, at least toward a synthetic substrate. On the other hand, random mutagenesis experiments have been carried out to improve the catalytic activity of mesophilic and thermophilic enzymes at low temperatures (Taguchi et al. 1998; Merz et al. 2000; Lebbink et al. 2000) and in the same context to obtain a higher thermostability of mesophilic enzymes (Giver et al. 1998; Akanuma et al. 1998, 1999; Spiller et al. 1999; Cherry et al. 1999; Zhao and Arnold 1999; Gershenson et al. 2000; Gonzalez-Blasco et al. 2000). Amazingly, whereas a decrease in stability was observed when a high catalytic efficiency at low temperatures was sought, matching perfectly the initial working hypothesis, mutants displaying an increased stability and an unchanged, or even increased, catalytic efficiency were, in contrast, obtained when the selective pressure was thermal stability. In fact, several generations of mutants were required to give rise to such an increase in both stability and activity, 13 and 7 mutations in the case of an esterase (Giver et al. 1998) and a peroxidase (Cherry et al. 1999), respectively.

On the other hand, Wintrode et al. (2000) have converted a mesophilic subtilisin-like protease, SSII, into its psychrophilic counterpart by a single mutation round; the k_{cat} of the first generation mutant P2G8 is 3.9 times that of the wild-type enzyme and 2.6 times that of the natural psychrophile S41. Another variant, P3C9, displays at 10°C a much greater k_{cat} (6.6 times) and k_{cat}/K_m (9.6 times) than those of the wild-type. Only four amino acid substitutions lead to this increase in cold activity, showing that SSII can rapidly adapt to low temperatures when strong selective pressure is applied. In common with the usual properties of natural cold enzymes, P3C9 is less thermostable than its mesophilic counterpart SSII. However, none of the mutations present in P3C9 are found in cold-adapted subtilisin S41. Furthermore, all but one mutation occurred at sites that are conserved between S41 and SSII. The authors rationalized this result on several grounds: (i) the number of all possible sequences for a 310-amino acid protein is vast; (ii) there are likely to be multiple routes to cold adaptation; (iii) the "directed evolution" selective pressure applied is different from that encountered by subtilisin in nature, which has to take into account multiple constraints; and (iv) the selection criteria were very stringent: only mutants showing improvements of ~20% or greater were allowed to proceed to the next generation, whereas in natural evolution, much smaller improvements could become fixed in the evolving population.

The authors also concluded that there is not a strict inverse relationship between stability and low-temperature activity, because the variant P7E1, which shows a nearly twofold increase in k_{cat}/K_m at 10°C, is actually more stable than the wild-type enzyme. This is consistent with previous results obtained when directed evolution was performed on psychrophilic subtilisin S41 (Miyazaki et al. 2000); its stability was increased ~500-fold with no loss of low-temperature activity. Again, however, these data have been obtained with small, synthetic substrates, which, as has been already discussed, can possibly fit with an adaptation strategy different from that required by large substrates.

Keeping this restriction in mind, it appears possible, at least in the laboratory, to increase both the activity of an enzyme at low temperatures and its stability at high temperatures. The authors suggest that the ultimate decrease in stability of naturally cold-adapted enzymes could be due to a random genetic drift during divergent evolution from a mesophilic ancestor facilitated by the flexibility requirement rather than to a physical or chemical incompatibility between the two properties. With respect to this idea, however, it appears difficult to explain why, in cold-adapted PGK and cold-adapted chitobiase, one part of the protein (the unstable part) would be subjected to genetic drift and not the other part (the stable part). The idea of a genetic drift could be extended to the case of thermophilic enzymes for which the first constraint is thermal stability (Wintrode et al. 2000; D'Amico et al., in preparation) and not the specific activity, which would also decrease through a random genetic drift. Another possible explanation of the thermal instability of cold-adapted enzymes could be negative selection: in cold-adapted organisms, highly stable enzymes would be resistant to turnover by normal cellular degradation mechanisms and could therefore accumulate and ultimately be harmful to the organisms (Somero 1995; Feller and Gerday 1997). This is, however, unlikely in the case of excreted proteins such as subtilisins.

Conclusions

From the growing body of available data, the adaptation of psychrophilic enzymes appears to follow different global

strategies to improve catalytic efficiency at low temperatures, either via an increase in the specific activity k_{cat} or, when appropriate, an improvement in the affinity for substrates (i.e., a decrease in K_m), or even by alteration of both parameters simultaneously. This is presumably achieved through an increase in the flexibility of either a selected part of or the complete protein structure, providing enhanced abilities to undergo the conformational changes required during catalysis at low temperatures. The structural modifications lead to a higher thermosensitivity when compared with the mesophilic and thermophilic homologues. Each cold-active enzyme adopts a specific strategy, depending on the mechanism of action, the potentialities of the 3-D structure in absorbing structural changes, and on other structural constraints imposed by the natural environment. However, two general routes do emerge: one, illustrated by the properties of a cold-adapted α-amylase, corresponds to a general decrease in the stability of the protein, giving rise to an increase in the overall plasticity of the molecular edifice, which is probably required for the accommodation of macromolecular substrates; the second, illustrated by the molecular characteristics of a cold-adapted phosphoglycerate kinase, consists in evolving a domain of the protein into a highly flexible unit while keeping another domain much more rigid, this in order to secure an appropriate affinity (K_m) of the protein for small substrates. This latter strategy is opposite to that elaborated for some thermophilic proteins that in addition to domains involved in catalysis and substrate binding also contain stabilizing domains (Fontes et al. 1995).

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References

Aghajari N, Feller G, Gerday C, Haser R (1998a) Crystal structures of the psychrophilic α-amylase from *Alteromonas haloplanctis* in its native form and complexed with an inhibitor. Protein Sci 7:564–572

Aghajari N, Feller G, Gerday C, Haser R (1998b) Structures of the psychrophilic *Alteromonas haloplanctis* α-amylase give insights into cold adaptation at a molecular level. Structure 6:1503–1516

Aittaleb M, Hubner R, Lamotte-Brasseur J, Gerday C (1997) Cold adaptation parameters derived from cDNA sequencing and molecular modelling of elastase from Antarctic fish *Notothenia neglecta*. Protein Eng 10:475–477

Akanuma S, Yamagishi A, Tanaka N, Oshima T (1998) Serial increase in the thermal stability of 3-isopropylmalate dehydrogenase from *Bacillus subtilis* by experimental evolution. Protein Sci 7:698–705

Akanuma S, Yamagishi A, Tanaka N, Oshima T (1999) Further improvement of the thermal stability of a partially stabilized *Bacillus subtilis* 3-isopropylmalate dehydrogenase variant by random and site-directed mutagenesis. Eur J Biochem 260:499–504

Alvarez M, Zeelen JP, Mainfroid V, Rentier-Delrue F, Martial JA, Wyns L, Wierenga RK, Maes D (1998) Triose-phosphate isomerase (TIM) of the psychrophilic bacterium *Vibrio marinus*. Kinetic and structural properties. J Biol Chem 273:2199–2206

Arpigny JL, Feller G, Davail S, Génicot S, Narinx E, Zekhnini Z, Gerday C (1994) Molecular adaptations of enzymes from thermophilic and psychrophilic organisms. In: Gilles R, (ed) Advances in comparative environmental physiology. Springer, Berlin, pp. 269–295

Arpigny JL, Lamotte J, Gerday C (1997) Molecular adaptation to cold of an Antarctic bacterial lipase. J Mol Catal B 3:29–35

Bentahir M, Feller G, Aittaleb M, Lamotte-Brasseur J, Himri T, Chessa JP, Gerday C (2000) Structural kinetic and calorimetric characterization of the cold-active phosphoglycerate kinase from the antarctic *Pseudomonas sp.* TACII18. J Biol Chem 275:11147–11153

Berchet V, Thomas T, Cavicchioli R, Russell NJ, Gounot AM (2000) Structural analysis of the elongation factor G protein from the low-temperature-adapted bacterium *Arthrobacter globiformis SI55*. Extremophiles 4:123–130

Berglund GI, Willasen NP, Horvick A, Smalas AO (1995) Structure of native pancreatic elastases from North Atlantic salmon at 1.61 Å resolution. Acta Cryst D51:925–937

Cherry JR, Lamsa MH, Schneider P, Vind J, Svendsen A, Jones A, Pedersen AH, (1999) Directed evolution of a fungal peroxidase. Nat Biotechnol 17:379–84

Clarke A (1983) Life in cold water: the physiological ecology of polar marine ectotherms. Oceanogr Mar Biol Ann Rev 21:341–453

D'Amico S, Gerday C, Feller G (2000) Structural similarities and evolutionary relationships in chloride-dependent alpha-amylases. Gene 253:95–105

Davail S, Feller G, Narinx E, Gerday C (1994) Cold adaptation of proteins. Purification characterization and sequence of the heat-labile subtilisin from the Antarctic psychrophile *Bacillus* TA41. J Biol Chem 269:17448–17453

Delille D, Bouvy M (1990) Relationship between viable and direct bacteriological counts in southern polar marine waters. Vie Milieu 40:281–284

Ditzel HJ, Itoh K, Burton DA (1996) Determinants of polyreactivity in a large panel of recombinant human antibodies from HIV-I infection. J Immunol 157:739–749

Feller G, Gerday C (1997) Psychrophilic enzymes: molecular basis of cold adaptation. Cell Mol Life Sci 53:830–841

Feller G, Lonhienne T, Deroanne C, Libioulle C, Van Beeumen J, Gerday C (1992) Purification characterization and nucleotide sequence of the thermolabile α-amylase from the Antarctic psychrotroph *Alteromonas haloplanctis* A23. J Biol Chem 267:5217–5221

Feller G, Payan F, Theys F, Qian M, Haser R, Gerday C (1994) Stability and structural analysis of α-amylase from the Antarctic psychrophile *Alteromonas haloplanctis* A23. Eur J Biochem 222:441–447

Feller G, Arpigny JL, Narinx E, Gerday C (1997a) Molecular adaptations of enzymes from psychrophilic organisms. Comp Biochem Physiol 118:495–499

Feller G, Zekhnini Z, Lamotte-Brasseur J, Gerday C (1997b) Enzymes from cold-adapted microorganisms. The class C β-lactamase from the Antarctic psychrophile *Psychrobacter immobilis* A5. Eur J Biochem 244:186–191

Feller G, d'Amico D, Gerday C (1999) Thermodynamic stability of a cold-active α-amylase from the Antarctic bacterium *Alteromonas haloplanctis*. Biochemistry 38:4613–4619

Fields PA, Somero GN (1998) Hot spots in cold adaptation: localized increases in conformational flexibility in lactate dehydrogenase A(4) orthologs of Antarctic notothenioid fishes. Proc Natl Acad Sci U S A 95:11476–11481

Fitter J, Heberle J (2000) Structural equilibrium fluctuations in mesophilic and thermophilic α-amylase. Biophys J 79:1629–1636

Fontes CM, Hall J, Hirst BH, Hazlewood GP, Gilbert HJ (1995) The resistance of cellulases and xylanases to proteolytic inactivation. Biotechnol 43:52–57

Forster J (1887) Uber einige Eigenschaften leuchtender Bakterien. Zentz Bacteriol Parasitenk Infekt Hyg 2:337–340

Gast K, Damaschun G, Desmadril M, Minard P, Muller-Frohne M, Pfeil W, Zirwer D (1995) Cold denaturation of yeast phosphoglycerate kinase: which domain is more stable? FEBS Lett 358:247–250

Genicot S, Rentier-Delrue F, Edwards D, Van Beeumen J, Gerday C (1996) Trypsin and trypsinogen from an Antarctic fish: molecular basis of cold adaptation. Biochim Biophys Acta 1298:45–57

Georlette D, Bentahir M, Claverie P, Collins T, D'Amico S, Dellile D, Feller G, Gratia E, Hoyoux A, Lonhienne T, Meuwis MA, Zecchinon L, Gerday C (2000a) Cold-adapted enzymes. Kluwer Academic Publishers BV, Dordrecht

Georlette D, Jonsson ZO, Van Petegem F, Chessa JP, Van Beeumen J, Hubscher U, Gerday C (2000b) A DNA ligase from the psychrophile *Pseudoalteromonas haloplanktis* gives insights into the adaptation of proteins to low temperatures. Eur J Biochem 267:3502–3512

Gerday C, Aittaleb M, Arpigny JL, Baise E, Chessa JP, Garsoux G, Petrescu I, Feller G (1997) Psychrophilic enzymes: a thermodynamic challenge. Biochim Biophys Acta 1342:119–131

Gerday C, Aittaleb M, Arpigny JL, Baise E, Chessa JP, François JM, Petrescu I, Feller G (1999) Cold enzymes: a hot topic. In: Margessi R and Schinner F (eds) Cold adapted organisms: ecology, physiology, enzymology, and molecular biology. Springer, Berlin, pp. 257–275

Gerday C, Aittaleb M, Bentahir M, Chessa JP, Claverie P, Collins T, D'Amico S, Dumont J, Garsoux G, Georlette D, Hoyoux A, Lonhienne T, Meuwis MA, Feller G (2000) Cold-adapted enzymes: from fundamentals to biotechnology. Trends Biotechnol 18:103–107

Gershenson A, Schauerte JA, Giver L, Arnold FH (2000) Tryptophan phosphorescence study of enzyme flexibility and unfolding in laboratory-evolved thermostable esterases. Biochemistry 39:4658–4665

Giver L, Gershenson A, Freskgard PO, Arnold FH (1998) Directed evolution of a thermostable esterase. Proc Natl Acad Sci U S A 95:12809–12813

Gonzalez-Blasco G, Sanz-Aparicio J, Gonzalez B, Hermoso JA, Polaina J (2000) Directed evolution of betaglucosidase A from *Paenibacillus polymyxa* to thermal resistance. J Biol Chem 275:13708–13712

Gounot AM, Russell NJ (1999) Physiology of cold-adapted microorganisms. In Margesin R and Schinner F (eds) Cold-adapted organisms: ecology, physiology, enzymology, and molecular biology. Springer, Berlin, pp. 33–55

Graumann P, Marahiel MA (1996) Some like it cold: response of microorganisms to cold shock. Arch Microbiol 166:293–300

Greener A, Callahan M, Jerpseth B (1997) An efficient random mutagenesis technique using an *E. coli* mutator strain. Mol Biotechnol 7:189–195

Hernandez G, Jenney FE, Adams M, Le Master D (2000) Millisecond time scale conformational flexibility in a hyper thermophile protein at ambient temperature. Proc Natl Acad Sci U S A 97:3166–3170

Herrmann L, Bowler BE, Dong A, Caughey WS (1995) The effects of hydrophilic to hydrophobic surface mutations on the denatured state of iso-1-cytochrome c: investigation of aliphatic residues. Biochemistry 34:3040–3047

Jaenicke R (1991) Protein stability and molecular adaptation to extreme conditions. Eur J Biochem 202:715–728

Jenks WP (1975) Binding energy specificity and enzymic catalysis: the Circe effect. Adv Enzymol 43:219–410

Jones PG, Inouye M (1994) The cold-shock response: a hot topic. Mol Microbiol 11:811–818

Karlsen S, Hough E, Olsen R (1998) Structure and proposed amino acid sequence of a pepsin from Atlantic cod (*Gadus morhua*) Acta Cryst D54:32–46

Kim SY, Hwang KY, Kim SH, Sung HC, Han YS, Cho YJ (1999) Structural basis for cold adaptation. Sequence biochemical properties and crystal structure of malate dehydrogenase from a psychrophile *Aquaspirillum arcticum*. J Biol Chem 274:11761–11767

Lebbink JH, Kaper T, Bron P, van der Oost J, de Vos WM (2000) Improving low-temperature catalysis in the hyperthermostable *Pyrococcus furiosus* beta-glucosidase CelB by directed evolution. Biochemistry 39:3656–3665

Lim J, Thomas T, Cavicchioli R (2000) Low temperature regulated DEAD-box RNA helicase from the Antarctic Archaeon *Methanococcoides bustonii*. J Mol Biol 297:553–557

Lonhienne T (2000) Cold-adaptation of chitinolytic enzymes secreted by an Antarctic marine bacterium. PhD Thesis, University of Liège

LonhienneT, Gerday C, Feller G, (2000) Psychrophilic enzymes: revisiting the thermodynamic parameters of activation may explain local flexibility. Biochim Biophys Acta 36284:1–10

Ma B, Kumar S, Tsai CJ, Nussinov R (1999) Folding funnels and binding mechanisms. Protein Eng 12:713–720

Merz A, Yee MC, Szadkowski H, Pappenberger G, Crameri A, Stemmer WP, Yanofsky C, Kirschner K (2000) Improving the catalytic activity of a thermophilic enzyme at low temperatures. Biochemistry 39:880–889

Miyazaki K, Wintrode PL, Grayling RA, Rubingh DN, Arnold FH (2000) Directed evolution study of temperature adaptation in a psychrophilic enzyme. J Mol Biol 297:1015–1026

Narinx E, Baise E, Gerday C (1997) Subtilisin from psychrophilic Antarctic bacteria: characterization and site-directed mutagenesis of residues possibly involved in the adaptation to cold. Protein Eng 10:1271–1279

Petrescu I, Lamotte-Brasseur J, Chessa JP, Ntarima P, Claeyssens M, Devreese B, Marino G, Gerday C (2000) Xylanase from the psychrophilic yeast *Cryptococcus adeliae*. Extremophiles 4:137–144

Qian M, Haser R, Buisson G, Duee E, Payan F, (1994) The active center of a mammalian alpha-amylase. Structure of the complex of a pancreatic alpha-amylase with a carbohydrate inhibitor refined to 2,2-Å resolution. Biochemistry 33:6284–6294

Rentier-Delrue F, Mande SC, Moyens S, Terpstra P, Mainfroid V, Goraj K, Lion M, Hol WG, Martial JA (1993) Cloning and overexpression of the triosephosphate isomerase genes from psychrophilic and thermophilic bacteria. Structural comparison of the predicted protein sequences. J Mol Biol 229:85–93

Russell NJ (1992) Physiology and molecular biology of psychrophilic micro-organisms. In: Herbert RA and Sharp RJ (eds) Molecular biology and biotechnology of extremophiles.. Blackie and Son, London, pp. 203–224

Russell NJ (2000) Toward a molecular understanding of cold activity of enzymes from psychrophiles. Extremophiles 4:83–90

Russell RJ, Gerike U, Danson MJ, Hough DW, Taylor GL (1998) Structural adaptations of the cold-active citrate synthase from an Antarctic bacterium. Structure 6:351–361

Shortle D, Chan HS, Dill KA (1992) Modeling the effects of mutations on the denatured states of proteins. Protein Sci 1:201–215

Smalas AO, Horvick A (1993) Structure determination and refinement of benzamidine inhibited trypsin from the North Atlantic salmon (*Salmo salar*) at 1.82 Å resolution. Acta Cryst D49:318–330

Smalas AO, Schrøder Leiros HK, Os V, Peder N (2000) Cold-adapted enzymes. Biotechnol Ann Rev 6:1–57

Somero GN (1995) Proteins and temperature. Annu Rev Physiol 57:43–68

Spiller B, Gershenson A, Arnold FH, Stevens RC (1999) A structural view of evolutionary divergence. Proc Natl Acad Sci U S A 96:12305–12310

Taguchi S, Ozaki A, Momose H (1998) Engineering of a cold-adapted protease by sequential random mutagenesis and a screening system. Appl Environ Microbiol 64:492–495

Taguchi S, Komada S, Momose H (2000) The complete amino acid substitutions at position 131 that are positively involved in cold adaptation of subtilisin BPN′. Appl Environ Microbiol 66:1410–1415

Tang KE, Dill KA (1998) Native protein fluctuations: the conformational-motion temperature and the inverse correlation of protein flexibility with protein stability. J Biomol Struct Dyn 16:397–411.

Thomas T, Cavicchioli R (1998) Archaeal cold-adapted proteins: structural and evolutionary analysis of the elongation factor 2 proteins from psychrophilic mesophilic and thermophilic methanogens. FEBS Lett 439:281–286

Varley PG, Pain RH (1991) Relation between stability dynamics and enzyme activity in 3-phosphoglycerate kinases from yeast and *Thermus thermophilus*. J Mol Biol 220:531–538

Villeret V, Chessa JP, Gerday C, Van Beeumen J (1997) Preliminary crystal structure determination of the alkaline protease from the Antarctic psychrophile *Pseudomonas aeruginosa*. Protein Sci 6:2462–2464

Wallon G, Lovett ST, Magyar C, Svingor A, Szilagyi A, Zavodszky P, Ringe D, Petsko GA (1997) Sequence and homology model of 3-

isopropylmalate dehydrogenase from the psychrotrophic bacterium *Vibrio* sp. I5 suggest reasons for thermal instability. Protein Eng 10:665–672

Wintrode PL, Miyazaki K, Arnold FH (2000) Cold adaptation of a mesophilic subtilisin-like protease by laboratory evolution. J Biol Chem 275:31635–31640

Wrba A, Schweiger A, Schultes V, Jaenicke R, Zavodsky P (1990) Extremely thermostable D-glyceraldehyde-3-phosphate dehydrogenase from the eubacterium *Thermotoga maritima*. Biochemistry 29:7584–7592

Yumoto I, Ichihashi D, Iwata H, Istokovics A, Ichise N, Matsuyama H, Okuyama H, Kawasaki K (2000) Purification and characterization of a catalase from the facultatively psychrophilic bacterium *Vibrio rumoiensis* S-1^T exhibiting high catalase activity. J Bacteriol 182:1903–1909

Zavodszky P, Kardos J, Svingor, Petsko GA (1998) Adjustment of conformational flexibility is a key event in the thermal adaptation of proteins. Proc Natl Acad Sci U S A 95:7406–7411

Zhao H, Arnold FH (1999) Directed evolution converts subtilisin E into a functional equivalent of thermitase. Protein Eng 12:47–53