Chapter 13
Fundamentals of Cold-Adapted Enzymes

Tony Collins, Frédéric Roulling, Florence Piette, Jean-Claude Marx, Georges Feller, Charles Gerday (✉) and Salvino D'Amico

Contents

13.1 Introduction

As already discussed in the preceding chapters, life at low temperatures is abundant, diverse and widespread, with organisms from all three domains of life being represented. Bacteria and archaea at thermal equilibrium with their environment are found to be preponderant, and these cold-adapted, or psychrophilic, microorganisms have been found to not only endure, but to flourish under the harsh conditions of permanently low-temperatures. In fact, for some, this environment is not only optimal, but mandatory for sustained cell proliferation, with moderate to high temperatures (e.g., >12°C) being deleterious (Xu et al. 2003c).

Clearly, adaptations at all levels of organisation, both structural and physiological, have occurred which allow these psychrophilic microorganisms to overcome key obstacles inherent to life at low temperatures (D'Amico et al. 2006a). The current knowledge of the adaptive features characteristic to psychrophilic organisms are addressed in this book (see Chaps. 11–19) with the present chapter being focused on the enzymes produced by these and on their adaptation to the cold. Here, the challenges imposed by low temperatures on enzyme activity and stability will be briefly introduced, whereafter the general characteristics of the cold-adapted enzymes hitherto investigated will be presented and discussed.

Charles Gerday
Laboratory of Biochemistry, University of Liege, Institute of Chemistry, B6, 4000 Liege, Belgium
e-mail: ch.gerday@ulg.ac.be

R. Margesin et al. (eds.) *Psychrophiles: from Biodiversity to Biotechnology.*

13.2 The low temperature challenge

Low temperatures have a strong negative effect on most biological reactions; reducing reaction rates, provoking alterations in the strength of enzyme-substrate interactions, inducing an increased viscosity of the solvent and an altered solubility of proteins, salts and gases and ultimately leading to protein cold-denaturation (Makhatadze and Privalov 1994; Georlette et al. 2004).

All chemical reactions, including enzymatic reactions, are strongly temperature dependent as described by the Arrhenius equation (Arrhenius 1889):

$$k_{cat} = Ae^{-Ea/RT} \tag{1}$$

From this equation, it can be clearly seen that any decrease in the temperature (T) leads to an exponential decrease in the enzymatic reaction rate (k_{cat}) with the magnitude of this decrease being dependent on the value of the activation energy for the reaction (*Ea*). Here, *R* is the universal gas constant (8.314 J mol^{-1} K^{-1}) and *A* is a frequency factor related to the frequency of collision of the reactants and to the probability of the reactants being in the appropriate orientation to react. In fact, the rate constant can also be expressed according to the transition state theory to give:

$$k_{cat} = \frac{\kappa k_B T}{h} e^{-\Delta G^*/RT} \tag{2}$$

From this and equation (1), one can also derive that (Garcia-Viloca et al. 2004; Collins et al. 2007):

$$A = \frac{\kappa k_B T}{h} e^{-\Delta s^*/R} e^1 \tag{3}$$

Thus, it can be seen that *A* is related to the activation entropy (ΔS^*). In the above equations, κ is the transmission coefficient expressing the probability that the activated state will not always give rise to the product, but will return to the ground state, k_B is the Boltzmann constant and *h* the Planck constant (Garcia-Viloca et al. 2004; Collins et al. 2007). The transmission coefficient κ can be negatively influenced by the viscosity of the medium through possible re-crossing and non-equilibrium effects so that the frequency factor *A* will also be dependent on the viscosity of the reaction medium, and the increase in this latter, concomitant to a temperature decrease, can indeed be quite significant. For example, the average cytoplasmic viscosity is augmented from a mean of 2.5 cP at 20°C (Mastro and Keith 1984) to approximately 5 cP at 0°C. This will obviously have a negative effect on enzyme activity, and such a viscosity increase has been shown to lead to an approximately 5-fold loss of activity for a lactate dehydrogenase at 25°C (Demchenko et al. 1989). Thus, it is important to keep this inverse relationship of viscosity with temperature and reaction rate in mind when monitoring temperature effects on enzyme activity. A model which takes this viscosity effect into account has even been recently proposed (Siddiqui et al. 2004).

The effect of low temperatures on enzyme–substrate binding is much less clear-cut than that on reaction rates, with both positive and negative outcomes being possible. In effect, electrostatic interactions are formed exothermically and are stabilized by low temperatures at least down to those temperatures where the dehydration of the individual ions can still be achieved. On the other hand, hydrophobic interactions are formed endothermically in the positive temperature range up to approximately 40°C and hence are destabilised at low temperatures. Thus, the effect of temperature (either detrimental or beneficial) is determined by the types and contributions of the bonds involved in substrate binding and hence by the particular enzyme and substrate concerned.

Finally, cold denaturation, while being a scantly studied phenomenon, is believed to be due to the hydration of charged, polar and non-polar amino acid residues that leads, in particular, to a weakening of critical hydrophobic forces. While this should typically occur at temperatures below the freezing point of water, it obviously poses a further predicament to enzymes produced by organisms inhabiting permanently low temperature environments (Makhatadze and Privalov 1994).

As a result of these diverse adverse effects, most biological reactions display an approximately 16- to 80-fold drop in activity on reducing the temperature from 37°C to 0°C. In contrast, psychrophilic microorganisms have been found to maintain relatively high metabolic fluxes at low temperatures, indicating that adaptation of the enzymatic repertoire has taken place to allow for appropriate reaction rates. Reported mechanisms of cold-adaptation include an increased enzyme production (Crawford and Powers 1992; De Vos et al. 1998), yet this is energetically expensive, and while an expression of specific isotypes adapted to different temperatures has also been reported (Lin and Somero 1995), this suffers the disadvantage of requiring the presence of multiple gene copies and is most apt for organisms requiring seasonal adaptation (e.g., fish and nematodes). Finally, the synthesis of enzymes specifically adapted to operate at permanently low temperatures is another putative cold-adaptation mechanism and this in fact appears to be the main physiological adaptation used by psychrophiles at the enzyme level.

We have attempted to tabulate all the currently known enzymes isolated from psychrophilic organisms, both prokaryotic and eukaryotic, and the listing of these can be found at http://www.ulg.ac.be/biochlab where both the identity of the host organism as well as the level of characterisation of the enzymes are also indicated. Only those enzymes purified from psychrophilic organisms inhabiting permanently low temperature environments have been listed and it currently appears that 108 prokaryotic and 51 eukaryotic enzymes have been isolated and studied. These originate from various sources and have been characterized to varying levels in terms of their physical, chemical, kinetic and structural properties. The majority of these have been found to have evolved strategies for overcoming the temperature challenge. In the subsequent sections, the principle results of the various studies of these enzymes and in particular the comparative studies of the activity, stability and flexibility will be presented and discussed.

13.3 Activity

In relation to activity, the dominant features of most cold-adapted enzymes are an increased specific activity (k_{cat}) or catalytic efficiency (k_{cat}/K_m) at low and moderate temperatures and a shift in the apparent optimal temperature towards low temperatures as compared to their mesophilic and thermophilic homologs (Fig. 13.1).

The apparent optimum temperature of an enzyme is obviously dependent on the experimental conditions used and in particular on the assay time. Nevertheless, analysis of the available data shows that the thermodependence of activity of psychrophilic enzymes is highly variable, with diverse apparent optimum temperatures for activity and both limited and broad temperature ranges being reported. For example, while apparent temperature optima around 30°C are preponderant, optima at approximately 15°C (Huston et al. 2000; Arnorsdottir et al. 2005) and as high as 64°C (Birolo et al. 2000) have also been reported. In fact, the defining feature of these cold-adapted enzymes is a shift in the apparent optimum temperature towards lower values and the retention of a high activity at low temperatures, in particular when compared to that of their mesophilic and thermophilic homologs (Bae and Philliops 2004). Examples include a psychrophilic xylanase (Collins et al. 2002b) and chitinase (Bendt et al. 2001) which retain, respectively, 60% and 40% of their

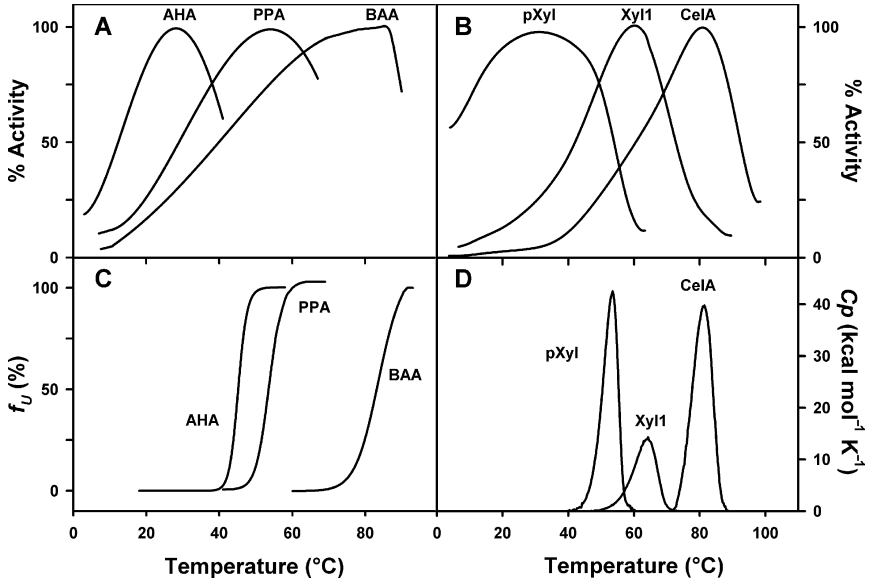

Fig. 13.1 Thermodependence of activity (**A** and **B**) and thermal stability as determined by fluorescence monitoring (**C**) and differential scanning calorimetry (**D**) of homologous α-amylases (**A** and **C**) and glycoside hydrolases (**B** and **D**). AHA, PPA and BAA are, respectively, psychrophilic, mesophilic and thermophilic α-amylases. pXyl, Xyl1 and CelA are, respectively, psychrophilic, mesophilic and thermophilic glycoside hydrolases. Taken from Georlette et al. (2004)

maximum activity at 5°C, and display apparent optimum temperature shifts towards lower temperatures of approximately 25°C. Indeed, this shift in the apparent optimum temperature for activity is related to the thermal lability of these enzymes (see Sect. 13.4), while the reduced temperature dependence and high activity at low temperatures is brought about by a reduction of the activation energy barrier for the reaction ΔG^* (i.e. the energy difference between the activated enzyme–substrate transition state and the ground state enzyme–substrate complex).

Extensive comparative thermodynamic analysis of the activity of psychrophilic, mesophilic and thermophilic enzymes (Lonhienne et al. 2000; Siddiqui and Cavicchioli 2006) indicates that, as compared to their higher temperature adapted homologs, the low ΔG^* of cold-adapted enzymes is characterized by a reduced activation enthalpy partially compensated for by a less favorable activation entropy. The decrease of the former reflects the lower dependence of these enzymes on temperature and indicates a reduction in the number or strength of weak interactions that are broken during activation. Accordingly, this favorable enthalpy change is the driving force for low temperature activity, but the magnitude of its effect is reduced by the unfavorable entropy change observed (i.e. $\Delta G^*=\Delta H^*-T\Delta S^*$). It is important to note that, while the entropy change on activation is often negative due to a more organised transition state, positive values are also encountered. Therefore, the unfavorable entropy change observed during the activation process in psychrophilic enzymes indicates, in the former case, the necessity for a greater change in order to reach the activated state (i.e. a higher negative entropy change as compared to mesophilic and thermophilic homologs) and, in the latter case, a lower degree of disordering (i.e. a lower positive entropy change). Both of these observations can be explained by a more disordered enzyme–substrate ground state complex for the cold-adapted enzyme as compared to its high temperature homologs. This is postulated to be the result of a more flexible enzyme structure leading to a broader distribution of conformational states for the ground state complex (Lonhienne et al. 2000).

In agreement with the apparent increased disorder of the enzyme–substrate ground state complex in psychrophilic enzymes, these have been found to be frequently characterized by an increased K_m in addition to a high k_{cat} (Collins et al. 2002a). Furthermore, this high K_m value frequently observed for psychrophilic enzymes may be conjectured as further proof of a highly flexible structure (Fields 2001), in particular at or around the active site, which perturbs the geometry and enzyme–substrate complementarity and leads to the observed decreased substrate affinity (i.e. increased K_m). Similarly, the frequently observed decreased affinity for various ions (Davail et al. 1994; D'Amico et al. 2006b), cofactors (Okubo et al. 1999) and inhibitors (Alvarez et al. 1998), and the reduced substrate specificity of some cold-adapted enzymes (Smalas et al. 2000), also points to an increased flexibility of the molecular edifice of these. Finally, and in a similar vein of thought, recent comparative studies of a psychrophilic α-amylase with its mesophilic counterpart has indicated that the former, in contrast to the latter, is capable of forming an enzyme–substrate–inhibitor ternary complex and has a higher activity on large macromolecular substrates but a decreased activity on small substrates (D'Amico et al. 2006b). These observations, in conjunction with the structural identity of the

catalytic cleft of these enzymes are also compatible with a more flexible active site in the psychrophilic enzyme.

For enzymes operating at high substrate concentrations, for example extracellular and in particular digestive enzymes (Narinx et al. 1997; Collins et al. 2002b), the high K_m value discussed above is of little importance as optimization of k_{cat} is the only relevant parameter. In contrast, for enzymes typically operating at low substrate concentrations, for example certain intracellular enzymes, both K_m and k_{cat} are important and indeed both of these parameters have been found to be optimized in a number of cases (Bentahir et al. 2000; Hoyoux et al. 2001; Lonhienne et al. 2001). Obviously, a different strategy must be employed by these proteins whereby the adaptive drift of the K_m is counteracted. For example, comparison of the kinetic parameters of a cold-adapted and mesophilic chitobiase (Fig. 13.2) reveals that these enzymes have been fine tuned so as to enable optimization of their kinetic parameters in accordance with their physiological temperature (Lonhienne et al. 2001). In particular, the crossed K_m plots for these enzymes illustrate the improved substrate binding for the psychrophilic enzyme on reduction of temperature, and this is believed to be achieved by the subtle replacement of two tryptophan residues (endothermic interactions) involved in substrate binding in the mesophile by polar residues (exothermic interactions) in the psychrophile, which, as discussed above, improves the enzyme-substrate interactions at low temperatures.

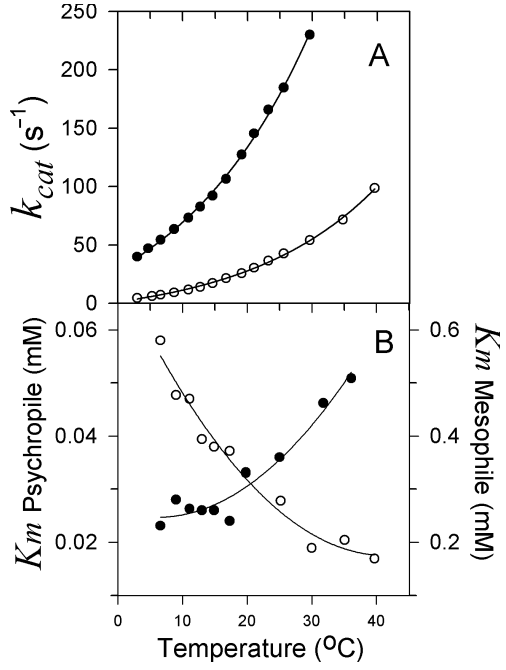

Fig. 13.2 Thermodependence of the k_{cat} (**A**) and K_m (**B**) for a psychrophilic (•) and mesophilic (o) chitobiase. Note the different scales used. Taken from Lonhienne et al. (2001)

A further interesting observation of a number of comparative studies is that both the K_m and k_{cat} of psychrophilic and mesophilic enzymes are often highly similar at their respective physiological temperatures. This gives rise to the corresponding state hypothesis suggested by Somero (1995) whereby enzymes have evolved so as that both of these parameters are optimized at the environmental temperature of the enzyme. However, this kinetic optimization has not always been possible and the kinetic parameters of some psychrophilic enzymes at their environmental temperature are sometimes found to be inferior to that of their high temperature homologs (Feller et al. 1992; Xu et al. 2003a; Xu et al. 2003b), hence pointing to an 'incomplete' adaptation of these enzymes to their environment.

13.4 Stability

In light of the essential role of protein stability in thermal adaptation, extensive studies have been performed in order to compare the conformational stability of psychrophilic enzymes with their mesophilic and thermophilic counterparts, and have shown that cold-adapted enzymes are generally the most unstable. As demonstrated by techniques such as fluorescence spectroscopy and differential scanning calorimetry (Fig. 13.3) their unfolding typically occurs at lower temperatures and the calorimetric enthalpy, $\Delta H_{cal,}$ i.e., the enthalpy of disruption of bonds involved in maintaining the protein structure, is generally reduced. Furthermore, thermal unfolding curves for cold-adapted enzymes are typically sharp and symmetrical and thereby reflect a highly cooperative process without any stable intermediate, while in contrast more stable enzymes exhibit distinct thermodynamic domains of varying stability (Fig. 13.3).

13.4.1 Reversible and irreversible unfolding

While the majority of proteins unfold irreversibly, some have been found to show completely reversible thermal denaturation and thus allow calculation of the stabilization energy (ΔG) curves as presented in Fig. 13.4. These curves represent the energy difference between the free energy of the unfolded state and that of the native state, in other words, they represent the energy required to unfold the molecular structure at each temperature. It can be clearly seen that the increase of the melting points of the more thermostable proteins is obtained by an uplifting of the stability curves and that this is mainly achieved through an increase of the stabilization enthalpy, hence demonstrating the major involvement of enthalpic factors in the stabilization of protein structures (Makhatadze and Privalov 1995; Kumar et al. 2001). Furthermore, the environmental temperatures of mesophiles and thermophiles are found to be above those corresponding to the ΔG_{max} and, consequently, it can be suggested that these enzymes use the thermal dissipative forces

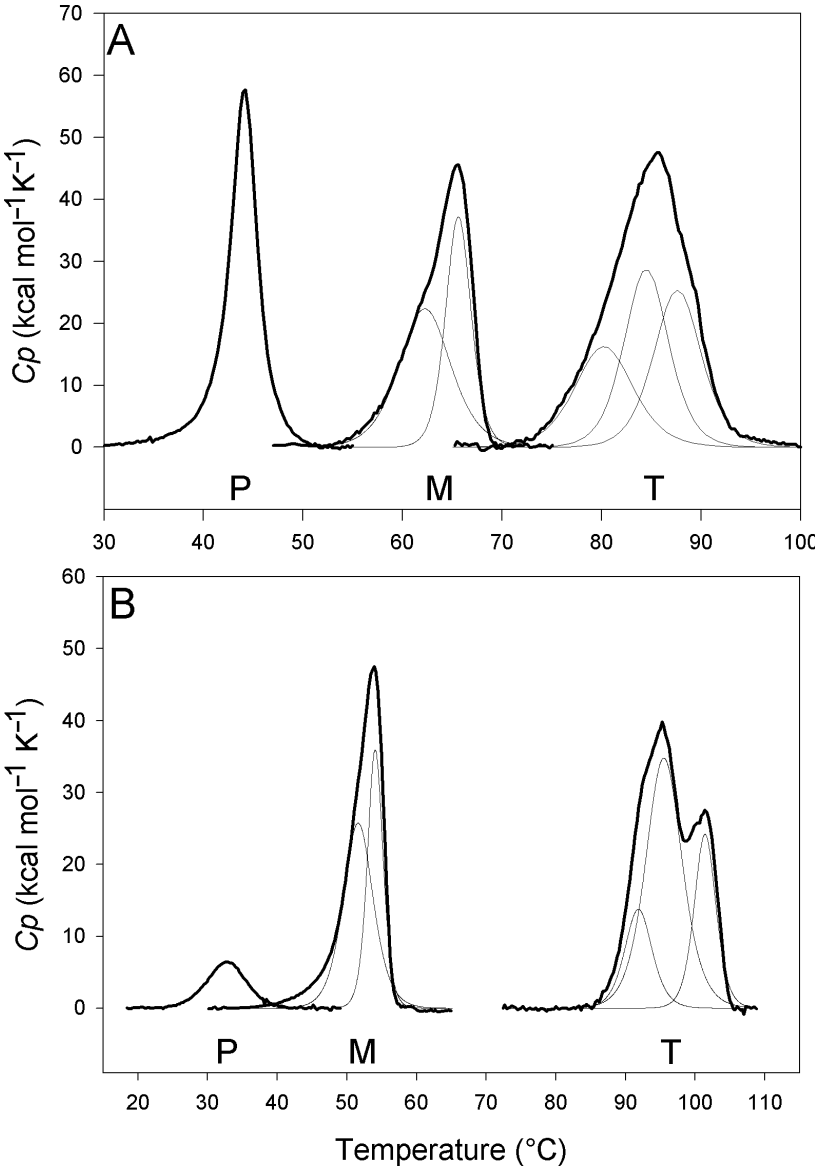

Fig. 13.3 Thermal unfolding of psychrophilic (*P*), mesophilic (*M*) and thermophilic (*T*) enzymes recorded by differential scanning calorimetry. **A** From left to right: α-amylases from *Pseudoalteromonas haloplanktis*, pig pancreas and *Bacillus amyloliquefaciens*. **B** From left to right: DNA ligases from *Pseudoalteromonas haloplanktis*, *Escherichia coli* and *Thermus scotoductus*. Deconvolution of the peaks into two or three domains for the mesophilic and thermophilic enzymes respectively are shown in thin lines. Taken with modification from D'Amico et al. (2001) and Georlette et al. (2003)

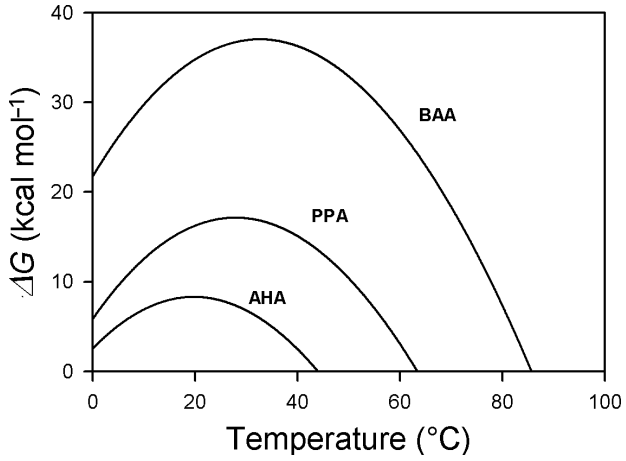

Fig. 13.4 Stabilization energy curves of three α-amylases: AHA, psychrophilic, PPA, mesophilic and BAA, thermophilic, as calculated from microcalorimetric data. Taken from D'Amico et al. (2003)

of high temperatures to promote the molecular motions needed for enzyme function. In contrast, for psychrophiles, the environmental temperatures are below that of the ΔG_{max}, indicating that molecular motions at low temperatures are gained from the hydration of ionic-, polar- and non-polar interactions, i.e. the factors ultimately leading to cold unfolding (Feller 2007). In fact, the stability curves shown in Fig. 13.4 also allow the prediction (by extrapolation) of a temperature of cold-unfolding and it can be seen that, contrary to what would be intuitively expected, cold-adapted enzymes are also the least resistant to cold denaturation. This poorly investigated observation must be kept in mind when considering the lower temperature limit of life and indeed further studies in this field are called for.

As has already been mentioned, the majority of proteins unfold irreversibly, thereby preventing extraction of thermodynamic equilibrium data but nevertheless allowing for determination of the activation parameters for these irreversible processes. Irreversible denaturation and/or inactivation have been found to be characterized by a low activation free energy value ($\Delta G^{\#}$) in cold-adapted enzymes (Collins et al. 2003; D'Amico et al. 2003; Georlette et al. 2003) and simply reflect the rapid unfolding of these proteins. The small differences observed in the $\Delta G^{\#}$ arise from large differences in the enthalpic and entropic contributions and are the consequence of classical enthalpy–entropy compensations. The high activation enthalpy ($\Delta H^{\#}$) for psychrophilic enzymes, as a result of the high slope of the Arrhenius plots used to calculate these parameters, simply reflects a higher cooperativity of the denaturation and inactivation processes in these enzymes. Furthermore, the lower value of the activation entropy ($\Delta S^{\#}$) for the thermostable enzymes suggests that the activated state is reached with less disordering and thus reflects the higher resistance of the thermophilic enzyme to irreversible denaturation.

13.4.2 Global versus local stability

From an evolutionary point of view, several studies have discussed the possibility that the instability of psychrophilic enzymes is the result of a random genetic drift due to the lack of a selective pressure on stability (Miyazaki et al. 2000; Wintrode et al. 2001). The fact is, however, that enzymes displaying a high stability as well as a high specific activity at low temperatures are not apparently found in nature, even though this appears to be possible to obtain in vitro, with small size synthetic substrates and after several rounds of random mutagenesis where a selective pressure on stability is imposed (Wintrode et al. 2001). Furthermore, it is important to note that while psychrophilic enzymes found in nature are generally characterized by a reduced stability, this destabilization is not always found to involve the whole protein structure and, indeed, a number of proteins with localized zones of reduced stability have been reported. Obviously this observation questions the possibility of random genetic drift playing a part in the poor stability of psychrophilic enzymes as it is improbable that this could affect only a specific part of the protein structure.

In particular, this destabilization in cold-adapted proteins appears to be concentrated at or near the active site. In multi-domain enzymes, such as the psychrophilic chitobiase (Lonhienne et al. 2001), phosphoglycerate kinase (Bentahir et al. 2000) and FKBP 22 (Suzuki et al. 2005), as well as in the precursor of the cold adapted α-amylase (Claverie et al. 2003), the catalytic domain is always found to be the least stable domain whereas other non catalytic domains can be as stable as that of the mesophilic homologs. Furthermore, although the thermal unfolding of the mature α-amylase is highly cooperative, it has been shown using transverse urea gel electrophoresis that the active site is the least stable structural entity in the presence of urea (Siddiqui et al. 2005).

Further strong support for this concept of a reduced stability at or near the active site can be found on comparing enzyme activity and stability (Collins et al. 2003; D'Amico et al. 2003; Georlette et al. 2003). As can be seen from Fig. 13.1, the initial loss of activity for the mesophilic and thermophilic enzymes corresponds quite well with the thermal protein unfolding, whereas in the case of the cold-adapted enzymes the activity decreases well before any unfolding process as recorded by fluorescence spectroscopy or microcalorimetry. This simply means that here the active site or the enzyme–substrate complex is more heat-labile than the whole protein structure and as a consequence the enzyme is inactivated several degrees before the melting temperature. This again points to a localized adaptation in psychrophilic enzymes whereby the active site and/or its surroundings are destabilized and may again be proposed to be due to the requirement for an increased flexibility of these specific regions for cold-adaptation.

13.5 Flexibility

From the previous discussions of the activity and stability of psychrophilic enzymes it is obvious that a number of studies indirectly point to the high activity and low stability of these to being a consequence of an increased flexibility of the whole or

of a particular part of the molecular edifice. Indeed, it is believed that this high flexibility, which derives from the observed low stability of psychrophilic enzymes, would allow for the molecular motions necessary for activity at a low energy cost, i.e. at low temperatures.

In this concept of an activity–stability–flexibility relationship, stability and activity can be experimentally demonstrated quite easily. In contrast, the determination of protein flexibility, mobility or plasticity, as it is also known, is much more difficult and, in particular, if it is limited to a small part of the whole structure. Moreover, we must consider the molecular motions in terms of amplitude as well as time scale. One approach which has been used to evaluate this peculiar characteristic of proteins has been to carry out a comparative study of fluorescence quenching in extremophilic enzymes so as to assess the propensity of the whole molecular structure to being penetrated by a small quencher molecule (acrylamide being the most frequently used). This technique gives an idea of all conformational opening processes occurring in the whole protein and averaged over a large time scale, and it has been successfully used to compare the flexibility of homologous Ca^{2+}-Zn^{2+} proteases (Chessa et al. 2000), xylanases (Collins et al. 2003), DNA ligases (Georlette et al. 2003), α-amylases (D'Amico et al. 2003) and cellulases (G. Sonan, personal communication). These experiments unequivocally demonstrated the decrease in protein permeability on going from psychrophilic to thermophilic enzymes.

Another approach is neutron scattering and this has been used to compare the mean macromolecular dynamics of whole crude extracts obtained from bacteria adapted to different environmental temperatures (Tehei et al. 2004). Here, the authors showed that at a given temperature, the resilience (which is approximately equivalent to rigidity) increased from psychrophile to thermophile. However, at their respective environmental temperatures, the measured resiliencies were similar and therefore in perfect agreement with the "corresponding state" theory (Somero 1995). Other methods which have been used more or less successfully to evaluate protein flexibility include hydrogen/deuterium exchange (Zavodszky et al. 1998; Svingor et al. 2001) and tryptophan phosphorescence (Fischer et al. 2000; Gershenson et al. 2000).

Finally, molecular dynamics studies are becoming increasingly common (Brandsdal et al. 1999; Olufsen et al. 2005; Papaleo et al. 2006; Spiwok et al. 2007) and a recent study of an elastase family (Papaleo et al. 2006) showed that (1) loop regions clustered around the active site are characterized by an enhanced flexibility in the cold-adapted enzyme, and (2) some scattered regions distant from the functional sites are more rigid in the cold-adapted enzyme, thus in perfect agreement with the theory we developed previously in this review.

Thus, it can be seen that while still suffering from the lack of a widely accepted technique to unequivocally characterise protein dynamics, it appears that many psychrophilic enzymes are characterised by an increased flexibility of at least a part of their structure and it is this increased flexibility, originating from the low stability of crucial parts of the molecular structures, which allows for the high activity at low temperatures.

13.6 Structural adaptations

The crystal structures of 22 psychrophilic enzymes have been reported up to now (Table 13.1) and many of these have been subjected to comparative structural analyses with their mesophilic and/or thermophilic homologs. The first observation from these studies is that the overall fold of proteins adapted to different thermal environments are very similar, and many residues, in particular those involved in catalysis, are found to be conserved (Aghajari et al. 1998b). A closer comparative examination does, however, reveal subtle structural modifications, with cold-adapted proteins being most frequently characterized by a reduced number and/or strength of stabilizing interactions. Indeed, any of a large number or combination of a variety of structural adjustments, such as a reduction of salt-bridges, hydrogen

Table 13.1 Cold-adapted enzymes for which the crystal structure has been determined

Enzyme	Host organism	Reference
Adenylate kinase	*Bacillus globisporus*	Bae and Phillips (2004)
Alkaline phosphatase	Bacterial strain TAB5	Wang et al. (2007)
Alpha-amylase	*Pseudoalteromonas haloplanktis*	Aghajari et al. (1998a)
Aspartate carbamoyltransferase	*Moritella profunda*	De Vos et al. (2007)
Beta-galactosidase	*Arthrobacter* sp. C2-2	Skalova et al. (2005)
Catalase	*Vibrio salmonicida*	Riise et al. (2007)
Cellulase	*Pseudoalteromonas haloplanktis*	Violot et al. (2005)
Citrate synthase	*Arthrobacter* sp. strain DS2-3R	Russell et al. (1998)
Lipase B	*Candida antarctica*	Uppenberg et al. (1994)
Malate dehydrogenase	*Aquaspirillium articum*	Kim et al. (1999)
Phosphatase (alkaline phosphatase)	*Pandalus borealis*	de Backer et al. (2002)
Phosphatase (protein-tyrosine phosphatase)	*Shewanella* sp.	Tsuruta et al. (2005)
Protease (alkaline metalloprotease)	*Pseudomonas* sp. strain TAC II 18	Aghajari et al. (2003)
Protease (anionic trypsin)	*Salmon salar*	Helland et al. (1998)
Protease (elastase)	*Salmon salar*	Berglund et al. (1995)
Protease (pepsin Iib)	*Gadus morhua*	Karlsen et al. (1998)
Protease (proteinase K-like)	*Serratia* sp.	Helland et al. (2006)
Protease (subtilisin-like serine proteinase)	*Vibrio* sp. PA-44	Arnorsdottir et al. (2005)
Protease (trypsin)	*Oncorhynchus ketav*	Toyota et al. (2002)
Triose phosphate isomerase	*Vibrio marinus*	Alvarez et al. (1998)
Uracil-DNA glycosylase	*Gadus morhua*	Leiros et al. (2003)
Xylanase	*Pseudoalteromonas* sp. strain TAH3a	Van Petegem et al. (2003)

bonds, core hydrophobicity, aromatic interactions, arginine and/or proline content, density of charged surface residues, an increased surface hydrophobicity and an increased clustering of glycine residues etc., have been observed, with each enzyme adopting a specific strategy (Feller et al. 1997; Smalas et al. 2000). Indeed, these are the main structural modifications involved in cold-adaptation and it is obvious how such alterations can give rise to a decreased stability but also an increased flexibility of the molecular structure and hence allow for the molecular motions required for activity at low temperatures.

In addition to the above common observations a number of other more enzyme specific structural adaptations have also been reported. These include a larger and more accessible active site (Russell et al. 1998; Aghajari et al. 2003) an improved electrostatics of and in the vicinity of the reactive centre (Brandsdal 2001; Leiros et al. 2003), a long and highly flexible linker region (Violot et al. 2005) and a reorganization of the quaternary structure (Skalova et al. 2005)

13.7 Conclusions

It is important to remember that while only cold-adapted enzymes have been discussed in this chapter, life at low temperatures requires a multitude of adaptations at all levels within the cell, from single molecules up to supra-molecular structures.

Enzymes achieve cold adaptation by means of fine tuning the activity, flexibility, stability balance. They efficiently catalyze reactions at low temperatures as a result of an inherent increased flexibility, in particular at or near the catalytic site and this enables the molecular motions necessary for activity in this low energy environment. On the other hand, this increased low temperature activity and flexibility is achieved at the expense of stability and frequently also an increased K_m. Of course there is a limit to this adaptation process since the stability of a protein cannot decrease below a certain limit, i.e. it must maintain a properly folded form and this is probably one of the reasons why enzyme adaptation appears incomplete for some proteins. Finally, it appears that cold-adaptation is mainly achieved by means of quite discrete structural modifications and primarily by removal of stabilizing interactions, with each enzyme using a specific strategy to achieve its goal.

Acknowledgements The authors wish to thank N. Gérardin, A. Dernier and R. Marchand for their skilful technical assistance and the French Institute for Polar Research (IPEV – Institut Polaire Français) for generously accommodating our research fellows at the French Antarctic station J.S. Dumont d'Urville in Terre Adelie. The generous support of the 'Region Wallonne' (contract FIRST EUROPE R0202/215266), the European Union (network contract CT970131) and the 'Fonds National de la Recherche Scientifique' is gratefully acknowledged. S D'Amico is a FNRS postdoctoral researcher. Frédéric Roulling and Florence Piette are holders of a FRIA fellowship.

References

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

Aghajari N, Feller G, Gerday C, Haser R (1998b) 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, Van Petegem F, Villeret V, Chessa JP, Gerday C, Haser R, Van Beeumen J (2003) Crystal structures of a psychrophilic metalloprotease reveal new insights into catalysis by cold-adapted proteases. Proteins 50:636–647

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

Arnorsdottir J, Kristjansson MM, Ficner R (2005) Crystal structure of a subtilisin-like serine proteinase from a psychrotrophic *Vibrio* species reveals structural aspects of cold adaptation. Febs J 272:832–845

Arrhenius S (1889) Uber die Reaktionsgeschwindigkeit bei der Inversion von Rohrzucker durch Säuren. Z Physik Chem 4:226–248

Bae E, Phillips GN Jr (2004) Structures and analysis of highly homologous psychrophilic, mesophilic, and thermophilic adenylate kinases. J Biol Chem 279:28202–28208

Bendt A, Huller H, Kammel U, Helmke E, Schweder T (2001) Cloning, expression, and characterization of a chitinase gene from the Antarctic psychrotolerant bacterium *Vibrio* sp. strain Fi:7. Extremophiles 5:119–126

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

Berglund GI, Willassen NP, Hordvik A, Smalas AO (1995) Structure of native pancreatic elastase from North Atlantic salmon at 1.61 A resolution. Acta Crystallogr D Biol Crystallogr 51:925–937

Birolo L, Tutino ML, Fontanella B, Gerday C, Mainolfi K, Pascarella S, Sannia G, Vinci F, Marino G (2000) Aspartate aminotransferase from the Antarctic bacterium *Pseudoalteromonas haloplanktis* TAC 125. Cloning, expression, properties, and molecular modelling. Eur J Biochem 267:2790–2802

Brandsdal BO, Heimstad ES, Sylte I, Smalas AO (1999) Comparative molecular dynamics of mesophilic and psychrophilic protein homologues studied by 1.2 ns simulations. J Biomol Struct Dyn 17:493–506

Chessa JP, Petrescu I, Bentahir M, Van Beeumen J, Gerday C (2000) Purification, physico-chemical characterization and sequence of a heat labile alkaline metalloprotease isolated from a psychrophilic *Pseudomonas* species. Biochim Biophys Acta. 1479:265–274

Claverie P, Vigano C, Ruysschaert JM, Gerday C, Feller G (2003) The precursor of a psychrophilic alpha-amylase: structural characterization and insights into cold adaptation. Biochim Biophys Acta 1649:119–122

Collins T, Claverie P, D'Amico S, Georlette D, Gratia E, Hoyoux A, Meuwis MA, Poncin J, Sonan G, Feller G, Gerday C (2002a) Life in the cold: psychrophilic enzymes. In: Pandalai SG (ed.), Recent Research Developments in Proteins. Transworld Research Network, Trivandrum, Vol. 1, pp. 13–26

Collins T, Meuwis MA, Stals I, Claeyssens M, Feller G, Gerday C (2002b) A novel family 8 xylanase: functional and physico-chemical characterization. J Biol Chem 277:35133–35139

Collins T, Meuwis MA, Gerday C, Feller G (2003) Activity, stability and flexibility in glycosidases adapted to extreme thermal environments. J Mol Biol 328:419–428

Collins T, D'Amico S, Marx J, Feller G, Gerday C (2007) Cold-Adapted Enzymes. In: Gerday C, Glansdorff N (eds.), Physiology and biochemistry of extremophiles. ASM Press, Washington, D.C., pp. 165–179

Crawford DL, Powers DA (1992) Evolutionary adaptation to different thermal environments via transcriptional regulation. Mol Biol Evol 9:806–813
D'Amico S, Gerday C, Feller G (2001) Structural determinants of cold adaptation and stability in a large protein. J Biol Chem 276:25791–25796
D'Amico S, Marx JC, Gerday C, Feller G (2003) Activity-stability relationships in extremophilic enzymes. J Biol Chem 278:7891–7896
D'Amico S, Collins T, Marx JC, Feller G, Gerday C (2006a) Psychrophilic microorganisms: challenges for life. EMBO Rep 7:385–389
D'Amico S, Sohier JS, Feller G (2006b) Kinetics and energetics of ligand binding determined by microcalorimetry: insights into active site mobility in a psychrophilic alpha-amylase. J Mol Biol 358:1296–1304
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
de Backer M, McSweeney S, Rasmussen HB, Riise BW, Lindley P, Hough E (2002) The 1.9 A crystal structure of heat-labile shrimp alkaline phosphatase. J Mol Biol 318:1265–1274
De Vos D, Xu Y, Hulpiau P, Vergauwen B, Van Beeumen JJ (2007) Structural investigation of cold activity and regulation of aspartate carbamoyltransferase from the extreme psychrophilic bacterium *Moritella profunda*. J Mol Biol 365:379–395
Demchenko AP, Ruskyn OI, Saburova EA (1989) Kinetics of the lactate dehydrogenase reaction in high-viscosity media. Biochim Biophys Acta. 998:196–203
Devos N, Ingouff M, Loppes R, Matagne RF (1998) Rubisco adaptation to low temperatures: A comparative study in psychrophilic and mesophilic unicellular algae. J Phycol 34:655–660
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, Arpigny JL, Narinx E, Gerday C (1997) Molecular adaptations of enzymes from psychrophilic organisms. Comp Biochem Physiol 118:495–499
Feller G (2007) Life at low temperatures: is disorder the driving force? Extremophiles 11:211–216
Fields PA (2001) Review: Protein function at thermal extremes: balancing stability and flexibility. Comp Biochem Physiol A Mol Integr Physiol 129:417–431
Fischer CJ, Schauerte JA, Wisser KC, Gafni A, Steel DG (2000) Hydrogen exchange at the core of *Escherichia coli* alkaline phosphatase studied by room-temperature tryptophan phosphorescence. Biochemistry. 39:1455–1461
Garcia-Viloca M, Gao J, Karplus M, Truhlar DG (2004) How enzymes work: analysis by modern rate theory and computer simulations. Science 303:186–195
Georlette D, Damien B, Blaise V, Depiereux E, Uversky VN, Gerday C, Feller G (2003) Structural and functional adaptations to extreme temperatures in psychrophilic, mesophilic, and thermophilic DNA ligases. J Biol Chem 278:37015–37023
Georlette D, Blaise V, Collins T, D'Amico S, Gratia E, Hoyoux A, Marx JC, Sonan G, Feller G, Gerday C (2004) Some like it cold: biocatalysis at low temperatures. FEMS Microbiol Rev 28:25–42
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
Helland R, Leiros I, Berglund GI, Willassen NP, Smalas AO (1998) The crystal structure of anionic salmon trypsin in complex with bovine pancreatic trypsin inhibitor. Eur J Biochem 256:317–324
Helland R, Larsen AN, Smalas AO, Willassen NP (2006) The 1.8 A crystal structure of a proteinase K-like enzyme from a psychrotroph *Serratia* species. FEBS J 273:61–71
Hoyoux A, Jennes I, Dubois P, Genicot S, Dubail F, Francois JM, Baise E, Feller G, Gerday C (2001) Cold-adapted beta-galactosidase from the Antarctic psychrophile *Pseudoalteromonas haloplanktis*. Appl Environ Microbiol 67:1529–1535

Huston AL, Krieger-Brockett BB, Deming JW (2000) Remarkably low temperature optima for extracellular enzyme activity from Arctic bacteria and sea ice. Environ Microbiol 2:383–388
Karlsen S, Hough E, Olsen RL (1998) Structure and proposed amino-acid sequence of a pepsin from atlantic cod (*Gadus morhua*). Acta Crystallogr D Biol Crystallogr 54:32–46
Kim SY, Hwang KY, Kim SH, Sung HC, Han YS, Cho Y (1999) Structural basis for cold adaptation. Sequence, biochemical properties, and crystal structure of malate dehydrogenase from a psychrophile *Aquaspirillium arcticum*. J Biol Chem 274:11761–11767
Kumar S, Tsai CJ, Nussinov R (2001) Thermodynamic differences among homologous thermophilic and mesophilic proteins. Biochemistry 40:14152–14165
Leiros I, Moe E, Lanes O, Smalas AO, Willassen NP (2003) The structure of uracil-DNA glycosylase from Atlantic cod (*Gadus morhua*) reveals cold-adaptation features. Acta Crystallogr D Biol Crystallogr 59:1357–1365
Lin JJ, Somero G (1995) Thermal adaptation of cytoplasmic malate dehydrogenases of eastern Pacific barracuda (*Sphyraena* spp): the role of differential isoenzyme expression. J Exp Biol 198:551–560
Lonhienne T, Gerday C, Feller G (2000) Psychrophilic enzymes: revisiting the thermodynamic parameters of activation may explain local flexibility. Biochim Biophys Acta 1543:1–10
Lonhienne T, Zoidakis J, Vorgias CE, Feller G, Gerday C, Bouriotis V (2001) Modular structure, local flexibility and cold-activity of a novel chitobiase from a psychrophilic Antarctic bacterium. J Mol Biol 310:291–297
Makhatadze GI, Privalov PL (1994) Hydration effects in protein unfolding. Biophys Chem 51:291–309
Makhatadze GI, Privalov PL (1995) Energetics of protein structure. Adv Protein Chem 47:307–425
Mastro AM, Keith AD (1984) Diffusion in the aqueous compartment. J Cell Biol 99:180–187
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
Okubo Y, Yokoigawa K, Esaki N, Soda K, Kawai H (1999) Characterization of psychrophilic alanine racemase from *Bacillus psychrosaccharolyticus*. Biochem Biophys Res Commun 256:333–340
Olufsen M, Smalas AO, Moe E, Brandsdal BO (2005) Increased Flexibility as a Strategy for Cold Adaptation: A comparative molecular dynamics study of cold- and warm-active uracil DNA glycosylase. J Biol Chem 280:18042–18048
Papaleo E, Riccardi L, Villa C, Fantucci P, De Gioia L (2006) Flexibility and enzymatic cold-adaptation: a comparative molecular dynamics investigation of the elastase family. Biochim Biophys Acta 1764:1397–1406
Riise EK, Lorentzen MS, Helland R, Smalas AO, Leiros H-KS, Willassen NP (2007) The first structure of a cold-active catalase from *Vibrio salmonicida* at 1.96 A reveals structural aspects of cold adaptation. Acta Crystallogr D Biol Crystallogr 63:135–148
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
Siddiqui KS, Bokhari SA, Afzal AJ, Singh S (2004) A novel thermodynamic relationship based on Kramers Theory for studying enzyme kinetics under high viscosity. IUBMB Life 56:403–407
Siddiqui KS, Feller G, D'Amico S, Gerday C, Giaquinto L, Cavicchioli R (2005) The active site is the least stable structure in the unfolding pathway of a multidomain cold-adapted alpha-amylase. J Bacteriol 187:6197–6205
Siddiqui KS, Cavicchioli R (2006) Cold-adapted enzymes. Annu Rev Biochem 75:403–433
Skalova T, Dohnalek J, Spiwok V, Lipovova P, Vondrackova E, Petrokova H, Duskova J, Strnad H, Kralova B, Hasek J (2005) Cold-active beta-galactosidase from *Arthrobacter* sp. C2-2

forms compact 660 kDa hexamers: crystal structure at 1.9A resolution. J Mol Biol 353:282–294
Smalas AO, Leiros HK, Os V, Willassen NP (2000) Cold adapted enzymes. Biotechnol Annu Rev 6:1–57
Somero GN (1995) Proteins and temperature. Annu Rev Physiol 57:43–68
Spiwok V, Lipovova P, Skalova T, Duskova J, Dohnalek J, Hasek J, Russell NJ, Kralova B (2007) Cold-active enzymes studied by comparative molecular dynamics simulation. J Mol Model 13:485–497
Suzuki Y, Takano K, Kanaya S (2005) Stabilities and activities of the N- and C-domains of FKBP22 from a psychrotrophic bacterium overproduced in *Escherichia coli*. FEBS J 272:632–642
Svingor A, Kardos J, Hajdu I, Nemeth A, Zavodszky P (2001) A better enzyme to cope with cold. Comparative flexibility studies on psychrotrophic, mesophilic, and thermophilic IPMDHs. J Biol Chem 276:28121–28125
Tehei M, Franzetti B, Madern D, Ginzburg M, Ginzburg BZ, Giudici-Orticoni MT, Bruschi M, Zaccai G (2004) Adaptation to extreme environments: macromolecular dynamics in bacteria compared in vivo by neutron scattering. EMBO Rep 5:66–70
Toyota E, Ng KK, Kuninaga S, Sekizaki H, Itoh K, Tanizawa K, James MN (2002) Crystal structure and nucleotide sequence of an anionic trypsin from chum salmon (*Oncorhynchus keta*) in comparison with Atlantic salmon (*Salmo salar*) and bovine trypsin. J Mol Biol 324:391–397
Tsuruta H, Mikami B, Aizono Y (2005) Crystal structure of cold-active protein-tyrosine phosphatase from a psychrophile, *Shewanella* sp. J Biochem (Tokyo) 137:69–77
Uppenberg J, Hansen MT, Patkar S, Jones TA (1994) The sequence, crystal structure determination and refinement of two crystal forms of lipase B from *Candida antarctica*. Structure. 2:293–308
Van Petegem F, Collins T, Meuwis MA, Gerday C, Feller G, Van Beeumen J (2003) The structure of a cold-adapted family 8 xylanase at 1.3 A resolution. Structural adaptations to cold and investigation of the active site. J Biol Chem 278:7531–7539
Violot S, Aghajari N, Czjzek M, Feller G, Sonan GK, Gouet P, Gerday C, Haser R, Receveur-Brechot V (2005) Structure of a full length psychrophilic cellulase from *Pseudoalteromonas haloplanktis* revealed by X-ray diffraction and small angle X-ray scattering. J Mol Biol 348:1211–1224
Wang E, Koutsioulis D, Leiros HK, Andersen OA, Bouriotis V, Hough E, Heikinheimo P (2007) Crystal Structure of Alkaline Phosphatase from the Antarctic Bacterium TAB5. J Mol Biol 366:1318–1331
Wintrode PL, Miyazaki K, Arnold FH (2001) Patterns of adaptation in a laboratory evolved thermophilic enzyme. Biochim Biophys Acta. 1549:1–8
Xu Y, Feller G, Gerday C, Glansdorff N (2003a) Metabolic enzymes from psychrophilic bacteria: challenge of adaptation to low temperatures in ornithine carbamoyltransferase from *Moritella abyssi*. J Bacteriol 185:2161–2168
Xu Y, Feller G, Gerday C, Glansdorff N (2003b) Moritella cold-active dihydrofolate reductase: are there natural limits to optimization of catalytic efficiency at low temperature? J Bacteriol 185:5519–5526
Xu Y, Nogi Y, Kato C, Liang Z, Ruger HJ, De Kegel D, Glansdorff N (2003c) *Moritella profunda* sp. nov. and *Moritella abyssi* sp. nov., two psychropiezophilic organisms isolated from deep Atlantic sediments. Int J Syst Evol Microbiol 53:533–538
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 USA 95:7406–7411