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Molecular basis of cold adaptation

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Cold-adapted, or psychrophilic, organisms are able to thrive at low temperatures in permanently cold environments, which in fact characterize the greatest proportion of our planet. Psychrophiles include both prokaryotic and eukaryotic organisms and thus represent a significant proportion of the living world. These organisms produce cold-evolved enzymes that are partially able to cope with the reduction in chemical reaction rates induced by low temperatures. As a rule, cold-active enzymes display a high catalytic efficiency, associated however, with a low thermal stability. In most cases, the adaptation to cold is achieved through a reduction in the activation energy that possibly originates from an increased flexibility of either a selected area or of the overall protein structure. This enhanced plasticity seems in turn to be induced by the weak thermal stability of psychrophilic enzymes. The adaptation strategies are beginning to be understood thanks to recent advances in the elucidation of the molecular characteristics of cold-adapted enzymes derived from X-ray crystallography, protein engineering and biophysical methods. Psychrophilic organisms and their enzymes have, in recent years, increasingly attracted the attention of the scientific community due to their peculiar properties that render them particularly useful in investigating the possible relationship existing between stability, flexibility and specific activity and as valuable tools for biotechnological purposes.

Keywords: cold adaptation; psychrophiles; extremophiles; enzymes; microcalorimetry; directed evolution

1. INTRODUCTION

Our anthropocentric point of view has resulted in the classification of cold-adapted organisms as extremophiles, even though environments of permanently cold temperatures (around 0 °C) abound on Earth, especially when one considers that these include not only the polar and alpine regions but also deep-sea waters. Psychrophiles, both prokaryotic and eukaryotic, have successfully colonized these environments and are able to grow efficiently at sub-zero temperatures. This adaptation requires a vast array of structural and physiological adjustments in order to counteract the reduction in chemical reaction rates due to the low temperature of the habitat.

2. THE LOW TEMPERATURE CHALLENGE

Temperature is one of the most important environmental factors for life as it influences most biochemical reactions. Low temperatures slow down and strongly inhibit chemical reaction rates catalysed by enzymes, the 'workhorses' of cell metabolism. The effect of temperature on chemical reactions is basically described by the Arrhenius equation: $k = A\mathrm{e}^{-Ea/RT}$, where k is the rate constant, A is the pre-exponential factor that depends on the reaction, E_a is the so-called activation energy, R is the gas constant (8.31 kJ mol^{-1}) and T is the temperature in kelvins. Accordingly, any decrease in temperature will induce an exponential decrease of the reaction rate, the extent of which depends on the value of the activation energy. The thermodependence of the activity can be approximately expressed by the Q_{10} value that is normally close to 2–3. This is the main factor preventing the growth, at low temperatures, of non-adapted organisms.

The relative effect of temperature on the activity of psychrophilic and mesophilic enzymes is illustrated in figure 1, which reveals three basic features: (i) psychrophiles synthesize enzymes with higher specific activity (k_{cat}) at low and moderate temperatures; (ii) the apparent maximal activity for cold-active enzymes is shifted towards low temperatures, reflecting their weak thermostability; and (iii) the adaptation is not apparently complete, as the specific activity displayed by psychrophilic enzymes around 0 °C, although high, remains generally lower than that of the mesophilic enzymes at their own environmental temperature.

3. KINETIC OPTIMIZATION

A possible strategy to counteract the negative effect of cold on the activity of an enzyme could be to synthesize more enzyme, but it can be easily understood that this would be energetically expensive. Therefore, the common strategy used to maintain sustainable activity at a permanently low temperature is to produce a cold-adapted

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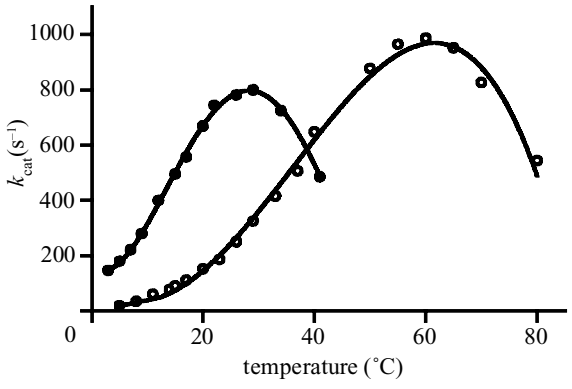

Figure 1. Comparison of the effect of temperature on the activity of two homologous enzymes. Curves representing the thermal dependence of the specific activity of the psychrophilic α-amylase from *Pseudoalteromonas haloplanktis* (closed circles) and of its thermostable homologue from *Bacillus amyloliquefaciens* (open circles).

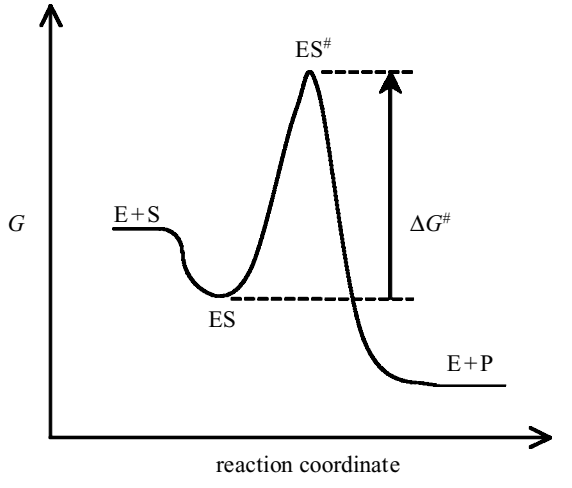

Figure 2. Transition state theory. Gibbs energy changes during a reaction. E, enzyme; S, substrate; P, product; $\Delta G^{\#}$, free energy of activation.

enzyme with enhanced catalytic efficiency k_{cat}/K_m (Feller & Gerday 1997). For extracellular enzymes that work at saturating substrate concentrations, adaptation consists mainly of increasing k_{cat} (Feller *et al.* 1994; Narinx *et al.* 1997; Petrescu *et al.* 2000). Alternatively, for secreted marine enzymes or intracellular enzymes that could face low substrate concentrations, a decrease in K_m (Michaelis–Menten constant) providing a higher substrate affinity could be useful (Kim *et al.* 1999; Bentahir *et al.* 2000; Georlette *et al.* 2000; Hoyoux *et al.* 2001). A compilation of available data (Smalas *et al.* 2000) indicates that cold-adapted enzymes optimize their catalytic efficiency by increasing k_{cat}, decreasing K_m or by changes in both parameters.

The turnover number k_{cat} corresponds to the maximum number of substrate molecules converted to product per active site per unit of time. In the Michaelis–Menten mechanism, the k_{cat} parameter is the first-order rate constant for the chemical conversion of the ES complex to enzyme and product. The transition state theory assumes that, during this conversion, a stable activated complex $ES^{\#}$ exists in equilibrium with the ground-state ES (figure 2). The relationships used to calculate the thermodynamic parameters of activation leading to $ES^{\#}$, $\Delta G^{\#}$ (free energy of activation), $\Delta H^{\#}$ (activation enthalpy) and $\Delta S^{\#}$ (activation entropy) can be found in Lonhienne *et al.* (2000). In the context of cold adaptation, a decrease in $\Delta G^{\#}$ seems crucial. The analysis of the differences between the activation parameters of cold-adapted enzymes and their mesophilic counterparts, namely $\Delta(\Delta G^{\#})_{p-m}$, $\Delta(\Delta H^{\#})_{p-m}$ and $\Delta(\Delta S^{\#})_{p-m}$, is especially striking (table 1). The small and negative $\Delta(\Delta G^{\#})_{p-m}$ values originate from differences in the enthalpic and entropic contributions and simply indicate, as expected, that psychrophilic enzymes are more active than their mesophilic homologues. As also expected, all psychrophilic enzymes studied so far display much lower $\Delta H^{\#}$ values, with the consequence that the temperature dependence of k_{cat} is buffered and thus the deleterious effect of low temperatures on enzyme reaction rates is moderate. As far as the entropic term is concerned, the analysis carried out on nine psychrophilic enzymes indicates that the difference between the activation entropy of a psychrophilic enzyme and that of the mesophilic counterpart is also always negative, whatever the sign (positive or negative) of the activation entropy. This feature leads to an antagonistic effect of the activation entropy so that the activation energy is not as low as would be expected from the decrease in activation enthalpy. It follows that the decrease of the activation enthalpy of a reaction catalysed by a psychrophilic enzyme can be considered as the main adaptive character to low temperatures. The corresponding decrease in activation energy is structurally achieved by a decrease in the number or in the strength of enthalpy-driven interactions that have to be broken during the activation steps. These interactions initially contribute to the stability of the protein folded conformation and, as a corollary, their alteration presumably gives rise to an increase in the flexibility of the structural domain of the enzyme involved in catalysis. As a consequence of active-site flexibility, the ground-state ES complex occupies a broader distribution of conformational states translated into an increased entropy of this state when compared with that of the mesophilic homologues, leading to a negative value of $\Delta\ (\Delta S^{\#})_{p-m}$, as already mentioned.

This phenomenon should possibly be accompanied by a weaker substrate binding strength and consequently by an evolutionary pressure on K_m in order to maximize the overall reaction rate. Psychrophilic enzymes, however, display identical substrate binding-site and active-site architecture when compared with their mesophilic homologues, as illustrated in the case of A_4-LDHs (lactate dehydrogenase) from South American and Antarctic notothenoid fishes (Holland *et al.* 1997; Fields & Somero 1998) and chloride-dependent α-amylases from Antarctic bacteria and pig pancreas (Qian *et al.* 1994; Aghajari *et al.* 1998*a*; D'Amico *et al.* 2000). An experimental demonstration of the relationship between k_{cat} and K_m was recently provided using cold-adapted and mesophilic α-amylases (D'Amico *et al.* 2001). Stabilizing weak interactions found in the porcine α-amylase, but absent in the cold-active α-amylase, were reintroduced by site-directed mutagenesis in the psychrophilic enzyme. As shown in figure 3, rigidifying the cold-active α-amylase tends to

Table 1. Activation parameters for the activity of psychrophilic and mesophilic enzymes.

enzyme	source	T (°C)	k_{cat} (s^{-1})	$\Delta G^{\#}$ (kJ mol^{-1})	$\Delta H^{\#}$ (kJ mol^{-1})	$T\Delta S^{\#}$ (kJ mol^{-1})	$\Delta(\Delta G^{\#})_{p-m}$ (kJ mol^{-1})	$\Delta(\Delta H^{\#})_{p-m}$ (kJ mol^{-1})	$T\Delta(\Delta S^{\#})_{p-m}$ (kJ mol^{-1})
trypsin	Greenland cod_p	5	82.0	57.7	33.0	−24.5	−2.5	−20.9	−18.3
	$bovine_m$		27.7	60.2	53.9	−6.2			
trypsin	Antarctic cod_p	15	0.97	70.5	40.6	−29.9	−0.9	−42.0	−41.2
	$bovine_m$		0.68	71.4	82.6	11.3			
amylase	*Alteromonas*$_p$	15	495.2	55.6	73.6	18.0	−4.1	−26.0	−21.9
	Bacillus$_m$		90.5	59.7	99.6	39.9			
subtilisin	*Bacillus* TA41$_p$	15	25.4	62.7	36.0	−26.7	−3.7	−10.0	−6.3
	B. subtilis$_m$		5.4	66.4	46.0	−20.4			
glu-6-phosphate dehydrogenase	Antarctic $fish_p$	0	106.5	56.1	36.8	−19.3	−2.1	−15.1	−13.0
	$human_m$		42.4	58.2	51.9	−6.3			
xylanase	Antarctic $yeast_p$	5	14.8	61.7	45.4	−16.3	−2.6	−4.5	−1.9
	$yeast_m$		4.9	64.3	49.9	−14.4			
chitobiase	*Arthrobacter*$_p$	15	98.0	59.5	44.7	−14.8	−4.0	−26.8	−22.8
	S. marcescens$_m$		18.0	63.5	71.5	8.0			
chitinase A	*Arthrobacter*$_p$	15	1.7	69.2	60.2	−9.0	2.0	−14.1	−16.1
	S. marcescens$_m$		3.9	67.2	74.3	7.1			
glutamate dehydrogenase	Antarctic $fish_p$	5	3.8	64.8	33.4	−31.4	−3.3	−25.6	−22.3
	$bovine_m$		0.9	68.1	59.0	−9.1			

Subscript p and m for psychrophile and mesophile, respectively.

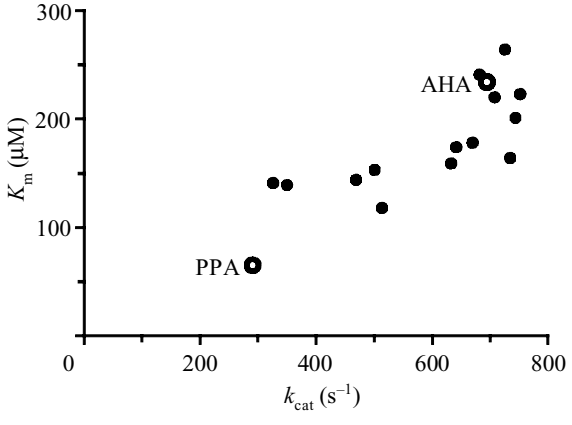

Figure 3. Correlation of kinetic parameters for wild-type (open circles) and mutant (filled circles) α-amylases. (AHA, psychrophilic α-amylase; PPA, mesophilic α-amylase.)

decrease both k_{cat} and K_m values revealing the correlation between these kinetic parameters.

4. STRUCTURAL ARCHITECTURE

The unique properties of cold-adapted enzymes, i.e. a high specific activity at low temperatures and a high thermosensitivity, can be useful in various biotechnological processes (Russell 1998; Gerday *et al.* 2000; Demirjian *et al.* 2001). Consequently, this has induced a growing interest in psychrophilic organisms and therefore the number of enzymes investigated has increased exponentially, leading to the elucidation of numerous primary structures (Russell 2000). Crystallographic structures of a number of cold-adapted enzymes have also been elucidated: α-amylase (Aghajari *et al.* 1998*a*,*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) and phosphoglycerate kinase (Mandelman *et al.* 2001). These 3D structures have provided new insights into the understanding of the molecular adaptations of cold-adapted enzymes. They have revealed that only minor structural modifications are needed to adapt a mesophilic homologue to cold temperatures. Moreover, as previously mentioned, all active-site residues involved in the reaction mechanisms are strictly conserved between homologous enzymes adapted to different temperatures. An important consequence is that the molecular changes responsible for cold adaptation have to be found elsewhere in the protein. The analysis of the 3D structures often reveals a better accessibility of the active site to the substrate. This larger opening of the catalytic cleft has been demonstrated from the model structure of Antarctic fish elastase (Aittaleb *et al.* 1997), from the structures of cold-active citrate synthase (Russell *et al.* 1998), Antarctic bacterial α-amylase (S. D'Amico, unpublished data) and the psychrophilic *Pseudomonas* Ca^{2+}, Zn^{2+} protease (N. Aghajari and R. Haser, personal communication). The better accessibility certainly helps to reduce the energy required for substrate accommodation and/or reaction product release in the case of macromolecular substrates (Merz *et al.* 2000).

A common trait of psychrophilic enzymes is their weak conformational stability. This requires a decrease in the culture temperature when expressing a recombinant cold-adapted enzyme in a mesophilic host such as *Escherichia coli* (Feller *et al.* 1998). The marked heat-lability is partly responsible for the shift of the apparent optimal temperature of activity shown in figure 1. The refined 3D structures and some structural models of psychrophilic enzymes have been quite useful in compiling an inventory of the structural factors possibly involved in cold-adaptation and also responsible for the low thermal stability. It was found that all structural factors currently known to stabilize the mesophilic protein counterpart

could be attenuated in strength and number in cold-active enzymes. This could possibly involve an increased number and clustering of glycine residues; a decrease in proline residues in loops; a reduction in arginine residues, capable of forming multiple electrostatic interactions and hydrogen bonds, and eventually a lowering of the number of ion pairs, aromatic interactions, hydrophobic interactions or hydrogen bonds in comparison with mesophilic homologues. Exhaustive analyses of these structural factors have been carried out previously (Feller *et al.* 1997; Feller & Gerday 1997). Obviously, each protein uses a few of these structural alterations in order to acquire the required flexibility to be more or less adapted to the temperature of the environment.

5. ACTIVITY–STABILITY–FLEXIBILITY RELATIONSHIPS

Low temperatures tend to improve the compactness of a protein by limiting the 'breathing' of the structure corresponding to micro-unfolding processes. Therefore, at low temperatures, a mesophilic protein will lose the mobility required for its catalytic activity. The current accepted hypothesis (Gerday *et al.* 1997; Zavodszky *et al.* 1998) suggests that psychrophilic enzymes have to increase their plasticity in order to perform catalysis at low temperatures, the enhanced plasticity being generated by the generally low stability of the protein structure. This balance between flexibility and stability represents one of the crucial points in the adaptation of a protein to environmental temperature. At present, the flexibility of a protein, especially that related to activity and/or stability, remains a difficult parameter to appreciate as the increase in flexibility can be limited only to a small but crucial part of the protein. A few methods have been used, more or less successfully, to evaluate the relative flexibility, including hydrogen/deuterium (H–D) exchange (Zavodszky *et al.* 1998; Svingor *et al.* 2001), tryptophan phosphorescence (Fischer *et al.* 2000; Gershenson *et al.* 2000) and tryptophan fluorescence quenching (J.-P. Chessa, D. Georlette and S. D'Amico, unpublished data). The problem first requires that the notion of flexibility is properly defined as something related directly to the specific activity of the enzyme. In other words, which type of flexibility is needed for the improvement of the activity? Is it a dynamic motion related to a specific time-scale? Is it a concept related to the amplitude of the deformation of the conformation of the protein at a given temperature? These uncertainties certainly explain why some thermophilic enzymes were found by H–D exchange techniques to be more flexible than their mesophilic and psychrophilic counterparts.

The relative flexibility of enzymes has been frequently suggested from structural comparisons of homologous proteins adapted to different temperature environments and the evaluation was mainly based on the difference in thermostability. It is clear that the modification of flexibility needed for improving the catalytic activity can occur only in specific regions of the protein structure. A significant increase in the overall flexibility of the protein could possibly lead to enzymes displaying a high specific activity at low temperature but also a weak affinity for the substrates and a very low stability. In this context, it has been shown that the psychrophilic α-amylase from *Pseudoaltero-*

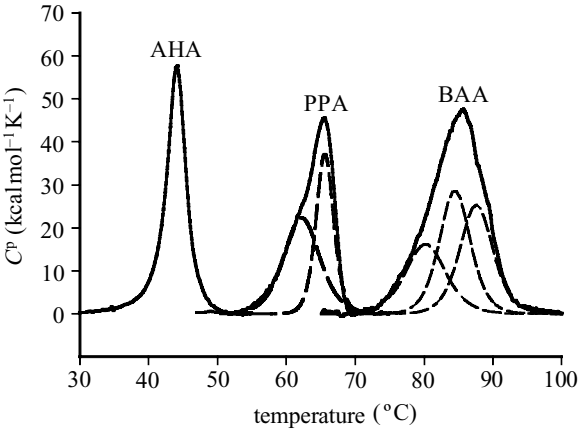

Figure 4. Thermal unfolding of three α-amylases recorded by differential scanning calorimetry. From left to right: thermograms of various α-amylases from the psychrophile *Pseudoalteromonas haloplanktis* (AHA), the mesophile, pig pancreas (PPA) and the thermostable one from *Bacillus amyloliquefaciens* (BAA). Deconvolution of the peaks into two or three domains for PPA and BAA respectively, are shown as dashed lines.

monas haloplanktis has reached the lowest possible stability of its native state (Feller *et al.* 1999). This has been notably demonstrated by DSC, which is a powerful tool to investigate the thermal unfolding of proteins. Unlike other methods, several thermodynamic parameters related to protein stability are recorded directly and not extrapolated from indirect data. Thermograms of heat-induced unfolding for psychrophilic, mesophilic and thermostable α-amylases are shown in figure 4 (Feller *et al.* 1999) from which a number of observations can be drawn. The low thermal stability of the cold-adapted protein is clearly illustrated by its low melting temperature, T_m (top of the peak that represents the temperature at which 50% of the protein is denaturated) of 44 °C. Unfolding enthalpy (ΔH_{cal}) can also be calculated from the area below the curve; this corresponds to the heat absorbed during the unfolding process. This parameter reflects the enthalpy of disruption of bonds involved in maintaining the protein structure and there is a clear trend for increasing it from cold-adapted to thermostable enzymes. It was also shown that the psychrophilic α-amylase unfolds reversibly and without any stable intermediate according to a highly cooperative process. By contrast, more stable α-amylases unfold irreversibly and the denaturation peaks exhibit distinct thermodynamic units or domains with different stability. The contribution of individual weak interactions in the behaviour of the psychrophilic α-amylase was analysed by site-directed mutagenesis (D'Amico *et al.* 2001). Fourteen mutants of this enzyme were constructed, each of them bearing an engineered residue forming a weak interaction found in mesophilic α-amylases but absent in the cold-active α-amylase. It was found that single amino acid side-chain substitutions can significantly modify the melting point T_m, the calorimetric enthalpy ΔH_{cal}, the cooperativity and reversibility of unfolding, the thermal inactivation rate constant and also the kinetic parameters k_{cat} and K_m (figure 3).

From the analysis of the data, it is clear that the adaptation to cold of the psychrophilic α-amylase consists of

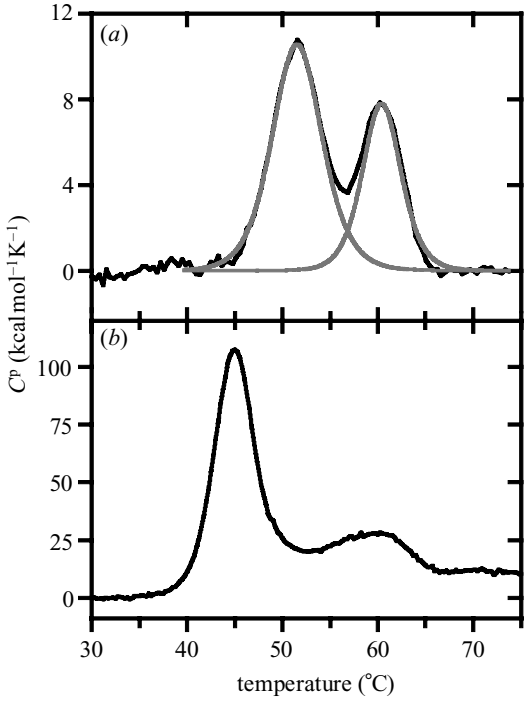

Figure 5. Thermal unfolding of psychrophilic enzymes with local flexibility. (*a*) Phosphoglycerate kinase, (*b*) chitobiase. Grey lines, deconvolution of the thermogram into two calorimetric domains.

a weakening of intramolecular interactions leading to an overall decrease of the thermostability of the protein. This in turn provides the appropriate plasticity around the catalytic residues necessary to adapt, more or less successfully, the catalytic efficiency of the enzyme to the low temperature of the environment. It is also clear that, in the case of the α-amylase, the limit of stability of the protein has been reached, precluding any further decrease of this stability, precluding therefore any further improvement of the flexibility of the protein following this strategy and therefore any further adjustment of the catalytic efficiency. This latter remains, therefore, at the usual environmental temperature of the Antarctic micro-organism, well below that displayed by the mesophilic counterpart at its own environmental temperature. We cannot of course anticipate that all cold-adapted enzymes exhibit a similar strategy and the following question is raised: is the high specific activity of a cold-adapted enzyme always related to a low stability of the enzyme? From the data obtained in our laboratory, from twelve psychrophilic enzymes investigated, the answer is yes. However, the analysis of the thermostability of these enzymes shows that the adaptation strategy can be different from that leading to a uniformly unstable protein (figure 5). As an example, the calorimetric curve for psychrophilic phosphoglycerate kinase appears to be composed of a heat-labile and a heat-stable domain (two denaturation peaks) (Bentahir *et al.* 2000) when compared with the fused transition observed in the case of the mesophilic counterpart from yeast. It has been hypothesized that the heat-labile domain provides the required flexibility around the active site and favours the reaction rate by reducing the energy cost of induced-fit mechanisms, whereas the heat-stable domain could improve substrate binding (as indicated by low K_m values) as a result of its rigidity. This is a strategy of compromise between the necessity to increase the specific activity at low temperatures (engineering the flexibility is apparently the simplest way to achieve this goal) and the necessity to retain a high affinity for the substrate, mandatory in the case of low substrate concentrations typical of the intracellular space. This fact is further emphasized by calorimetric experiments carried out on a psychrophilic chitobiase (Lonhienne *et al.* 2001). The DSC thermograms also display a heat-labile and a heat-stable domain corresponding to the catalytic and to the galactose-binding domains, respectively. These results demonstrate that heat-lability is not simply the result of a lack of selective pressure in cold environments but seems to be the consequence of the improved plasticity required around the active site.

6. DIRECTED EVOLUTION

Recently, random mutagenesis techniques using essentially error-prone PCR or, in a few cases, mutating strains (Roovers *et al.* 2001) have been used to study the thermal adaptation of different enzymes. Proteins were engineered by directed evolution experiments composed of mutagenesis steps, recombination and screening processes aiming to identify interesting mutants on the basis of an altered property such as thermostability. The advantage of directed evolution lies in the possibility to select a specific property that will be subjected to selective pressure. Therefore, this technique appears to be the ideal tool to establish understanding of the relationship, if there is any, between stability, activity and flexibility. To our knowledge, only one psychrophilic enzyme, subtilisin S41 (Davail *et al.* 1994), has been submitted to such an experiment (Miyazaki *et al.* 2000; Wintrode *et al.* 2001). Following several generations of mutagenesis/recombination and screening, a variant with 13 mutations was found to exhibit a higher thermostability as well as a higher activity at low temperatures. Even if this seems to be in contradiction with the current hypothesis of thermal adaptation, it should be mentioned that in this particular case, mutant libraries were screened simultaneously for both of these parameters. However, the mutated enzyme is also characterized by a decrease in the K_m value, an increased affinity for calcium, additional salt bridges and an apparently decreased conformational flexibility. All of these results fit perfectly with the model of activity–flexibility–stability relationships discussed above (see § 5). In addition, the kinetic parameters have been measured using a synthetic substrate, succ-AAPF-pNa, and it remains to be demonstrated using natural substrate that the specific activity, at low temperature, of the mutant enzyme, will also be high. In support of this, in the specific case of subtilisin, the thermodependence of the activity of the enzyme, psychrophilic or not, towards natural or synthetic substrates is quite different (Narinx *et al.* 1997). Therefore, the higher specific activity of the mutated enzyme at low temperature can simply reflect the fact that succ-AAPF-pNa is a better substrate for the mutated enzyme than for the wild-type enzyme.

Other directed evolution experiments have focused on mesophilic and thermophilic proteins. When a higher catalytic activity at low temperatures was sought (Taguchi

et al. 1998; Lebbink *et al.* 2000; Merz *et al.* 2000; Roovers *et al.* 2001), a decreased stability as well as a higher (supposed) flexibility was induced, thus perfectly matching the initial hypothesis. By contrast, when the selection pressure was thermal stability (Akanuma *et al.* 1998, 1999; Giver *et al.* 1998; Spiller *et al.* 1999; Zhao & Arnold 1999; Gershenson *et al.* 2000; Gonzalez-Blasco *et al.* 2000), mutants with higher stability and unchanged or even enhanced catalytic activity were found, in contradiction to the hypothesis that a high stability induces a low catalytic efficiency. However, as has been observed for the fungal peroxidase (Cherry *et al.* 1999) and other enzymes, stabilized mutants selected from the first generations exhibited a lower activity and several mutations were required in the more stable and active mutants. Within the limits of what we have discussed above concerning the use of synthetic substrates to evaluate the specific activity of enzymes, there would be apparently no physical or chemical constraint that could prevent a stable enzyme from also being active at low temperatures. It is, of course, possible that the stability of an enzyme can be achieved by means other than increasing the stabilization enthalpy supposed to rigidify an enzyme. In this context, it has been shown that the high stability of the β-glycosidase from the hyperthermophile *Sulfolobus solfataricus* (Aguilar *et al.* 1997; Carrea & Colombo 2000) is partly achieved by the insertion of a large number of water molecules in hydrophilic cavities, contributing to an increase in the entropy of the native state and the resilience of the site. Via this mechanism of stabilization, mutated enzymes, through an improvement of the resilience of an appropriate site of the protein, could also improve their catalytic efficiency. The fact is, however, that enzymes displaying a high stability as well as a high flexibility and activity are not found in nature, even if this appears possible in the laboratory. It has been proposed that the low thermal stability of cold-adapted enzymes results from a random genetic drift originating from the lack of selective pressure (Miyazaki *et al.* 2000). By contrast, the microcalorimetric studies described in § 5 have indicated that the active site is always in the less stable region of the protein, the other domain remaining even more stable than the mesophilic homologue. Why would the genetic drift only affect one part of the protein?

7. CONCLUSION

We can therefore conclude that even though important advances have been made in the field, the debate regarding why naturally evolved psychrophilic enzymes systematically associate high activity with low thermal stability remains a hot topic. At this stage, it seems that cold-adapted enzymes require an improved flexibility of the structural moiety involved in the catalytic activity, whereas other regions not implicated in catalysis can retain a certain rigidity. We have to keep in mind that we have only a limited view of the living world and of the numerous parameters that characterize the environment of cold-adapted organisms and, as a consequence, their enzymes. Our working hypotheses derive from an oversimplified model attempting to explain the effect of temperature and of its selective pressure on the conformation of a protein. It is clear, however, that other chemical, ecological, physical and physiological parameters are involved and force the protein to adopt, towards a simple parameter, a strategy that has to also take into account all of the other characteristics of the environment. It is not therefore surprising to note that mutated enzymes obtained by directed evolution using a high specific activity at low temperature as the selective parameter display structural adaptations that are different from those observed in a natural environment.

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Discussion

M. A. Marahiel (*Department of Chemistry, Philipps-Universität Marburg, Marburg, Germany*). I would like to make a general comment about the environment of these enzymes. We are really excluding the environment in the cytosol or extracellularly from our account of stability and activity, and there are substances in the cytosol which may also be influencing their activity. Can we now take this out of consideration?

C. Gerday. You are perfectly right. In the case of the cold α-amylase, there is no problem because its environmental temperature is −1 °C and I do not think there is any point discussing that. Now, in the case of the phosphoglycerate kinase the problem is quite different and some compatible solute may contribute to stabilize the enzyme against cold denaturation. The maximum stability in the case of the cold-adapted α-amylase is obtained at 17 °C, far beyond the usual environmental temperature of the enzymes, so it is possible, but I do not know anything about a compatible solute able to stabilize the cold adapted enzyme at low temperature. It is difficult to determine the cold denaturation because you have to use urea or guanidinium chloride to move the stability curve above 0 °C. It is possible but it is tricky.

R. Russell (*Centre for Biomolecular Studies, University of St Andrews, St Andrews, UK*). Does the α-amylase undergo a conformational change upon substrate binding?

C. Gerday. Very very little.

R. Russell. Because I know that phosphoglycerate kinase (PGK) undergoes a large conformation change.

C. Gerday. Oh yes, it is a hinge-bending mechanism.

R. Russell. So should your two strategies be based on whether it undergoes conformational change or not?

G. Warren (*School of Biological Sciences, Royal Holloway, University of London, Egham, UK*). How long will it be before you can design a mesophilic enzyme that has no psychrophilic counterpart and make it psychrophilic?

C. Gerday. I would probably never succeed. I think there are too many constraints to take into account when you rationally analyse a 3D structure because the enzyme is not only modulated by cold, it is also modulated by the type of substrate available. So that is the reason why our rational approach is always, well, almost a failure, 50% success is not a lot. But we can proceed slowly and it is clear in the case of the α-amylase that one single mutation can give rise to a significant improvement of the melting temperature and possibly also of the stabilization energy. But you have to be lucky.

G. Warren. It seems like a 50% success rate is pretty good. It is better than evolution.

C. Gerday. Another approach has been directed evolution. You introduce random mutation and select your protein on the basis of, say, stability. Frances Arnold has shown that sometimes she ends up with not only more stable enzymes but also more activity. This does not fit with the initial compromise between stability and flexibility.

Anon. Do we know enough of the theoretical basis such that it would be possible to do this *in silico* by modelling?

C. Gerday. In my opinion, there is no chance because we do not know even the basis of thermal stability. I would think that probably Doctor Russell will agree with me. We are coming close but still there are some contradictions. The introduction of water into the structure can correspond to a dramatic increase in the stability of thermophilic enzymes.

G. Warren. Any more questions specifically for Professor Gerday? In that case I would like to invite the audience to discuss any of this morning's talks and also the general direction in ways of approaching the molecular basis of cold adaptation. Is there anything else we should be doing?

C. Gerday. I would like to have a good definition of flexibility in relation with the specific activity. Which type of flexibility is needed to get a good specific activity? Usually, flexibility is approached by the study of hydrogen/deuterium exchange related to the dynamic of the molecule. Dynamics and flexibility are not, in my opinion, identical notions. Dynamics is related to time, but the flexibility needed for the specific activity is not maybe related to time, but in fact to the possible amplitude of the resilience of the site.

M. A. Marahiel. I would like to make another comment in the possibility of distribution of charge in the surface. In certain mesophilic and cryoproteins there are real differences in the surface charge as well as the hydrophobicity and I do not know what this means in the context of ions or molecules in the cell which are present in the environment. So this is maybe the communication in cryo environments between proteins and their environment, which is highly salt or highly concentrated with other cryoprotective substances.

But to go back to Professor Gerday's point, presumably if you have the right model system you could use NMR techniques to study the global flexibility, and then do NMR at different temperatures to solve the structure?

C. Gerday. To come back to the point raised by Doctor Marahiel, it is true that we have observed in almost all our psychrophilic enzymes an increase in the negative charge on the surface of the protein. The initial thought was that this was in fact necessary to increase the interaction with the solvent. But I do not think this really is a good reason because our enzymes are originating from sea water and so we do not know whether this negative charge is also involved in some sort of halophilic rather than psychrophilic effect.

G. Warren. How might one approach testing that question?

C. Gerday. Well, we have to find probably another enzyme from another organism which is not in contact with salt and see whether or not there is the same type of structural convergence in the adaptation to low temperature.

Anon. Concerning the physiological significance, I am

wondering how far the stability of the isolated protein is worth looking at? If we are looking at plants, for instance, you can freeze a plant to a temperature where it is dead and there is no evidence that there is any damage to soluble proteins. So I think that the question is really how far the intrinsic stability of the proteins is really limiting for the organism, and I would think that in most cases there are so many other things around in the cell that would stabilize the proteins that the intrinsic stability of the proteins really is not limited.

C. Gerday. I believe you need this low stability because this instability may be necessary to give the appropriate flexibility at the low temperature of the environment.

Anon. In evolutionary terms, a price is paid by reaching more flexibility, but the benefit may be small because the physiological result of reducing protein stability may not be very significant.

C. Gerday. Well it is necessary, so it is significant.

Anon. What I mean is that pressure may be on optimizing activity and if you reduce stability that may not carry a large load for the organisms. This is because there may be enough other stabilizing molecules around that would stabilize the protein, so for the organism, reducing stability may not be a big price to pay.

C. Gerday. Yes, but you are again forgetting that there are extracellular enzymes. But you are right.

G. Warren. There is also the question of the type of organism you are considering. A plant has to go between −8 and +30 °C. There are different constraints on its enzymes compared with one of these bacteria that spends its life at +1 °C.

C. Gerday. I agree that the instability may be a result of the absence of selective pressure but I disagree with the idea that it is the only thing that explains the instability of cold enzymes.

G. Warren. I would like to ask a quick question related to antifreezes. Does any of this make any sense to a person interested in antifreeze structure. What are the implications of it?

P. L. Davies (*Department of Biochemistry, Queen's University, Ontario, Canada*). When we look at the antifreeze proteins in fish and insects, we are looking at very small proteins. They do not have very much of a hydrophobic core and many of them are extensively disulphide bonded, particularly the two insect antifreezes that would function at –10 and −30 °C. So it may well be that they have adapted to function at a low temperature by using disulphide bonds for stability.

GLOSSARY

3D: three dimensional
DSC: differential scanning calorimetry
ES: ensyme–substrate
NMR: nuclear magnetic resonance