Invited Review

MARINE BIOTECHNOLOGY

Cold-Adapted Enzymes from Marine Antarctic Microorganisms

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Received: 24 July 2006 / Revised: 9 September 2006 / Published online: 29 December 2006

Abstract

The Antarctic marine environment is characterized by challenging conditions for the survival of native microorganisms. Indeed, next to the temperature effect represented by the Arrhenius law, the viscosity of the medium, which is also significantly enhanced by low temperatures, contributes to slow down reaction rates. This review analyses the different challenges and focuses on a key element of life at low temperatures: cold-adapted enzymes. The molecular characteristics of these enzymes are discussed as well as the adaptation strategies which can be inferred from the comparison of their properties and three-dimensional structures with those of their mesophilic counterparts. As these enzymes display a high specific activity at low and moderate temperatures associated with a relatively high thermosensitivity, the interest in these properties is discussed with regard to their current and possible applications in biotechnology.

Keywords: Antarctic — biotechnology — cold adaptation — psychrophiles

Introduction

The Antarctic marine environment is characterized by a constant average temperature of about −1°C. This constitutes a challenging condition for the survival of native microorganisms. Low temperature has two main physicochemical effects. First, the rate of chemical reactions decreases exponentially according to the Arrhenius law: $k_{cat} = A \cdot \exp(-E_a/RT)$, in which k_{cat} is the catalytic constant; A is the preexponential factor, also known as the frequency factor and closely related to the activation entropy of the reaction; E_a, is the activation energy; R is the gas constant; and T is the temperature in Kelvin. Second, temperature has also a strong effect on the viscosity of the medium, thereby contributing to further slow down reaction rates. It is therefore rather surprising that the cell densities of microorganisms in these extreme environments are close to those prevailing in temperate oceans. Indeed, in the Antarctic, average bacterial cell densities of 10^5–10^6 cells/ml were found in the coastal waters of Adelie Land (Delille, 1990) and in the Weddell Sea (Grossman, 1994). These numbers are very close to those reported for the surface waters of the North Atlantic (Herndl et al., 2005). Thus, psychrophilic bacteria have successfully developed adaptations enabling them to thrive at low temperatures. One can expect that this adaptation to cold involves modifications of the cytoplasmic membrane so as to maintain the appropriate permeability. Adjustments in the control of supramolecular assembly processes and of secondary structures of nucleic acids are also necessary in order to secure appropriate rates of transcription and translation. Finally, the enzymes, either extra- or intracellular, have to be finely tuned to compensate for the freezing effect of low temperatures on the three-dimensional structure. The effects of low temperatures on membranes, supramolecular assembly, and nucleic acids have been discussed elsewhere and are not treated here. Readers are referred to the following reviews and articles: for membranes (Ray et al., 1994; Russell, 1997; Chintalapati et al., 2004), for supramolecular assembly (Willem et al., 1999; Pucciarelli and Miceli, 2002), and for nucleic acids (Somkuti, 1981; Dalluge et al., 1997; Fukunaga et al., 1999; Sahara et al., 1999; Eriksson et al., 2002; Thomas and Cavicchioli, 2002; Gualerzi et al., 2003; Duilio et al., 2004a). One also has to consider the effects of cold-shock proteins which have important roles in the control of nucleic acid dynamics, in the protection of proteins, and possibly in other functions still to be unravelled (Hebraud and Potier, 1999; Phadtare et al., 1999; Wouters et al., 2001; Ermolenko and Makhatadze,

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2002; Inouye and Phadtare, 2004; Phadtare, 2004; Yoshimune et al., 2005). The complete genomes of *Desulfotalea psychrophila* (Rabus et al., 2005), *Colwellia psychrerythraea* (Methe et al., 2005), and *Pseudoalteromonas haloplanktis* TAC125 (Medigue et al., 2005) have been sequenced and partially analyzed. These sequenced genomes together with proteomic studies provide valuable information about cold adaptation (Imbert and Gancel, 2004; Seo et al., 2004). In this review, we focus on cold-adapted enzymes produced by marine microorganisms thriving in permanently cold environments such as Antarctic sea waters.

Challenges at Low Temperatures

The Temperature Factor. Chemical reaction rates are very sensitive to temperature changes, although the degree of sensitivity exponentially depends on the value of the activation energy of the reaction, as illustrated by the Arrhenius law. This empirical law can be rewritten in an equivalent but more theoretical form:

$$k = \kappa k_B T/h \ \exp\left(-\Delta G^* \Big/ RT\right), \qquad (1)$$

in which k is the catalytic constant, k_B is the Boltzmann constant, T is the temperature in Kelvin, ΔG^* is the activation energy, R is the gas constant, and κ is the transmission coefficient which is in general misleadingly considered to be equal to 1. From this equation, one can readily appreciate the importance of the value of the activation energy on the reaction rate. Indeed, if one assumes a value of ΔG^*=40 kJ/mol, a decrease of only 10% of this value will increase the reaction rate by a factor of nearly 2. This underlines the importance of catalysts such as enzymes, the basic action of which is to decrease the activation energy of a chemical reaction. A very efficient enzyme will bring the activation energy close to zero, in which case the exponential term will tend to 1 and the reaction will become nearly independent of temperature. An important consequence is that the lower the activation energy, the lower the thermodependence of the reaction rate. This has to be considered as of prime importance in the context of cold adaptation, because one way to render the rate of a chemical reaction appropriate at low temperatures is to develop an enzyme that either decreases the activation enthalpy of the reaction or increases the activation entropy, because: $\Delta G^* = \Delta H^* - T\Delta S^*$.

The Viscosity Factor. The coordinates of an enzyme-catalyzed reaction in its simplest form are expressed as follows: $E + S \Leftrightarrow ES \Leftrightarrow ES^* \Rightarrow E + P$, also known as the transition state theory. Every situation perturbing the conversion of the activated enzyme-substrate complex ES* into E and P, or bypassing the ES* state (tunneling effect) will lead to a variation of the transmission coefficient κ in equation (1).

This coefficient has recently been reanalyzed and the following expression has been developed (Garcia-Viloca et al., 2004): $\kappa = \gamma_t = \Gamma_{(T)} \cdot \kappa_{(T)} \cdot g_{(T)}$. In this equation, $\Gamma_{(T)}$ stands for a possible recrossing of the energy barrier leading to the return of the ES* complex to E and S: $\Gamma_{(T)}$ is therefore equal to or less than 1 and the reaction rate will be negatively affected. $\kappa_{(T)}$, different from the κ found in equation (1), takes into account possible tunneling effects, meaning that some of the enzyme-substrate complexes with a free energy lower than the required activation energy could nevertheless lead to product formation: $\kappa_{(T)}$ can be greater than or equal to 1. Finally, $g_{(T)}$ expresses the possible deviation of the system from the assumed equilibrium distribution; it will therefore be less than or greater than 1. Thus, depending on the physicochemical state of the environment of the catalyzed reaction, the transmission coefficient in equation (1) has a high probability of being different from 1.

In the context of cold adaptation, the viscosity of the medium certainly influences the transmission coefficient. The viscosity of the cytoplasm of mammalian cells was found to change, on average, from 5 cP at 0°C to 2.5 cP at 20°C (Mastro and Keith, 1984) and the influence of viscosity on the reaction rate of enzyme-catalyzed reactions has been analyzed by Demchenko et al. (1989). Lactate dehydrogenase was selected as the reference enzyme and the influence of the viscosity of the aqueous medium on the reaction rates was measured in both directions, reduction of pyruvate and oxidation of lactate, after adjustment of the viscosity to different values using compatible solutes such as sucrose, glycerol, and ethylene glycol. The authors unambiguously demonstrated that the rate of lactate oxidation (V_{max}) decreases from 8.5 relative units in low viscosity buffer to 1.5 units in a 44% sucrose solution, which has a viscosity of about 6 cP. Similar effects were observed with other additives and showed that low temperatures, by affecting the viscosity of the medium, can bring the value of the transmission coefficient well below unity, and can further depress reaction rates to a significantly higher extent than that predicted by the Arrhenius law.

Table 1. Catalytic properties and activation parameters of various psychrophile enzymes compared to those of their mesophilic counterparts.

Enzyme	*Source*	*T* (°C)	k_{cat} (s^{-1})	T_{opt} (°C)	ΔG^* (*kJ/mol*)	ΔH^* (*kJ/mol*)	$T\Delta S^*$ (*kJ/mol*)	$\Delta\Delta H^*$ (*p-m*) (*kJ/mol*)	$T\Delta\Delta S^*$ (*p-m*) (*kJ/mol*)	*References*
Amylase	Psychro	10	294	28	57.68	34.7	−23.0	−11.7	−10.9	D'Amico et al. 2003a,b
	Meso		97	54	58.52	46.4	−12.1			
Chitinase	Psychro	15	1.7	–	69.2	60.2	−9.0	−14.1	−16.1	Lonhienne et al., 2001a
	Meso		3.9	–	67.2	74.3	+7.1			
Chitobiase	Psychro	15	3.8	–	59.5	44.7	−14.8	−26.8	−22.8	Lonhienne et al. 2001b
	Meso		0.9	–	63.5	71.5	+8.0			
Cellulase	Psychro	4	0.18	37	71.6	46.2	−25.4	−19.6	−13.0	Garsoux et al., 2004
	Meso		00.1	56	78.2	65.8	−12.4			
Subtilisin	Psychro	15	25.4	40	62.0	36.0	−26.5	−10.0	−6.3	Davail et al., 1994
	Meso		5.4	60	66.0	46.0	−20.2			
Xylanase (bacterium)	Psychro	10	515.5	35	54.0	21.0	−3.3	−37.0	−3.1	Collins et al., 2003
	Meso		59.5	62	60.0	58.0	−2.0			
Xylanase (yeast)	Psychro	5	14.8		52.3	45.4	−7.0	−4.5	−2.3	Petrescu et al., 2000
	Meso		4.9		54.6	49.9	−4.7			

Nutrition. In terms of nutrition, marine micro organisms are in a peculiar context. Although some dissolved inorganic and organic components of small size can be more or less directly absorbed by the organisms, many of the vital carbon- and/or nitrogen-containing compounds are in the form of macromolecular structures in the natural environ ment. To access these substrates, microorganisms must secrete enzymes capable of hydrolyzing these compounds, and must be able to absorb the hydrolysate before its dilution in the medium. It is clear that the extracellular enzymes cannot be directly secreted in the liquid environment but are produced after the attachment of the micro organism to the substrate; in the same context, the liberated nutrients should be directly absorbed by the cell or in some manner kept in place.

This general situation is independent of temperature, but new challenges are added when we consider that polar marine environments may freeze. First, in a frozen environment, microorganisms will have to avoid dehydration and freezing of the intracellular space; second, nutrient fluxes will be limited; and third, the secreted enzymes should be protected against cold denaturation (Privalov, 1990). Besides the production of enzymes adapted to cold, exopolysaccharides (EPS) produced by the microorganisms probably play a crucial role in this situation. These components are high molecular mass carbohydrates produced by many marine bacteria, either in the form of soluble compounds found in the environment and/or tightly associated with the bacterial cell (Decho, 1990; Nichols et al., 2005). They contain three or four different monosaccharides and are particularly rich in uronic acid, which gives these compounds a net negative charge; most importantly, they are highly hydrated because EPS gels consist of up to 99% water. Their role is to act on the chemical and physical environment of the cell, notably favoring the adhesion of the bacteria to surfaces, but also trapping the extracellular enzymes, substrates, and products. In addition, they can act as cryoprotectants: in their free form, they decrease the freezing point of sea water and contribute in this way to keeping water available to the cell; this process is also enhanced by their high water binding capacity. They also offer a protective shell to extracellular enzymes, preserving the catalysts from cold denaturation and enabling them to sustain their activity at subzero temperatures, according to the exclusion principle defined by Timasheff (1992), and as described for other polymers such as polyethylene glycol and dextrans. Indeed, the role of EPS seems to be quite important and strongly associated with cellular temperature

stress; interestingly, an Antarctic *Pseudoalteromonas* strain from sea ice produced 30 times more EPS at −2°C than at 20°C (Mancuso Nichols et al., 2004).

General Properties of Cold-Adapted Enzymes

Various cold-adapted enzymes from Antarctic marine bacterial strains have been studied in detail, but we will discuss only those for which the activation parameters have been determined and compared to those of mesophilic homologs; their properties are summarized in Table 1 (see also Siddiqui and Cavicchioli, 2006). First, the specific activity of these enzymes, with only a few reported exceptions, is much higher than that of their mesophilic counterparts, with differences sometimes exceeding a factor 10. One exception is the chitinase produced by an Antarctic *Arthrobacter* strain which was compared to its mesophilic counterpart from *Serratia marcescens* (Lonhienne et al., 2001a). A soluble preparation of chitin from crab shell was used as substrate, and because chitin from different origins displays significantly different structures, we believe that the substrate specificity of the respective enzymes could possibly explain the unexpected data. This case is mentioned to underline the difficulties of comparing enzymes adapted to different temperature environments, as often the enzymes originate from phylogenetically distant organisms and display different substrate specificities.

The higher specific activity of psychrophilic enzymes is reflected by the significantly lower values of the activation energy ΔG^*. The analysis of the enthalpic and entropic terms shows that, as expected, the activation enthalpy (ΔH^*) of cold-adapted enzymes is lower than that of their mesophilic counterparts, rendering these enzymes less dependent on temperature changes. The activation entropy values ($T\Delta S^*$) indicate that there is a systematic compensation effect leading to an attenuation of the difference between the ΔG^* values of psychrophilic and mesophilic enzymes. Indeed, the difference between the activation entropy of the psychrophilic and mesophilic enzymes is always negative ($T\Delta\Delta S^*$), independent of the sign of the entropy changes associated with the activation processes. Usually, these entropic changes are negative (Table 1) and correspond to a more ordered state of the activated complexes. The higher negative values of the entropic changes of the cold-adapted enzymes already suggest that their initial conformation is highly disordered as compared to their mesophilic counterparts. For some enzymes, the activation entropy is positive and this can be due to a higher disorder of the activated state or to a redistribution of water molecules participating in the activation process. However, in any case, the $T\Delta S^*$ values of psychrophilic enzymes remain lower than those of their mesophilic counterparts and this also points to a larger distribution of the basal conformational states of cold-adapted enzymes (D'Amico et al., 2003a).

The apparent optimum temperature (T_{opt}) of psychrophilic enzymes is lower than that of the mesophilic enzymes because the former display a lower thermal stability, so that, in general, the apparent optima are shifted toward low temperatures by 10 to 20°C. The thermal instability of cold-adapted enzymes has been demonstrated by unequivocal techniques such as fluorescence spectroscopy and differential scanning calorimetry (D'Amico et al., 2002; Collins et al., 2003; Georlette et al., 2003a). In some examples, thermal denaturation appears fully reversible such as for the α-amylase from *Pseudoalteromonas haloplanktis* (Feller et al., 1999). This has allowed for the calculation of the stabilization energy, ΔG_{stab}, which is the energy necessary to unfold the molecular structure at a given temperature, but also corresponds to the difference between the free energy of the unfolded state and that of the native state. Figure 1 shows that for the psychrophilic, mesophilic, and thermophilic enzymes, the maximum stabilization energy is reached around room temperature; this is presumably due to the hydrophobic effect (hydrophobic interactions are a crucial component of protein stability), which is maximum around this temperature (Kumar et al., 2002). The higher melting temperatures of the mesophilic and thermophilic α-amylases (ΔG_{stab}=0), when compared to the psy-

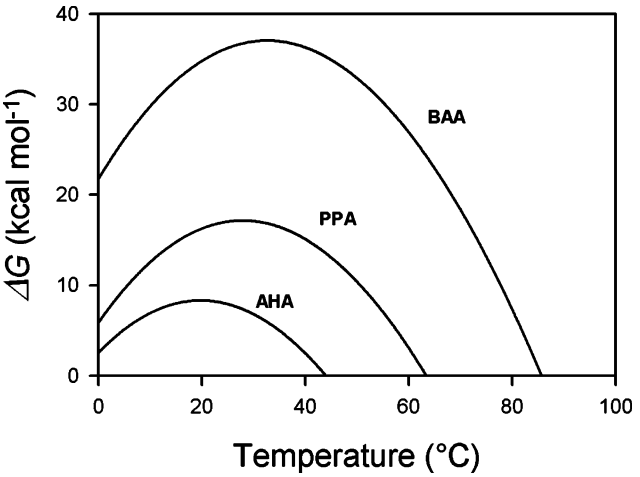

Figure 1. Stabilization energy of three α-amylases are shown: AHA, psychrophilic; PPA, mesophilic; and BAA, thermophilic, as calculated from microcalorimetric data.

chrophilic enzyme, are reached mainly by a lifting of the stability curve of the cold-adapted enzyme. This is achieved by an increase of the stabilization enthalpy, as demonstrated by the respective values of the calorimetric enthalpy ΔH_{cal}, that is, an increase in the number or/and of the strength of the weak bonds participating in the stabilization process, these include hydrogen bonds, ionic, hydrophobic, and Van der Waals interactions. From these curves (Figure 1), one can also deduce that the cold-adapted enzyme will be more prone to cold denaturation, a phenomenon driven mainly by the hydration processes of ionic, polar, and nonpolar bonds. This is easily understandable since less of these bonds are involved in the stabilization of cold-adapted enzymes as compared to mesophilic ones, and their formation involves a component requiring a positive modification of the enthalpy (unfavorable absorption of heat) known as the desolvation penalty, which is necessary to make these groups available for a new combination. In the case of the cold-adapted α-amylase, the maximum conformational stability ΔG_{stab} is around 15 kJ/mol at 17°C, close to that (ΔG_{stab}=14 kJ/mol at 15°C) of cold-adapted dihydrofolate reductase from a deep sea microorganism *Moritella profunda* (Hata et al., 2004), which also shows a reversible thermal unfolding. These values, close to the limit preceding the unfolding of the three-dimensional structure, once again reflect the low stability of these enzymes. Finally, the reversibility of the thermal unfolding of some cold-adapted enzymes has been attributed to the fact that on unfolding, a lower number of hydrophobic groups are exposed to the aqueous solvent, preventing or limiting the irreversible aggregation process typical of more stable proteins.

The Flexibility Concept. There is now a general consensus attributing the high specific activity of psychrophilic enzymes at low temperatures to a significant improvement of their flexibility, leading to a large distribution of conformational isomers, only slightly differing in free energy, and in equilibrium with each other (D'Amico et al., 2003a). This concept originates from the fact that the stable and rigid edifices of thermophilic enzymes show no or a very limited activity at low temperatures. Indeed, such temperatures contribute to increase further the rigidity of heat-stable enzymes and renders impossible the activation process which requires a remodeling of the enzyme's structure, that is, some bonds have to be broken to allow the accommodation of the substrate. We can easily imagine that a rigid structure will necessitate the absorption of a higher amount of energy from the surrounding environment when compared to a more flexible one.

In the case of thermophilic enzymes, the high temperature of the environment will enable, through a positive value of the stabilization entropy ΔS_{stab}, a sufficient lowering of ΔG_{stab} so as to secure the appropriate flexibility. On the contrary, in the case of psychrophilic enzymes, a negative stabilization enthalpy, which gives rise to the observed reduced ΔG_{stab}, allows for an appropriate flexibility at its environmental temperature. This negative ΔH_{stab} issues from the hydration process mentioned above and is compensated for by a negative ΔS_{stab}, which itself results from the large initial entropy of the native state and the reduced entropy of the unfolded state induced by the precise ordering of water molecules around the hydrated groups.

Conceptually, psychrophilic and mesophilic enzymes should display a similar specific activity at their respective environmental temperature, and thus also a comparable flexibility (Fields and Somero, 1998). This concept is strongly supported by a recent neutron scattering experiment. It was shown that in whole cell extracts, in which proteins represent 70% of the dry weight (Tehei et al., 2004), from three closely related bacteria adapted to different temperatures, the resilience (rigidity) at room temperature increased with the physiological temperature of the microorganisms, from 0.2 N/m in psychrophiles to 0.6 N/m in hyperthermophiles. However, at the respective environmental temperatures, the measured resiliencies were very similar.

In cold-adapted enzymes, flexibility thus appears to play a crucial role in catalysis by securing the accommodation of the substrate at low temperature, as well as by facilitating the movement of water molecules and the release of products. In this context, an interesting observation has been made: the thermal inactivation of cold-adapted enzymes often precedes the unfolding of the structure (Collins et al., 2002; D'Amico et al., 2003a; Georlette et al., 2003a). This indicates that above a certain temperature threshold, the active site is unable to accommodate the substrate, although no modification of the three-dimensional structure could be recorded by fluorescence spectroscopy or differential scanning calorimetry. Thus, the active site seems to be very heat sensitive, yet the residues involved in catalysis and substrate binding are in general conserved when compared to their mesophilic counterparts for which the thermal inactivation coincides with the loss of conformation (Figure 2). Further, the increase in flexibility of psychrophilic enzymes can be distributed throughout the structure or can be limited to only crucial parts of the molecule, as

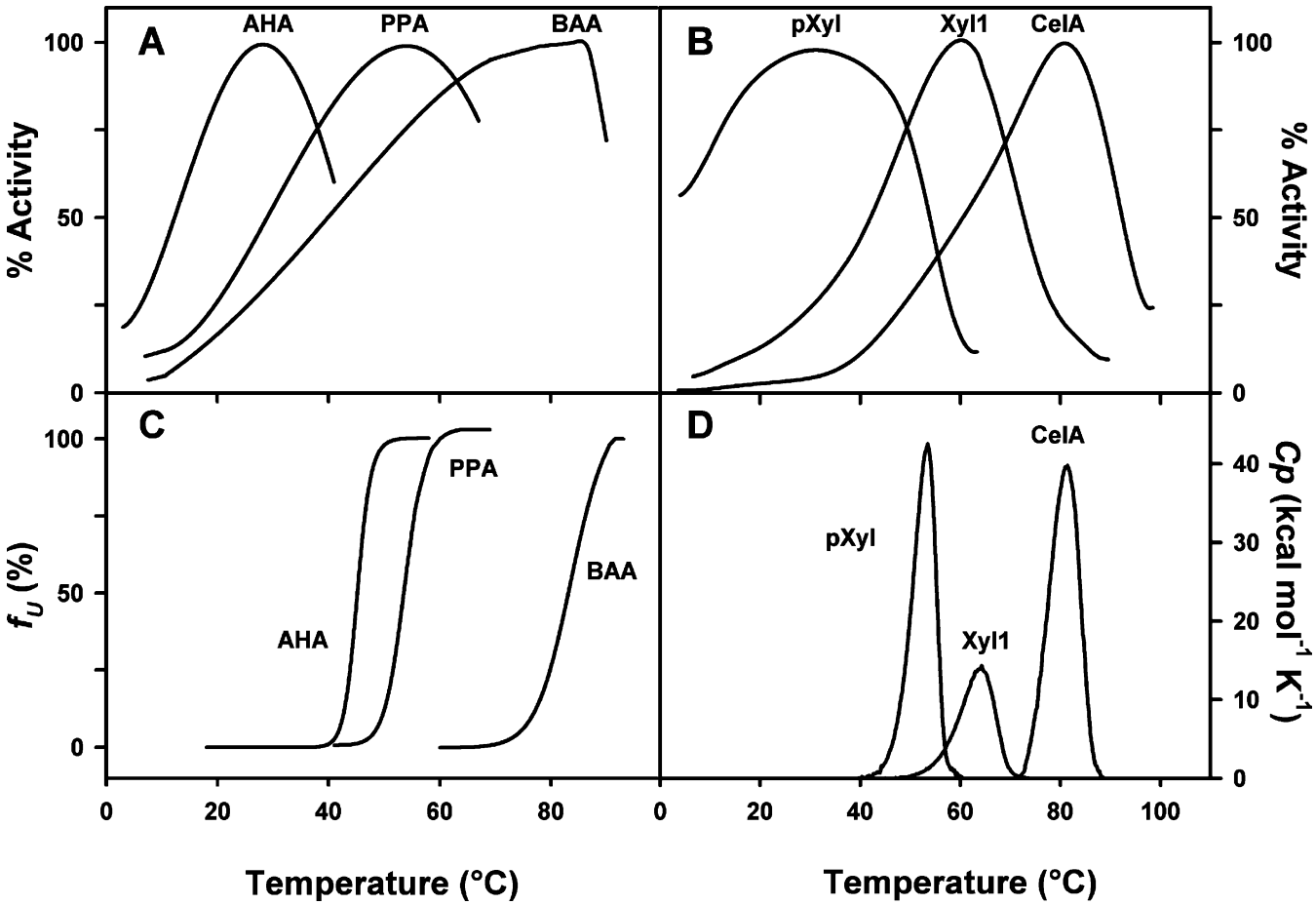

Figure 2. Thermodependence of activity (AB, upper panel), and unfolding process, as recorded by fluorescence spectroscopy (C), and differential microcalorimetry (D), of cold-adapted α-amylase (AHA), mesophilic α-amylase from pig pancreas (PPA), thermostable α-amylase from *Bacillus amyloliquefaciens* (BAA), family 8 cold-adapted xylanase from Antarctic *Pseudoalteromonas haloplanktis* (pXyl), family 11 mesophilic xylanase from *Streptomyces* sp. S38(Xyl1), and family 8 thermostable endoglucanase from Clostridium thermocellum (CelA). The apparent maximal activities of cold-adapted enzymes, AHA and pXyl, are reached well before any significant structural changes.

was recently illustrated for a phosphoglycerate kinase from an Antartic strain of *Pseudomonas aeruginosa* (Zecchinon et al., 2005). However, if cold-adapted enzymes do indeed improve their activity through an increase in flexibility, originating from a decrease in stability, one can expect that, being already close to the limit of stability, these enzymes cannot further adapt. Thus, they will not be able to achieve the same performance at low temperatures as their mesophilic counterparts display at their environmental temperatures. Indeed, as illustrated in most cases by the experimental data the adaptation appears to be incomplete.

Flexibility is not an easily measured parameter because it is related not only to the amplitude of the possible relative movements between structural components but also to the multiplicity of configurations that can be adopted (static flexibility) and to the interval of time necessary to switch from one configuration to the other (dynamic flexibility). When crystal structures are available, the temperature factors or B-factors can be taken as indexes of a possible disorder in a particular region of the protein. The relative B-factors, corresponding to the ratio between the temperature factor of one atom and the average value of all the others, can be quite useful in detecting a particularly dynamic or flexible region. When two enzymes from different environmental temperatures need to be compared, the situation is more complicated because various parameters such as the temperature of crystallization, the temperature at which the diffraction patterns have been obtained, the position of the molecules in the crystal unit, their possible interactions, and so forth, can affect the B-factor values. Data in agreement with the flexibility concept have however been reported for malate dehydrogenases from psychrophilic and thermophilic microorgan-

isms (Sun-Yong et al., 1999). More recently, a very good correlation between the B-factors, the activity and the stability of adenylate kinases from psychrophilic, mesophilic, and thermophilic *Bacillus* sp. has been reported (Bae and Phillips, 2004). Some other experimental techniques, such as amide hydrogen-deuterium exchange followed by nuclear magnetic resonance, mass spectrometry, and Fourier transform infrared spectroscopy (FTIR) have provided controversial data. In fact, the time scale of the exchange is very important and has to be related to that of the catalytic process in order to find a correlation between exchange rate, stability, and the catalytic process. Results in agreement with the concept have been reported for alcohol dehydrogenases (Liang et al., 2004) and mesophilic and thermophilic 3-isopropylmalate dehydrogenases (Zavodszky et al., 1998).

Fluorescence quenching experiments of tryptophan residues, using acrylamide as a dynamic quencher, have provided a good correlation between stability, flexibility, and the catalytic efficiency. Here, the decrease of emitted fluorescence of the fluorophores (Trp) is due to the diffusive collision of the quencher with the fluorophores. This process reflects the ability of the quencher to penetrate the structure and is therefore related to the micro-unfolding of the enzyme, this being viewed as an index of flexibility. Experiments based on this technique were performed with Ca^{2+}–Zn^{2+} proteases (Chessa et al., 2000), α-amylases (D'Amico et al., 2003a), DNA ligases (Georlette et al., 2003a), xylanases (Collins et al., 2003), and cellulases (Sonan G., personal communication) adapted to different thermal environments. In all cases, a higher flexibility was found for the psychrophilic enzymes when compared to mesophilic and thermophilic counterparts, as well as to mutants stabilized by genetic engineering.

Site-Directed Mutagenesis

From the comparison of the aforementioned three-dimensional structures and model structures of cold-adapted enzymes with their mesophilic and/or thermophilic counterparts, some hypotheses related to cold-adaptation have been proposed. They are based on amino acid substitutions which are supposed to contribute to the low stability of psychrophilic enzymes. This approach is of course risky, since *in vivo*, the enzyme structure is modulated through numerous constraints, the effect of which is impossible to evaluate. It is also predictable that the absence of any selective pressure from heat on the structure could also give rise to some genetic drift, further obscuring the real significance of the observed amino acids changes. In this context, site-directed mutagenesis appears to be an interesting tool to discriminate between the amino acid substitutions involved or not in cold adaptation. This technique has been applied to a few psychrophilic enzymes and the conclusions of the most recent experiments are summarized hereafter. The comparison of the cold-adapted citrate synthase from an *Arthrobacter* strain with the homologous enzyme from the hyper-thermophile *Pyrococcus furiosus* led to the conclusion that part of the adaptation could reside in a better accessibility of the active site of the cold-adapted enzyme. Two alanine residues at the entrance of the site were thus replaced with an arginine and a glutamate residue occupying respectively similar positions in the thermophilic enzyme; an additional loop found in this enzyme was also introduced in the cold-adapted counterpart. These changes did not significantly alter the specific activities when compared to the wild-type form, but in the case of the double mutant, the K_m value was considerably lower for acetyl-CoA, presumably due to the fact that in *P. furiosus* the arginine residue is one of the ligand of the phosphate group of the substrate. The thermostability of the mutants was, however, significantly increased. The introduction of a loop of three residues (SKG) in the active site resulted in a drastic increase of the K_m value and in a reduction of about 70% of the k_{cat} value with no modification of the thermostability. These data underline the difficulty of the rational approach even though a high-resolution three-dimensional structure is available (Gerike et al., 2001). Numerous single mutations were also introduced in a cold-adapted α-amylase from the Antarctic strain *Pseudoalteromonas haloplanktis*, mimicking the situation found in more stable homologous α-amylases. All mutations are located far from the active site but, as expected, the overall trend was a decrease in both k_{cat} and K_m, corresponding to a rigidification of the molecular structure (D'Amico et al., 2001). The introduction of an additional disulfide bridge found in the more stable enzyme from pig pancreas (1) decreases the k_{cat} twofold as well as the K_m; (2) increases the overall rigidity of the structure as demonstrated by fluorescence quenching; and (3) transforms the single thermal unfolding transition of the wild-type enzyme into two distinct calorimetric domains, one with a high thermostability and the other with a lower thermal stability than that of the wild type enzyme, indicating that although the extra disulfide bridge contributes to significantly increase the stability of one part of the

molecule it nevertheless creates some constraints in an other part (D'Amico et al., 2002). However, when associated with five other single mutations, the disulfide bridge mutant displays a thermodynamic stability curve close to that of the mesophilic enzyme with a T_m value improved by 5.4°C. As expected, the k_{cat} value is half that of the wild-type enzyme and the affinity of the mutated enzyme toward its substrate is improved by a factor close to four (D'Amico et al., 2003b). Glycine residues have often been cited as a means to improve the flexibility of protein structure, providing more amplitude to the relative movements between secondary structures. Indeed, in some cases the proportion of this residue in psychrophilic enzymes is higher than in mesophilic counterparts. In an attempt to rigidify the molecular structure of the cold-adapted chitinase from the Antarctic *Arthrobacter* sp. TAD20, three glycine residues were replaced by amino acids found in more stable enzymes; the mutation G93P contributes to increase the melting temperature by 1.2°C and the activity of the mutant is, as predicted, lower. In contrast, the mutant G254P is by contrast extremely unstable but highly active, displaying an activation energy half that of the wild-type enzyme. The mutant G406Q is less thermostable, displays higher activation energy, and is less active than the wild type. Although some of these data confirm the inverse relationship existing between the rigidity of an enzyme structure and the activity of the enzyme, the mutant G406Q demonstrates that the structural environment of the mutated glycine residue can render this relation much more complex than expected. Another mutant, N198K, after introduction of an additional salt bridge, shows a higher melting temperature and a reduced activity, corroborating the general concept (Mavromatis et al., 2003). Site-directed mutagenesis was also applied to a cold-active esterase from the Antarctic *Psychrobacter* sp. Ant300. Here again, a glycine residue in a loop close to the active site was replaced by a proline residue and a rigidification was expected. Indeed the mutant G244P shows a drastically improved thermal stability at 40°C ($t_{1/2}$=11.6 h versus $t_{1/2}$=16 min) whereas the k_{cat} was significantly reduced especially when large substrates such as pNP-hexanoate and pNP-octanoate were used (Kulakova et al., 2004). The observed shift in the specificity also underlines the necessity to evaluate the performance of mutated multisubstrate enzymes by using as many substrates as possible. Too often indeed the catalytic properties of mutants of enzymes such as esterases, lipases, and proteases, for example, are measured with only one synthetic and small size substrate. This, undoubtedly, can give rise to erroneous conclusions regarding the possible relationship between thermal stability and specific activity. From these selected examples, it is clear that site-directed mutagenesis can help in detecting the amino acid substitution involved in the adaptation of a psychrophilic enzyme but it is also clear that the specific environment of the selected residue can complicate the interpretation of the data. Sometimes synergistic mutations are necessary to render more obvious the inverse relationship between thermal stability and specific activity. In this context, an experiment exploring the role of a glycine cluster in the adaptation to cold of an alkaline phosphatase from an Antarctic strain is exemplary: indeed, the replacement of G261 by an alanine residue gives rise to a mutant with a lower thermal stability due to steric hindrance, but also to a lower specific activity. However, when this mutation was associated with the substitution Y269A, predicted to remove the spatial constraint created by this level by the alanine residue, the double mutant showed, as expected, a much higher thermal stability and a reduced catalytic efficiency. It was concluded that indeed G261 is involved in cold adaptation since in other mesophilic and thermophilic counterparts position 269 is occupied by less bulky residues, such as glycine and serine, than Y269 in the cold-adapted enzyme (Mavromatis et al., 2002).

Cold-Adapted Enzymes as Tools in Biotechnology

The properties of cold-adapted enzymes make them potentially valuable alternatives to their mesophilic counterparts. Indeed, the interest of a high specific activity at low temperatures is obvious, whereas the relatively high thermosensitivity provides the possibility of rapidly inactivating these enzymes in complex mixtures using, for example, mild heat treatment and preserving in this way product quality. This property could be particularly useful in those industrial processes in which the contact of the enzyme with the substrates to be transformed and products should be limited in time so as to prevent excessive or deleterious action. Examples include meat tenderizing with proteases and stone-washing in the textile industry, in which the excessive action of cellulases could lead to the loss of mechanical resistance of the cotton fibers.

Several reviews have already been devoted to the use of cold-adapted microorganisms and their enzymes in biotechnology: Margesin and Schinner (1994), Margesin and Schinner (1999), Gerday et al. (2000), Allen et al. (2002), and Cavicchioli et al. (2002).

As Additives in Detergents. This industry should be mentioned first, as the market for enzymes such as proteases, lipases, amylases, and cellulases, which are commonly used as additives in detergents, represents about 40% of the total sale of enzymes. Cold-active detergents are commonly advertised because washing is now frequently carried out at environmental temperatures. Cold-adapted enzymes offer therefore a high potential in this type of application but production costs still limit their use at present. Patents involving enzymes such as proteases from cold-adapted bacteria have already been filed: Baeck and Quamrul, ZA9610820; Mikio and Katsuhisa, WO9743406; Quamrul and Eiichi, US6200793; Quamrul and Eiichi, WO9730172; and Eiichi, ZA9601237.

As Additives in the Food Industry. Meat tenderizing with proteases has already been mentioned; other examples are cold-active β-galactosidases, which constitute interesting alternatives for lactose removal in milk so as to enhance digestibility and sweetness; their high specific activities at low temperature are their main advantage and they can be used during transport and storage of milk at low temperatures. Patents filed in connection with this application are: in 2002, Gerday and Hoyoux, EP1261697; in 2004, Hoyoux and François, US6727084, and in 2005, Gerday and Hoyoux, US2005196835. An interesting application, protected by patents WO2004023879 (Dutron et al.), and WO200587916 (Georis et al.), is the successful use of highly cold-active bacterial xylanases from marine Antarctic waters in the baking industry, favoring an increased bread volume and improved crumb quality (Collins et al., 2006).

Other potential applications that could be developed in the near future include the use of pectinases (Truong et al., 2001) in the fruit juice industry for enhancing extraction yield, clarification, and taste. Cold-active lipases could be useful for the development of various tastes and flavors owing to their high specific activity and unique specificities. Animal feed can also constitute a vast application field for these enzymes in terms of improvement of the digestibility and assimilation. One can also think about applying these enzymes for the synthesis of various valuable peptides, fatty acids, and polysaccharides by reverse hydrolysis in low water conditions, taking advantage of the high flexibility of these enzymes to further decrease water activity and improve yields. This process can also be extended to the pharmaceutical and chemical industries for the production of compounds with high added value.

As Additives in the Textile Industry. Besides the application of cold-adapted cellulases during the stone-washing process and the production of denim fabric, one can also add the treatment of cotton garments by these easily inactivated cellulases during the washing cycle to restore softness and to reduce pill formation, a process known as biopolishing.

As New Tools in Molecular Biology. It is worth mentioning that the first cold-adapted enzyme from Antarctic microorganisms to have been fully characterized is an alkaline phosphatase catalyzing the hydrolysis of the 5′-phosphate of oligonucleotides. The authors had, as early as in 1984, already underlined the interest of such an enzyme in the radioactive end-labeling of nucleic acids using radioactive ATP and T4 polynucleotide kinase. Indeed, the cold-adapted phosphatase offers the unique advantage, contrary to its mesophilic counterparts, of being rapidly inactivated by mild heat treatment prior to the use of the kinase, thereby allowing higher yields and preserving product integrity (Kobori et al., 1984).

The coupling of oligonucleotides using cold-adapted DNA ligases also seems promising as the ligation yield is much higher at low temperatures. Cold-adapted DNA ligases, such as those already described (Georlette et al., 2003b), could offer a significant advantage over mesophilic enzymes poorly active at low temperatures.

These applications are only a few examples of the tools that are or will be provided by cold-adapted microorganisms. Bioremediation processes utilizing these organisms for the degradation of soils and waters contaminated by hydrocarbons, heavy metals, and other xenobiotic compounds have already been investigated (Margesin and Schinner, 1999). In addition, studies are also underway for low-temperature expression systems using psychrophilic hosts, which should allow for a reduction in the formation of inclusion bodies and an increase in the expression of correctly folded proteins under soluble form (Duilio et al., 2004b) due to the weakening of hydrophobic interactions at low temperatures.

Conclusions

From biodiversity to biotechnological aspects, scientific investigations related to cold-adapted microorganisms remain rather limited and have expanded

only in recent years, particularly as a result of the support of the European Union. Cold environments have to be investigated further so as to discover and to characterize new strains, cultivable or not. Their enzymes, key features of their adaptation to these extreme environments, should be isolated, cloned, and characterized to gain further insight into the strategies of the adaptation to cold, but also to evaluate their biotechnological potential. New genomes need to be sequenced, annotated, and analyzed because at the present, only two genomes, that of *Colwellia psychrerythraea* from Arctic marine sediments (Methe et al., 2005) and that of *Pseudoalteromonas haloplanktis* from Antarctic seawaters (Medigue et al., 2005), have been sufficiently analyzed to enable detection of some of the more specific adaptations to cold which can cover numerous genes and gene clusters. Among these adaptations, the factors responsible for the adjustment of membrane fluidity are of prime importance, whereas the large diversity of factors contributing to limit the toxicity of highly concentrated dissolved oxygen has been already investigated. These studies indicate a high content of enzymes involved in oxygen consumption such as desaturases, superoxide dismutases, and catalases whereas an unusual feature observed in the Antarctic *Pseudoalteromonas haloplanktis* is the elimination of the ubiquitous molybdopterin-dependent metabolism which is usually responsible for the production of reactive oxygen species. Nevertheless, further genome sequences are needed to detect whether there are some general trends in cold-adaptation or, if on the contrary, each microorganism has its own specific strategy. Proteomic analyses are also progressing and are required to establish the relationships that should exist between the expression of regulatory proteins and the environmental temperature.

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