Chapter 15
Fundamentals of Cold-Active Enzymes

Charles Gerday

Abstract Cold-active enzymes are produced by organisms adapted to permanently cold habitats. Due to the depressive effect of low temperatures on reaction rates, these enzymes have to be adapted to secure appropriate reaction rates in those organisms that often thrive in environments characterized by temperatures close or below the freezing point of water. They are encountered in all prokaryotic or eukaryotic organisms adapted to cold such as microorganisms, invertebrates, insects and fish originating from the Arctic and Antarctic zones, as well as from alpine regions, glaciers or permafrost zones. They are characterized by a high specific activity at low temperatures, in any case higher than that of their mesophilic and thermophilic counterparts. This higher specific activity is generally accompanied by a decrease in thermal stability illustrated by a shift of the apparent optimum towards low temperatures, and by an important decrease in the thermodynamic stability characterized by a significantly lower stabilization enthalpy. The generally low stability induces an increase in the flexibility of the overall edifice or of crucial zones for activity of the molecular structure. There is apparently a continuum in the adaptation since some enzymes display extreme adaptation illustrated by a severe shift of the activity towards low temperatures whereas others are moderately adapted. This probably depends on their position in a metabolic pathway, on their intracellular or extracellular localization, on the environmental temperature and on the evolutionary history of the organisms.

Keywords Psychrophiles · Cold adaptation · Psychrophilic enzymes · Protein stability

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15.1 Introduction

The first significant report related to cold-active enzymes dates back to more than a quarter of a century with the publication of the properties of a heat-labile alkaline phosphatase isolated from an Antarctic bacterium (Kobori et al. 1984). This paper can be considered as a seed paper since the two main properties of cold-active catalysts were already correctly described, i.e., a high specific activity at low temperature and a rather high-temperature sensitivity. The biotechnological potential of this enzyme was also underlined since this cold-active enzyme could be, contrary to calf intestinal ATPase, rapidly inactivated by mild heat treatment offering a very convenient method for the 5′-end radio-labelling of DNA fragments. Cold-active enzymes are produced by psychrophiles, a term introduced by Schmidt-Nielsen (1902), but their existence in cold environments was considered as hypothetical due to the fact that their optimum growth temperature was generally above 20 °C. They were considered as cold tolerant or psychrotolerant rather than cold loving or psychrophiles (Ingraham and Stokes 1959). This confusion still persists nowadays since some investigators erroneously consider that the so-called optimum temperature based on the rate of growth is the best possible temperature for microorganisms. It was the merit of Hess (1934) to point out that numerous bacteria, although their maximum growth rate occurred around 20 °C, were true psychrophiles simply because in broth cultures, the largest cell populations were obtained at 5 °C rather than at 20 °C. This was confirmed by more recent works such as that of Margesin (2009) who stated that «cultivation temperatures close to the maximum growth temperature are not appropriate for studying psychrophiles». It was also shown that the production of extracellular enzymes was the highest at temperatures close to that of the environment of the microorganism and far below the so-called optimum temperature (Feller et al. 1994). An overview on the concept of psychrophily and psychrotolerance is reported in Chap. 1.

The adaptative modifications of enzymatic properties as a function of environmental temperature have been discussed long time ago by pioneer scientists such as Peter Hochachka and George Somero (Hochachka and Somero 1973;

Somero 1977). They defined three possible strategies for the adaptation of enzymes to low temperatures: first, the organism can increase the concentration of the enzyme in the cell or secreted; second, the activity can also be modulated by increasing the substrate-binding ability; and third, the organism can modify in a permanent way the catalytic efficiency of the enzyme. The first strategy is, at first sight, improbable due to its energy cost in a cold environment; the second strategy will not be efficient in the case of enzymes working at saturated substrate concentration that probably concerns most of the extracellular enzymes. In the case of intracellular enzymes, it has been shown that the K_m values of phosphoenolpyruvate for pyruvate kinases of fishes adapted to different temperatures were the lowest in the range of temperatures usually experimented by the organisms and that at the respective cell temperatures, the substrate-binding affinities were close to each other (Somero 1977). So, only the third strategy, i.e., the production of enzymes specifically adapted to low temperatures, seemed to be the only one appropriate. These first information, as well as an easier accessibility to Arctic and Antarctic sites, have stimulated the interest for these enzymes and in the 90s several groups were formed, thanks to the support of the European Union, and focused on psychrophiles with the aims to shed some light on the fundamentals aspects and on the biotechnological potential of these extreme organisms. Therefore, significant progresses in the understanding of the molecular adaptation to cold of cold-active enzymes were made. In this context, the characteristics of a cold-active xylanase from the Antarctic yeast, *Cryptococcus adeliensis* (formerly *Cryptococcus adeliae*), was investigated in 1996 (Petrescu et al. 2000). When compared to its mesophilic counterpart, *C. adeliensis,* a maximum cell density was obtained by cultivation at 4 °C and exceeded by sixfold the cell density reached by *C. albidus* at the same temperature. By contrast, at 20 °C, the cell density obtained with the mesophilic strain was 2.5 times as large as that of the psychrophilic organism illustrating the cold-adapted character of *C. adeliensis*. Concomitant differences were observed at the level of the respective catalytic properties of the xylanases with the cold-active enzyme displaying a k_{cat} at 5 °C three times as high as that of the mesophilic counterpart. Interestingly also, the cold-active enzymes displayed an activation energy, ΔG^*, about 5 % lower than that of the mesophilic enzyme. This mainly resulted from a significantly lower activation enthalpy, ΔH^*, partially compensated by a more negative value of the activation entropy ΔS^*. The cold-active enzyme was also rapidly inactivated after exposure at 30 °C contrarily to the mesophilic enzyme.

15.2 Catalytic Properties and Substrate Binding

Few years ago, (Garcia-Viloca et al. 2004), a generalized expression of reaction rate for enzyme-catalysed reactions, based on the transition state theory developed by Eyring (1935), was introduced. This expression, $k_{cat} = \gamma_{(T)} k_B T/h \exp(-\Delta G^*/RT)$, through its coefficient $\gamma_{(T)}$, takes into account the possibility that the activated

complex ES* could recross the energy barrier under the influence of various parameters among which the viscosity of the medium should play an important role in inducing a value for $\gamma_{(T)}$ that could differ from 1 in the case of cold-active enzymes. This coefficient, an extended version of the old transmission coefficient κ, has been usually neglected, but the intracellular space represents a very crowded medium with viscosity increasing from 2.5 cP at 20 °C to 5.0 cP at 0 °C. This high viscosity affects reaction rates as demonstrated by studies on lactate dehydrogenases (Demchenko et al. 1989) and alkaline phosphatase (Homchaudhuri et al. 2006). In this latter study, it was also demonstrated that, next to the influence of viscosity, the high fractional cell volume occupancy by the numerous macromolecules could also severely influence reaction rates of enzyme-catalysed reaction in reducing the frequency of enzyme–substrate encounter. Therefore, the catalytic parameters of cold-active enzymes measured in test tubes are probably far from those prevailing in cells. Taking these considerations apart, numerous investigations on cold-active enzymes have clearly demonstrated that these enzymes display a specific activity usually much higher than their mesophilic and *a fortiori* thermophilic counterparts at low and moderate temperatures as shown in Fig. 15.1. This figure concerns, from left to right, the temperature dependence of the activity of ornithine transcarbamylase (OTCase) from psychrophilic (*Moritella abyssi*) and mesophilic bacteria (*Escherichia coli*) and hyperthermophilic archaea (*Pyrococcus furiosus*) (Xu et al. 2003). One can see that the apparent optimum temperature of the OTCases of these microorganisms considerably differs from each other with a difference of nearly 40 °C between the psychrophilic and mesophilic enzymes. The specific activity of the cold-active enzyme is much higher than that of the mesophilic counterpart in the range of 0–30 °C, and the enzyme is also rapidly inactivated by heat since the apparent optimum does not exceed 25 °C. A large majority of cold-active enzymes displays similar properties as shown in Table 15.1. In a limited number of cases, the specific activity of the cold-active enzyme does not exceed that of the mesophilic one as demonstrated by

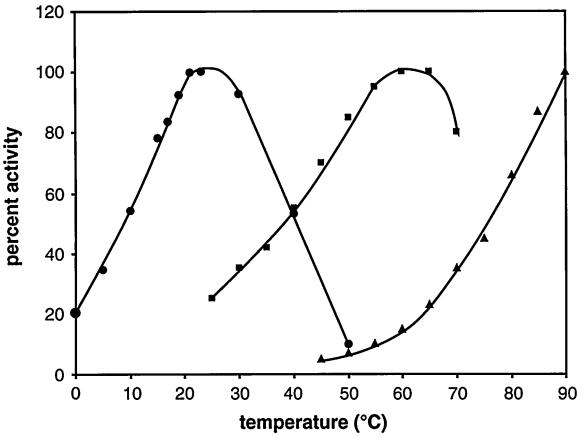

Fig. 15.1 Relative activities of psychrophilic (*Moritella abyssi*; *black circles*) mesophilic (*E. coli*; *black squares*) and thermophilic (*Pyrococcus furiosus*; *black triangles*) OTCases as a function of temperature. Reproduced with permission from Xu et al. (2003)

Table 15.1 K_m and k_{cat} values of some psychrophilic (P) and mesophilic (M) enzymes

Enzyme/organism	T (°C)	K_m	k_{cat} s^{-1}	Reference
Alpha-amylase				D'Amico et al. (2001)
P: *Pseudoalteromonas haloplanktis*	25	234 μM	294 (10 °C)	
M: Pig pancreatic	25	65 μM	97	
Cellulase				Garsoux et al. (2004)
P: *Pseudoalteromonas haloplanktis*	4	600 μM	0.18	
M: *Erwinia chrysanthemi*	4	200 μM	0.01	
DNA ligase				Georlette et al. (2000)
P: *P. haloplanktis*	18	0.30 μM	0.034	
M: *Escherichia coli*	18	0.18 μM	0.004	
Endonuclease I				Altermark et al. (2007)
P: *Vibrio salmonicido*	0.5	246 mM	9.41	
M: *Vibrio cholerae*	0.5	118 mM	1.03	
Isocitrate dehydrogenase				Watanabe et al. (2005)
P: *Colwellia maris*	15	62 mM	70.8	
M: *Escherichia coli*	15	3.3 mM	22.0	
Lactate dehydrogenase				Coquelle et al. (2007)
P: *Champsocephalus gunnarii*	0	0.16 mM	230	
M: *Squalus acanthias*	0	~0.3	72	
Ornithine transcarbamylase				Xu et al. (2003)
P: *Moritella abyssi*	30	45 mM	690	
M: *Saccharomyces cerevisiae*	30	0.9 mM	235	
Subtilisin				Narinx et al. (1997)
P: *Bacillus* sp. (Antarctic)	5	26 μM	32	
M: Subtilisin Carlsberg	5	6 μM	18	

arginine kinase that shows an activity similar to that of the mesophilic enzyme (Suzuki et al. 2012). This type of anomaly has also been mentioned in the case of chitinase (Lonhienne et al. 2001a), and isocitrate reductase (Fedoy et al. 2007), for example. In this latter case, the thermal stability also seems higher than that of the mesophilic counterpart. These data tend to indicate that the enzyme from the psychrophilic bacterium *Desulfotalea psychrophila* is not well adapted to low temperature. It would be interesting to compare the catalytic properties of other enzymes from *D. psychrophila* to those of homologous enzymes from the mesophilic bacterium *Desulfitobacterium hafniense* to see whether this apparently incomplete adaptation to cold can be extended to other enzymes and possibly to the whole organism. In the case of chitinase, the type of substrate is probably important since chitins are structurally very different from each other. As far the arginine kinase from the deep-sea clam *Calyptogena kaikoi* is concerned, the significance of the data is uncertain since, at 25 °C, the activation enthalpy is, as expected, lower than that of the mesophilic enzyme from clams of the genus *Corbicula.* This favourable term is, however, over-compensated by a much more

negative activation entropy, the difference leading to a less favourable activation energy. As this enzyme is intracellular, one has in fact to consider the physiological efficiency k_{cat}/K_m rather than the k_{cat} alone because these enzymes are not probably working at saturated substrate concentration. If this is taken into consideration, the cold-active arginine kinase is still, at 10 °C, three times less efficient than the mesophilic enzyme.

Also interesting in Table 15.1 are the values of the Michaelis constant, K_m. With the possible exception of lactate dehydrogenase from the icefish *Champsocephalus gunnarii,* the K_m of cold-active enzymes are systematically higher than the values observed in the case of mesophilic counterparts and the tendency is the same for extracellular and intracellular enzymes. The larger values can be translated into a lower affinity of the cold-active one, provided that one neglects the rate constants not directly involved in the dissociation of the enzyme–substrate complex. In the case of intracellular enzymes, the catalytic efficiency or physiological efficiency is better evaluated by the ratio K_m/k_{cat}. That leads to largely spread situations, favourable for cold-active endonuclease and lactate dehydrogenase but unfavourable for DNA ligase, Isocitrate dehydrogenase and OTCase. So, the usual increase in the rate constant, k_{cat}, of cold-active enzyme is not systematically large enough to compensate for the loss of substrate-binding affinity.

That could indicate that the adaptation of some cold-active enzymes is not complete and depends on the enzyme concerned and probably also on the evolution history of the organism. For extracellular enzymes, the situation appears more favourable since they are probably generally working at saturated substrate concentrations. As already discussed by Somero (1977), the K_m values are rather constant in the range of temperature experimented by the organism but can rapidly increase for temperatures exceeding this zone. This loss in substrate-binding affinity contributes to fix the limit of the thermal tolerance of living organisms. The nearly systematic increase in the K_m values of cold-active enzymes is not fortuitous and constitutes a consequence of the structural modifications needed to improve the catalytic efficiency of these enzymes at low temperatures.

15.3 The Activity Challenge

A quick look to the modern form of the Eyring equation mentioned above indicates that the best way to improve the catalytic efficiency of enzymes at low temperature would be to further decrease the activation energy ΔG^* of the catalysed reaction when compared to the reaction catalysed by the mesophilic counterpart. Simply because the rate constant is exponentially related to the activation energy and that even a small decrease in this value would considerably affect the velocity of the reaction. The activation energy is related to the energy changes necessary to transform the ground state of the enzyme–substrate complex into an activated state. This eventually leads to the transformation of substrate into product. The energy change resulting from the interaction of the enzyme with the

substrate has also to be taken into consideration since it is associated with a negative energy change that places the enzyme substrate complex in an unfavourable deep pit of energy as shown in Fig. 15.2. As the K_m values of cold-active enzymes are higher than their mesophilic counterparts, one can consider that the ground state of the ES complex of the cold-active enzyme has a higher free energy level that is favourable in limiting the energy change necessary to reach the activated state. The fact that the ground state of the complex ES occupies a higher level of energy could also be favourable for the activation of ES into ES*. Indeed, if less weak bonds or looser bonds are implicated in the formation of the complex ES, less costly structural modifications will be necessary to reach the activated state. That could lead to the situation represented in Fig. 15.2 showing a significant decrease in the activation energy in the case of the cold-active enzyme. Experimentally, the activation energy of some cold-active enzymes has been measured and compared to that of their mesophilic counterpart and the data are presented in Table 15.2. The activation energy ΔG^* of cold-active enzymes is systematically lower that that of mesophilic homologues to an extent ranging roughly from 1 (amylases) to 10 % (cellulases and bacterial xylanases). These rather moderate figures result from an important decrease in the activation enthalpies ΔH^*, that, for example, amounts to 25 % in the case of the amylases, systematically compensated by a much more negative value of the activation entropy ΔS^*. This indicates that the activated state of the psychrophilic enzymes requires a much more important reordering of the ground state. The activation entropy values of three mesophilic enzymes, i.e., the chitobiase, endonuclease and lysozyme, are positive. That could mean that contrarily to the other enzymes, the activation state ES* is characterized by more disorder than the ground state or, alternatively, that the rearrangement of water molecules, under the form of a release, induces a positive value of the entropy change in the course of the activation. The negative values of the activation entropy of the other cold-active enzymes confirm that they display a

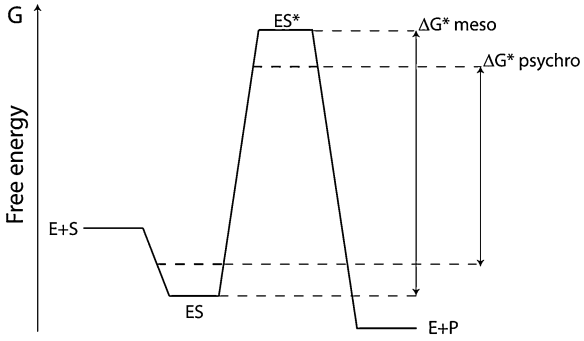

Fig. 15.2 Energetics of the activation of an enzyme-catalysed reaction for mesophilic enzyme (*continuous line*) and psychrophilic counterpart (*dotted line*). The activation in psychrophilic enzymes is rendered easier by a decrease in the affinity of the enzyme for the substrate (higher level of ES) and by a possibly lower energetic level of ES*

Table 15.2 Activation parameters of a few psychrophilic enzymes (P) compared to those of mesophilic counterparts (M)

Enzyme	Type	Temp. (°C)	ΔG* (kJ mol^{-1})	ΔH* (kJ mol^{-1})	TΔS* (kJ mol^{-1})	Reference
Amylase	P	10	57.7	34.7	−23.0	D'Amico et al. (2003)
	M		58.5	46.4	−12.1	
Cellulase	P	4	71.6	46.2	−25.4	Garsoux et al. (2004)
	M		78.2	65.8	−12.4	
Chitobiase	P	15	59.5	44.7	−14.8	Lonhienne et al. (2001b)
	M		63.5	71.5	+8.0	
Endonuclease	P	5	62.8	33.4	−29.4	Altermark et al. (2007)
	M		67.9	74.0	+6.1	
Lysozyme	P	25	45.1	31.9	−13.2	Sotelo-Mundo et al. (2007)
	M		46.2	49.4	+3.2	
Subtilisin	P	15	62.0	36.0	−26.5	Davail et al. (1994)
	M		66.0	46.0	−20.2	
Xylanase (bacteria)	P	10	54.0	21.0	−33.0	Collins et al. (2003)
	M		60.0	58.0	−2.0	
Xylanase (yeast)	P	5	52.3	45.3	−7.0	Petrescu et al. (2000)
	M		54.6	49.9	−4.7	

rather loose structure as suggested by the nearly systematic increase in the K_m values. So the high catalytic efficiency of cold-active enzymes seems to be associated with a rather open structure that has also been associated with a certain loss of specificity of these enzymes. Indeed multi-substrate cold-active enzymes seem to display a broader specificity when compared to their mesophilic counterpart. This has been demonstrated in the case of elastases (Smalas et al. 2000), alcohol dehydrogenase (Tsigos et al. 1998) and α-amylases (D'Amico et al. 2006). These enzymes have the propensity to more easily accept bulky substrates but are less efficient in the case of small-size substrates.

The notion of flexibility in relation with the high specific activity and the generally low thermal stability of cold-active enzymes has been introduced long time ago and notably by Somero (1977). This correlation has been demonstrated in a large majority of cases, but a more refined view of the linkage between the catalytic properties and overall thermal stability of enzymes is probably now emerging. In fact, the catalytic efficiency of a protein is mainly related to the mobility–flexibility of crucial parts of the molecular edifice. An improvement of this flexibility usually leads to an overall decrease in the thermal stability but in a

limited number of cases this can occur without significant modifications or visible changes of the thermal stability. Intuitively, one can indeed think that substitutions of amino acids in existing loops, for example, can modify the conformational mobility–flexibility of regions crucial for catalysis without altering the thermal stability. A clear relationship between the activity and local flexibility in psychrophilic and mesophilic enzymes has been recently demonstrated in carbonic anhydrases. It was shown that the cold-active enzyme with a higher specific activity displays a higher flexibility in the region that controls the folding of the protein contrarily to the mesophilic counterpart (Chiuri et al. 2009). EPR spectroscopy has also shown that the catalytic function of a cold-active alkaline phosphatase was closely related to the relative mobility of the helix carrying cysteine 67 and nucleophilic serine 65 (Heidarsson et al. 2009). In subtilisin-like proteases, the relative flexibility of a beta-hairpin close to the active site correlates well with the differential activity of psychrophilic, mesophilic and thermophilic enzymes, but in this example, the homologous enzymes also display a different thermostability (Tiberti and Papaleo 2011).

Protein dynamics obviously play a crucial role in catalytic processes; a systematic investigation using molecular dynamics of 5 pairs of psychrophilic and mesophilic or thermophilic enzymes has shown that amino acid sequences common to both enzymes are generally more flexible in cold-active enzymes, this in relation with differences in the rate or amplitude of opening of the respective active sites. However, in the case of α-amylases, the overall flexibility of the mesophilic enzyme was higher than that of the psychrophilic one due to the insertion of additional loops. It was also shown that the flexibility of orthologous xylanases was approximately the same at the respective apparent optimum temperatures (Spiwok et al. 2007). Experiments using NMR relaxation have also demonstrated that in adenylate kinases, the rate-limiting step was strictly correlated with flexibility in relation with the opening and closing of the active-site lids that shows slower rates in the thermophilic enzyme when compared to the mesophilic counterpart (Wolf-Watz et al. 2004). An apparent increase in the flexibility of a region important for the catalytic efficiency was noticed in the isocitrate dehydrogenase from the cold-adapted *D. psychrophila* and that seems to be induced by the formation of a methionine cluster, but the catalytic efficiency of this enzyme is lower than that of the mesophilic counterpart from *D. hafniense*. Its activation energy ΔG^* is also higher at 5 °C as well as its thermostability. The K_m of the cold-active enzyme for isocitrate is strongly affected by temperatures over 25 °C and is, in any case, higher than that of the mesophilic counterpart (Fedoy et al. 2007). The interpretation of these data seems rather complex since, apart from the K_m value and possibly the local flexibility, this enzyme does not show the usual characteristics of a cold-adapted enzyme, i.e., a higher catalytic efficiency at low temperature and a lower thermal stability. As already mentioned above, it would certainly be interesting to study the biochemical characteristics of other enzymes from this organism. The notion of flexibility is rather complex since the term can refer to structural modifications as a function of time which can be assimilated to a «respiration» of the molecular edifice or to the amplitude of the

conformational changes. Both are probably important for the accommodation of the substrates at low temperature and the release of products. The flexibility of proteins has been evaluated by various techniques. Molecular dynamics simulations (MDS) have been applied with a certain success in the case for instance of subtilisin-like proteases from organisms living in different temperature environments (Tindbaek et al. 2004; Tiberti and Papaleo 2011). It was shown that the higher catalytic efficiency of cold-active enzymes was correlated to a higher flexibility of regions important for catalysis, itself correlated to an evolution towards a lower thermal stability from high to low-temperature environments. Recent investigations using MDS have corroborated the idea that the adaptation to cold, in terms of function, is correlated with an improvement of the flexibility of crucial regions in various proteins such as chitinases (Ramli et al. 2012), frataxins (Roman et al. 2013), haemoglobins (Stadler et al. 2012) and beta-tubulins (Chiappori et al. 2012). It has also been shown from studies carried out on homologous 3-isopropylmalate dehydrogenases and thermolysin-like proteases that, at their respective environment temperature, enzymes from mesophilic and thermophilic organisms maintain a balance between their overall rigidity and local flexibility important for their catalytic properties (Radestock and Gohlke 2011) in agreement with the strong correlation between resistance to unfolding and evolutionary adaptation temperature (Hochachka and Somero 2002). Amide hydrogen–deuterium exchanges have provided controversial data. For example, a psychrophilic 3-isopropylmalate dehydrogenase was found to be more rigid than its mesophilic counterpart (Svingor et al. 2001). The «anomalies» observed with the H/D exchange techniques may be due to the use of non-relevant temperatures or time scales. The temperature factor or B-factor has also been used to evaluate the relative flexibility of proteins. It represents the spread of electron densities and can be calculated from known three-dimensional structures. Average B-factors are not systematically correlated with the activity-flexibility relationship (Sun-Yong et al. 1999) but relative B-factors representing the ratio between the B-factors of local parts of the proteins and the average B-factor apparently correlate well with the relative activity and thermostability of psychrophilic and mesophilic enzymes (Russell et al. 1998; Sun-Yong et al. 1999). A recent systematic comparison of the B-factors of twenty pairs of psychrophilic and mesophilic enzymes has demonstrated that psychrophilic enzymes are more flexible in 5-turn and strand secondary structures; they have also larger average cavity sizes and these cavities are lined with an increased frequency of acidic groups (Paredes et al. 2011). Fluorescence quenching of trytophane fluorescence using acrylamide has been used successfully to evaluate the relative flexibility of psychrophilic and mesophilic enzymes. The data have shown that psychrophilic enzymes are much more permeable than their mesophilic counterparts in the case of Ca^{2+}-Zn^{2+} protease (Chessa et al. 2000), α-amylase (D'Amico et al. 2003; Cipolla et al. 2012), DNA ligase (Georlette et al. 2000, 2003), xylanase (Collins et al. 2003), cellulase (Sonan et al. 2007), aminopeptidase (Huston et al. 2008) and thermolysin (Xie et al. 2009). An example of the comparison of tryptophane fluorescence quenching of psychrophilic and mesophilic enzyme is shown in Fig. 15.3. One can see that the cold-active

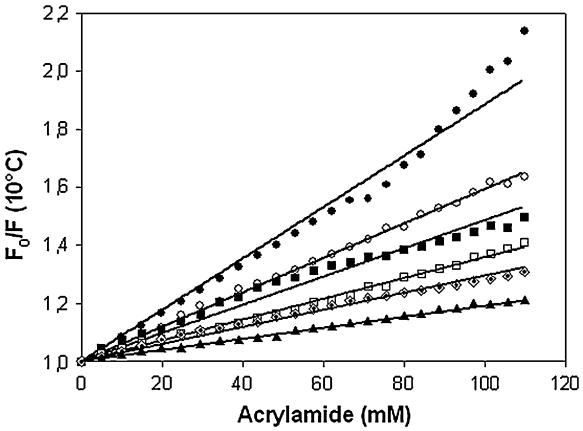

Fig. 15.3 Quenching of tryptophane fluorescence at 10 °C of cold-active cellulase from Antarctic *Pseudoalteromonas haloplanktis* (Cel5G, *black dots*) and variants differing by a shorter length of the linker region (Cel5GΔ1P, *open circles*; Cel5GΔ2P, *black squares*) as well as mutants characterized by higher melting points (Cel5GΔ2PmutCC, *open squares*; $Cel5G_{CM}$, *diamonds*) and mesophilic cellulase from *Erwinia chrysanthemi* (Cel5A, *black triangles*). F_0/F represents the ratio between the fluorescence in the absence (F_0) and presence (F) of acrylamide at various concentrations. Adapted from Sonan et al. (2007)

cellulase presents a much more opened structure when compared to the mesophilic enzyme. Moreover, the mutants that are characterized by a progressive shortening of the linker region (Cel5GΔ1P, Cel5GΔ2P), which induces a progressive increase in the melting point, display a more compact structure, further enhanced in the case of the variant Cel5GΔ2P mutCC carrying an additional disulphide bridge and of the catalytic module $Cel5G_{CM}$. This once again illustrates the good correlation that generally exists between the low stability and flexibility of cold-active enzyme responsible for their higher catalytic efficiency at low temperature (Sonan et al. 2007).

15.4 Conformational and Thermodynamic Stability

The analysis of the thermodependence of the activity of cold-active enzyme, as illustrated in Fig. 15.1, indicates that these enzymes are inactivated at temperatures lower than that of their mesophilic counterparts, and in consequence, they seem to display a lower thermal stability. This thermal stability has been investigated by numerous techniques from the evaluation of the residual activity after exposure for a certain time at a given temperature to biophysical techniques such as circular dichroïsm, fluorescence spectroscopy or microcalorimetry. The method using residual activity is only valid if one demonstrates that the unfolding is irreversible at all temperature tested; an example of the use of this technique is

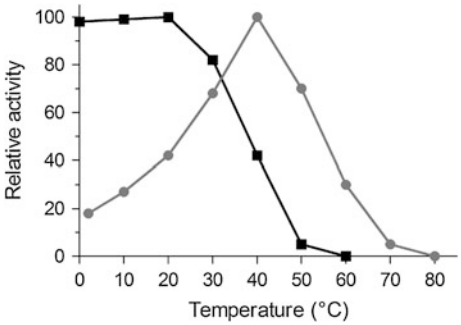

Fig. 15.4 Activity as a function of temperature of polygalacturonase from the Antarctic yeast *Cystofilobasidium capitatum* (formerly *Cystofilobasidium lari-marini*) S3B (*grey circles*) and residual activities of the enzyme after exposure at various temperatures (*black squares*). Note that at the so-called optimum temperature, around 40 °C, the enzyme is already partially unfolded. Adapted from Birgisson et al. (2003)

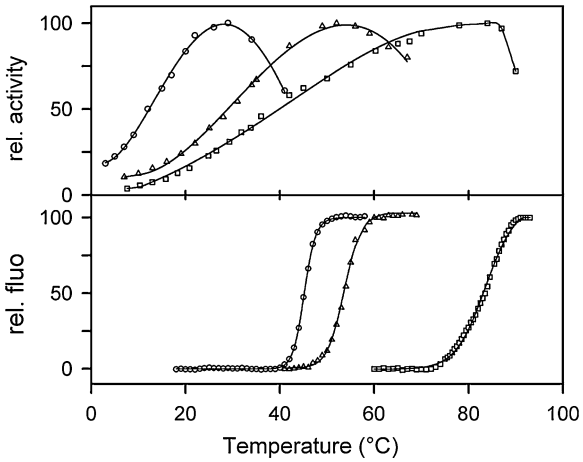

Fig. 15.5 *Upper panel* Relative activity as a function of temperature of psychrophilic (*open circles*), mesophilic (*open triangles*), and thermophilic (*open squares*) α-amylases. *Lower panel* Concomitant thermal unfolding as recorded by fluorescence spectroscopy. Worth noting is the fact that the thermal inactivation of the psychrophilic enzyme occurs without any significant modification of the three-dimensional structure contrary to mesophilic and thermophilic counterparts. Adapted from Georlette et al. (2004)

shown in Fig. 15.4. In this figure, the thermodependence of the activity of a cold-active polygalacturonase from Icelandic yeast (Birgisson et al. 2003) is compared to the thermal stability of the enzyme exposed for one hour at the respective temperatures. One can see that the enzyme starts to unfold at a temperature around 25 °C, far below the so-called optimum temperature which is around 40 °C. This figure clearly demonstrates that the so-called optimum temperature is only an

apparent optimum since at this temperature, the half-life of the enzyme is rather short. Another illustration of the thermodependence of the activity and stability of a cold-active enzyme is shown in Fig. 15.5. That concerns a cold-active α-amylase for the Antarctic bacterial strain *Pseudoalteromonas haloplanktis* (AHA) as compared with the mesophilic enzyme from pig pancreas (PPA) and the thermophilic counterpart from *Bacillus amyloliquefaciens* (BAA). The upper panel represents the relative activities and the lower panel the thermal unfolding as followed by fluorescence spectroscopy (D'Amico et al. 2003). A striking feature of these experiments is the fact that the inactivation of the cold-active enzymes precedes the unfolding of the structure. In the mesophilic and thermophilic enzymes, the apparent optimum is strictly correlated to the unfolding transition. That can mean that the active site is very sensitive to temperature or that the ES complex is destabilized before any significant or visible modification of the three-dimensional structure of the enzyme. Such phenomenon has been observed in other cold-active enzymes such as for instance xylanases (Collins et al. 2003, 2007) and DNA ligases (Georlette et al. 2003).The thermal sensitivity of the active site and/or of the ES complex is in agreement with the increase in K_m values reported for cold-active enzymes. In general, the residues lining the catalytic cavities and involved in the interaction of the substrate are strictly conserved. This has been notably demonstrated by comparison of the crystallographic structures of the psychrophilic α-amylase from an Antarctic bacterium with that of the homologous enzyme from pig pancreas. The superimposition of the active site of the two enzymes has shown that the 24 residues forming the catalytic cleft have a position strictly conserved in both enzymes (Aghajari et al. 1998, 2002). Therefore, the flexibility of the catalytic region should originate from other regions of the protein. In other cases, the catalytic cavities have been altered so as to confer a larger opening of the catalytic region of psychrophilic enzymes that favours the accommodation of larger substrates and facilitates the release of products. This has been achieved through the modification, for example, of electric potentials that favours the attraction and orientation of substrates in citrate synthase (Russell et al. 1998), malate dehydrogenase (Kim et al. 1999), uracyl-DNA glycosylase (Leiros et al. 2003) and trypsin (Gorfe et al. 2000). In a cold-active Ca^{2+}-Zn^{2+} protease, an additional Ca^{2+} pulls the backbone lining the entrance of the site and offers in this way a much better accessibility for the substrate (Aghajari et al. 2003). In cold-active iron superoxide dismutases, it has been shown that the residues forming the active site and the active-site channel are more mobile than in mesophilic counterparts (Merlino et al. 2010).

The thermodynamic stability of some cold-adapted proteins has been investigated assuming that the unfolding is a reversible two-state process defined by the equilibrium

$$\Delta G_{N-U} = -RT \ln K_{N-U} \quad \text{with} \quad K_{N-U} = [U]/[N]$$

For a protein mainly in a native state, ΔG_{N-U} is a positive value and can be defined as the energy necessary to unfold the protein. The thermodynamic stability

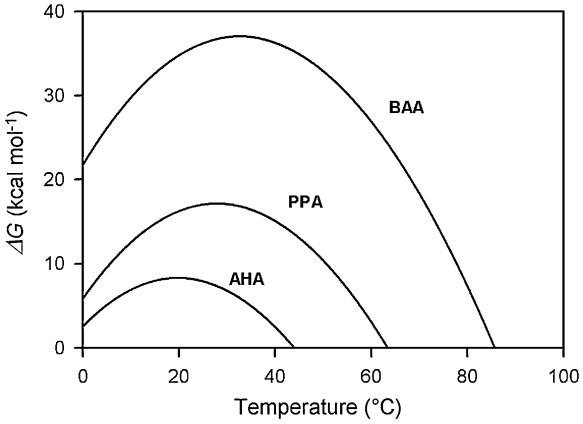

Fig. 15.6 Stabilization energy as a function of temperature of psychrophilic α-amylase (*AHA*) from the Antarctic strain *Pseudoalteromonas haloplanktis*, of mesophilic enzyme from pig pancreas (*PPA*) and of the thermophilic counterpart (*BAA*) from *Bacillus amyloliquefaciens.* Adapted from D'Amico et al. (2003)

of enzymes can be evaluated by non-calorimetric techniques making use of chemical denaturants (Pace and Laurents 1989; Talla-Singh and Stites 2008) or more easily in a microcalorimeter in which the heat absorbed during the unfolding of the protein is measured and defined as ΔH_{cal}. It represents the energy necessary to disrupt the weak bonds that stabilize the three-dimensional structure and appears as a peak often symmetrical in the case of cold-active enzymes but often unsymmetric and flattened in the case of mesophilic and thermophilic counterparts. ΔH_{cal} is equivalent to the area limited by the peak and this area significantly increases from psychrophiles to thermophiles. The symmetry is indicative of a pronounced cooperativity of unfolding and the transition appears in this case sharp contrarily to that of mesophilic and thermophilic proteins that show a flattened profile distributed over a rather broad range of temperatures (Feller 2010). As a function of temperature, the stabilization energy or free energy of unfolding is described by an equivalent of the Gibbs–Helmholtz equation:

$$\Delta G_{(T)} = \Delta H_{cal}(1 - T/T_m) + \Delta C_p(T - T_m) - T\Delta C_p \ln(T/T_m)$$

In this equation, T_m is the so-called melting temperature, located at the top of the peak, and defined as the temperature of half denaturation where $[N] = [U]$, ΔH_{cal} has been defined above and is equivalent to the van't Hoff enthalpy in a two-state model, T is a given temperature, and ΔC_p, the heat capacity change occurring during the transition from the native to the unfolded state. The calculation of the stabilization energy, over a temperature range where the native state is in excess over the unfolded state, gives access to stability curves as shown in Fig. 15.6. These curves, which are parabola-shaped, have been established for cold-active α-amylase AHA, mesophilic α-amylase PPA and thermophilic α-amylase BAA. One can see that over the whole range of temperature, the cold-active enzyme presents a much lower

thermodynamic stability than its mesophilic and thermophilic counterparts, even in the low-temperature range. This has been attributed to the lower number or/and strength of the weak bonds that stabilizes the structure. A second striking feature is that the maximum stability for the three orthologous enzymes is found in the rather narrow range of 20–30 °C. This is due to the hydrophobic effect found to be maximum around room temperature (Kumar et al. 2002). Cold denaturation is also more easily achieved in the case of the psychrophilic enzyme with a T_m prediction around −10 °C. On the left side of the curve, the stabilization enthalpy and stabilization entropy are negative. That means that the residual stability of the cold-active enzyme near the environment temperature, around 0 °C (Antarctic sea water), is secured through the negative value of the stabilization entropy resulting from the hydration of the hydrophobic groups of non-polar amino acids that contributes to decrease the entropy of the unfolded state (Dias et al. 2010). Cold denaturation occurs with a release of heat contrarily to heat denaturation. It is also interesting to note that at temperatures corresponding to the most stable conditions, around 20 °C, the cold-active enzyme is marginally stable since the energy required for unfolding is around 30 kJ mol^{-1}. The value drops to around 8 kJ mol^{-1} at 0 °C, the equivalent of about 2 weak bonds.

15.5 Determinants of Cold Adaptation and Mutagenesis

It has been shown that the flexibility of crucial parts of the structure of cold-active enzymes is generally, but not probably always, accompanied by a decrease in the thermal stability of the protein structure. To understand cold adaptation, it is necessary to define the structural changes that are directly or indirectly involved in the adaptation to cold. The problem is rendered more complicated by the high number of neutral mutations resulting from genetic drift that leads to large differences in the amino acid sequences of homologous enzymes produced by related species. The elucidation of the three-dimensional structure of several cold-active enzymes by X-ray crystallography, more than thirty actually, as well as the production of reliable models based on the comparison of the amino acid sequences of mesophilic and psychrophilic proteins, has allowed to detect, in a first approach, the amino acid substitutions that could lead to a decrease in the thermal stability of the molecular structure of cold-active enzymes. Again, only a few of them are intuitively involved in the adaptation to cold since, in low-temperature environments, the absence a high selective pressure on thermal stability can enhance the importance of the genetic drift.

The structural parameters involved in the thermal stability of proteins have been extensively discussed elsewhere (Vieille and Zeikus 2001), and their implications in the adaptation of cold-active enzymes have already been discussed in many reviews (Somero and Low 1976; Feller et al. 1997a; Marshall 1997; Feller and Gerday 1997, 2003; Russell 2000; Smalas et al. 2000; Gerday et al. 2000; Feller 2003, 2010; Hoyoux et al. 2004; Georlette et al. 2004; Siddiqui and Cavicchioli

2006; Marx et al. 2007; Collins et al. 2007, 2008), and there is a consensus to agree that the enhanced flexibility of crucial parts of these enzymes is mainly secured through a weakening of intramolecular weak bonds that stabilize the structure of mesophilic counterparts associated or not to entropic factors that tend to increase the entropy of the folded state. In general, however, the structural changes leading to cold adaptation are rather discrete since the three-dimensional structure of psychrophilic and mesophilic homologous enzymes can be nearly superimposed. Hydrophobic interactions play important roles in driving the folding of proteins in aqueous media and in stabilizing the nascent polypeptide chain. In cold-active enzymes, the number of hydrophobic interactions can be lower and their strength can also be affected by a decrease in the size of the hydrophobic groups and a reduction in the number or/and size of hydrophobic clusters that contribute to decrease the compactness of the molecule in creating cavities that can be occupied by water molecules (Paredes et al. 2011). In other cases, there is an increase in the proportion of hydrophobic groups at the surface of the protein; this induces a destabilization of the molecular edifice through the reduction in the overall entropy of the system through the formation of clathrate-like structures around these groups. The stabilizing effect of salt bridges can be altered through a modification of the orientation of ionized groups and of their reduction in number (Papaleo et al. 2007). The presence of ionized groups carrying similar charge, in general negative, can also modify the stability through repulsion and by increasing the interaction with the solvent (Narinx et al. 1997; Feller et al. 1999; Adekoya et al. 2006). In cold-active uracil-DNA N-glycosylase, however, a more positively charged surface near the active site seems to be important for the adaptation to cold (Moe et al. 2004). In citrate synthases, psychrophilic, mesophilic and thermophilic enzymes have increasingly stronger electrostatic stabilization of the transition state (Bjelic et al. 2008). Some cold-active enzymes are also poorer in arginine residues and display a lower Arg/Arg + Lys ratio (Adekoya et al. 2006). This in relation with the fact that arginine can develop more electrostatic interactions with surrounding amino acids than lysine. α-helices can be assimilated to macroscopic electrostatic dipoles that carry a net positive charge at their N-terminal end and a net negative charge at their C-terminal end (Serrano and Fresht 1989). These secondary structures can be stabilized by various electrostatic interactions in their vicinity that can be weakened in psychrophilic enzymes such as in cold-active trypsins (Leiros et al. 2000). Also the hydrogen bonding network is often weakened in psychrophilic enzymes and found responsible for the increase in flexibility of these enzymes (Xie et al. 2009). The structure of loops connecting secondary structures seems also important in conferring the appropriate flexibility to some cold-active enzymes; their length can be increased, their level of interactions with the internal moiety can be lowered, and their flexibility can be enhanced thanks to the deletion of proline residues (Feller et al. 1997b; Gudmundsdottir 2002; Matsuura et al. 2002). Metal binding can also be involved through the reduction in binding affinity or deletion of site (Narinx et al. 1997; Almog et al. 2003). The deletion of disulphide bridges can also improve the flexibility and activity of cold-active

enzymes; this has been demonstrated in cold-active α-amylase and alkaline phosphatase (D'Amico et al. 2003; Asgeirsson et al. 2007; Papaleo et al. 2007).

Following the comparison of the three-dimensional structures of psychrophilic and mesophilic proteins, mutation experiments were carried out in order either to transform psychrophilic enzymes into mesophilic counterparts or to try to force mesophilic enzymes to adopt psychrophilic characteristics.

In a first attempt, random mutagenesis was applied to mesophilic subtilisin BPN' (Taguchi et al. 1998) and a mutant *m63* carrying three mutations, V72I, A92T, G131D, was obtained. It displayed, at 10 °C, an activity, towards the synthetic small-size substrate s-AAPF para nitroanilide, identical to that of the wild-type mesophilic enzyme; only the K_m was twice lower than that of subtilisin BPN'. The activity was not tested on large-size substrates and its thermal stability was slightly lower than that of the original enzyme. More success was obtained later on with the multiple mutant *m 51* also carrying three mutations, A31T, A88 V and A98T (Taguchi et al. 1999). Its specific activity at 10 °C was 1.5 higher than that of subtilisin BPN'; also towards s-AAPF-pNA, the K_m was slightly lower than that of the original enzyme and the thermostability was apparently unchanged. Another cold-active subtilisin (S41) from Antarctic *Bacillus* sp. (Davail et al. 1994) was submitted to random mutagenesis followed by in vitro recombination. Mutant libraries were screened to identify enzymes that displayed greater thermal stability without sacrificing low-temperature activity. After several generations and recombinations, a variant, 3-2G7, carrying 7 mutations was obtained. The specific activity towards a small-size synthetic substrate, s-AAPF-*p*Na, displayed a threefold increase at all temperatures tested with no modification of the K_m values. A higher thermostability was also observed (Miyazaki et al. 2000). This variant was further engineered to give the subtilisin mutant 8-4A9 carrying 13 amino acid substitutions distributed throughout the structure leading to an increase in the affinity for Ca^{2+}. The midpoint of unfolding was about 3 °C higher than the variant 3-2G7 with a specific activity at 10 °C slightly lower than that of 3-2G7 but nearly twice as high as that of the wild-type S41. The K_m fors-AAPF-*p*Na steadily decreased from S41 to 3-2G7 and 8-4A9 (Wintrode et al. 2001). These experiments tended to demonstrate that it was quite possible to increase the thermal stability of an enzyme while keeping its high catalytic activity at low temperature; in other words that the two properties were not necessarily inversely related. It is, however, worth noting that these data have been obtained with non-natural, synthetic and small-size substrates. If it seems possible to modify, by directed evolution, enzymes in such a way to display a higher thermostability associated with a higher specific activity at low temperature towards artificial substrate, the situation is probably much more complicated when natural large-size substrates are concerned. We have indeed learned that the protease mutant 3-2G7 in fact displays a much lower activity than the wild-type S41 cold-active enzymes when natural and large-size substrates such as proteins were used. That does not mean that in vitro it is not possible to increase the thermostability of a cold-active enzyme while preserving or even increasing its catalytic efficiency at low temperature. A cold-active subtilisin, S39, extremely similar to the S41 enzyme, was also submitted to

site-directed mutagenesis with the aim to mainly try to increase its stability (Narinx et al. 1997). Several mutations were introduced first to restore a salt bridge found in subtilisin BPN', a hydrophobic interaction found in subtilisin Carlsberg, a disulphide bridge present in aqualysine, a mutation N136S supposed to increase the specific activity towards large-size substrates and a mutation T85D to exchange a poor Ca^{2+} ligand for a strong one. Most of these mutations had a positive effect on the thermostability of the enzyme, especially the mutation T85D, involved in the Ca^{2+} coordination of calcium-binding site 1 that increased the half-life at 50 °C by more than 50 min. As expected, the affinity of this mutant for calcium was drastically improved. At 5 °C, the specific activity of this mutant towards the synthetic substrate s-FAAF-*p*Na was 4 times as high as that of the wild-type enzyme; the K_m value was not affected. The specific activity was, however, only slightly increased when azocasein was used as substrate. In another cold-active subtilisin-like proteinase, Ser-Ala and Xaa-Pro mutations were introduced to produce single, double and triple mutants. Most of these mutations, especially the Xaa-Pro exchanges, led to an increase in the thermostability of the variants with a concomitant decrease in the specific activities (Arnorsdottir et al. 2007). A thermophilic subtilase was also engineered in order to produce cold-active variants (Zhong et al. 2009). All successful single mutations were located within or near the active site and a variant carrying four amino acid substitutions showed a sixfold increase in specific activity towards casein in the temperature range of 15–25 °C, a decrease in thermal stability, and a shift of its apparent optimum towards low temperature by approximately 15 °C. Interestingly, this multiple mutant was less active towards the synthetic substrate s-AAPF-*p*NA due to a large increase in K_m and decrease in k_{cat}. This again underlines the importance, before drawing robust conclusions, of selecting appropriate substrates when studying multi-substrate enzymes by mutagenesis.

Directed evolution was also applied to a thermostable esterase from *B. subtilis* using p-nitrobenzyl acetate as substrate (Giver et al. 1998). After several generations, mutants of higher thermostability were obtained; they show a specific activity slightly higher, about 20 %, than that of the wild-type enzyme. In this case, the natural substrates are unfortunately unknown.

The molecular basis of the adaptation to cold of a psychrophilic α-amylase was also investigated by site-directed mutagenesis. Fourteen amino acid substitutions, including double mutations were introduced with the aim to mimic specific structural characteristics found in mesophilic and thermophilic homologous enzymes (D'Amico et al. 2001, 2002) under the form of hydrogen bonds, salt bridges, helix dipole stabilization, hydrophobic interactions and reinsertion of a disulphide bridge that connects domain A and B in mesophilic counterpart. The highest contribution to stability, in terms of both T_m and ΔH_{cal}, was obtained by the introduction of an electrostatic interaction; a double aromatic interaction increased the stabilization energy by a factor of two at 10 °C whereas the reinforcement of hydrophobic clusters within the hydrophobic core of the enzyme induced the production of multiple calorimetric domains similar to those existing in the mesophilic counterpart from pig pancreas. Most of the introduced mutations

aiming to increase the stability were efficient but led to a decrease in k_{cat} and K_m. Any attempts to further increase the catalytic efficiency at low temperature through a decrease in stability gave rise to unfolded forms suggesting that the cold-active α-amylase has reached a limit in stability precluding any improvement of the specific activity via a decrease in thermal stability. The incorporation of a disulphide bridge found in the mesophilic enzyme decreased the specific activity by a factor of two at 5 °C as well as the K_m and showed a microcalorimetric profile similar to that of the mesophilic counterpart with, however, a first T_m transition lower than that of the mesophilic α-amylase. This indicates that the introduction, in the cold-active enzyme, of this disulphide bridge leads to a stabilization of some parts of the cold-adapted protein but also induces an unfavourable structural constraint in another part of this protein. Multiple mutants of this cold-active enzyme were also recently produced to force the cold-active enzyme to display mesophilic characteristics (Cipolla et al. 2012); the specific activity of the variants was lowered whereas the thermal stability was drastically improved with a concomitant decrease in the flexibility of the mutants. It was concluded that these mutants can be considered as structural intermediates between their parents psychrophilic and mesophilic enzymes. The effects of these mutations, single and multiple, on the molecular dynamics of the protein, were recently investigated (Papaleo et al. 2011), and it was shown that the above-mentioned mutants displayed a reduced flexibility in various regions, not only near the active-site and substrate-binding groove, but also at long distance such as in domain C that did not carry any mutation.

Several other cold-active enzymes have been the target of site-directed mutagenesis in order to shed some light on the residues involved in the adaptation. One can mention the work on a cold-active lipase in which the substitutions of three polar residues in the lid region by amino acid residues conserved in homologous mesophilic lipases gave rise to an increase in stability and a modification of the substrate specificity with a preference for C8 rather than C4 fatty acid chains. A substitution, Ser-Gly, increased the specificity for C12 fatty acid chain associated with a decrease in stability this probably in relation with a better accessibility of the active site and improvement of the flexibility in this region (Santarossa et al. 2005).

An interesting study on the adaptation to cold of an alkaline phosphatase from an Antarctic bacterium was carried out by directed evolution to identify amino acid residues involved in the adaptation and stability of this protein (Koutsioulis et al. 2008). Three thermostable and six thermolabile variants were obtained. All the variants showing an increase in stability were also characterized by an increase in the T_m of the first calorimetric transition and persistence, as in the case of wild type, of secondary transitions at higher temperatures; their specific activity at 15 °C was also lower than that of the parent enzyme and this was associated, as expected, with a higher activation energy. By contrast, the two variants, H135E and double mutant H135E/G149D, displaying an activity about 1.5 higher and a much higher K_m than that of the wild-type enzyme had a nearly symmetrical microcalorimetric profile with only one detectable transition shifted towards

higher temperature by about 6 °C when compared to the first transition of the parent enzyme. This latter, however, displayed a calorimetric domain with a T_m about 7 °C higher than those of both variants. It has been suggested that the mutation H135E distorts the Mg^{2+} binding site, decreases the affinity for the metal and increases the flexibility of the site presumably through the destabilization of the domain showing the highest thermostability in the wild-type enzyme. In the double mutant, the mutation G149D apparently improves the mobility of residue R148 located in a loop that facilitates the phosphate/substrate coordination during catalysis. Also, after incubation at 60 °C, these two more active variants were also more rapidly inactivated than the wild-type enzyme suggesting an increase in flexibility of the active site.

In a cold-active isocitrate lyase, the replacement of Ala 214 by a Ser residue found in some mesophilic enzyme and close to the Tim barrel involved in substrate binding induced a decrease in the catalytic activity associated with an increase in the thermostability of the mutant presumably due to the two hydrogen bonds formed between Ser and Gln119. It was concluded that Ala 214 was implicated in cold adaptation by providing more flexibility to the active site (Sato et al. 2008).

These experiments of mutagenesis indicate that, in most cases, the high specific activity of cold-active enzymes is related to an improvement of the flexibility of crucial parts of the molecular structure that induces a high thermal sensitivity of the active site and a generally better accessibility for the substrate at low temperatures. The attempts to try to simultaneously increase the thermostability and activity at low temperature have been hardly successful. Directed evolution has produced more stable and more active variants but essentially with multi-substrate enzymes and small-size and artificial substrates, and it remains to be demonstrated that the mutants are also active on large-size substrates at low temperature. One mutated cold-active subtilisin, carrying only one mutation was, however, found more stable and more active on a large-size substrate, azocasein (Narinx et al. 1997), and interestingly, two mutants of a cold-active alkaline phosphatase were found more active than the wild-type enzyme and showed a symmetrical calorimetric profile synonymous of a high cooperativity of unfolding. Although the first transition of the wild-type enzyme had a T_m lower than that of the mutants, these variants had lost the most thermostable calorimetric domain and appear to be more rapidly inactivated than the wild-type alkaline phosphatase. This is correlated to the fact that many cold-active enzymes also show a high cooperativity on unfolding whereas heat inactivation often precedes any significant changes in the three-dimensional structure.

15.6 Conclusions

Psychrophilic organisms living in permanently cold habitats have developed numerous adaptations that allowed them to successfully thrive in low-temperature environments. Their enzymes are a key feature of this adaptation. They are much

more active than their mesophilic counterparts at low and moderate temperatures and their high specific activity is undoubtedly due to an improvement of the flexibility of the active site or/and of other regions of the structure indirectly involved. This is induced by rather discrete structural modifications which can be located at long distance from the active site and which generally lead to a higher thermal instability of these enzymes, often associated with an even faster heat inactivation. If the general strategy adopted by nature consists in a weakening of the intramolecular forces that stabilize the structure of their mesophilic and *a fortiori* thermophilic counterparts, this strategy appears to be specific to each enzyme; depends on the position of the enzyme within a metabolic pathway; on its localization, intra-or extra-cellular; on its structural modification capability; on the environment of the organism and on its evolution history. As a consequence, there is a continuum in the adaptation to low temperatures; some cold-active enzymes indeed display high specific activity associated with low T_m while others show rather high T_m and stronger thermal dependence like their mesophilic counterparts.

References

Adekoya OA, Helland R, Willassen NP, Sylte I (2006) Comparative sequence and structure analysis reveal features of cold adaptation of an enzyme in the thermolysin family. Proteins 62:435–439

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

Aghajari N, Roth M, Haser R (2002) Crystallographic evidence of a transglycosylation reaction: ternary complexes of a psychrophilic α-amylase. Biochemistry 41:4273–4280

Aghajari N, Van petegem F, Villeret V, Chessa J-P, 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

Almog O, Gonzales A, Klein D, Greenblatt HM, Braun S, Shoham G (2003) The 0.93Å crystal structure of spericase: a calcium-loaded serine protease from *Bacillus sphaericus*. J Mol Biol 332:1071–1082

Altermark B, Niiranen L, Willassen NP, Smalas AO, Moe E (2007) Comparative studies of endonuclease I from cold-adapted *Vibrio salmonicida* and mesophilic *Vibrio cholerae*. FEBS J 274:252–263

Arnorsdottir J, Helgadottir S, Thorbjarnardottir SH, Eggertsson G, Kristjansson MM (2007) Effect of selected Ser/Ala and Xaa/Pro mutations on the stability and catalytic properties of a cold adapted subtilisin-like serine proteinase. Biochim Biophys Acta 1774:749–755

Asgeirsson B, Adalbjörsson BV, Gylfason GA (2007) Engineered disulfide bonds increase active-site local stability and reduce catalytic activity of a cold-adapted alkaline phosphatase. Biochim Biophys Acta 1774:679–687

Birgisson H, Delgado O, Garcia-Arroyo L, Hatti-Kaul R, Mattiasson B (2003) Cold-adapted yeasts as producers of cold-active polygalacturonases. Extremophiles 7:185–193

Bjelic S, Bransdal BO, Aqvist J (2008) Cold adaptation of enzyme reaction rates. Biochemistry 47:10049–10057

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

Chiappori F, Pucciarelli S, Merelli I, ballarini P, Miceli C, Milanesi L (2012) Structural thermal adaptation of β-tubulins from the Antarctic psychrophilic protozoan *Euplotes focardii*. Proteins 80:1154–1166

Chiuri R, Majorano G, Rizello A, del Mercato LL, Cingolani R, Rinaldi R, Maffia M, Pompa PP (2009) Exploring local flexibility/rigidity in psychrophilic and mesophilic carbonic anhydrases. Biophys J 96:1586–1596

Cipolla A, Delbrassine F, da Lage JL, Feller G (2012) Temperature adaptations in psychrophilic, mesophilic and thermophilic chloride-dependent alpha-amylases. Biochimie 94:1943–1950

Collins T, Meuwis M-A, Gerday C, Feller G (2003) Activity, stability and flexibility in glycosidases adapted to extreme thermal environments. J Mol Biol 338:419–428

Collins T, D'Amico S, Marx J-C, Feller G, Gerday C (2007) I cold-adapted enzymes. In: Gerday C, Glansdorff N (eds) Physiology and biochemistry of extremophiles. ASM press, Washington DC, pp 165–179

Collins T, Roulling F, Piette F, Marx J-C, Feller G, Gerday C, D'Amico S (2008) Fundamentals of cold-adapted enzymes. In: Margesin R, Schinner F, Marx J-C, Gerday C (eds) Psychrophiles from biodiversity to biotechnology. Springer, Berlin, pp 211–227

Coquelle N, Fioravanti E, Weik M, Vellieux F, Madern D (2007) Activity, stability and structural studies of lactate dehydrogenases adapted to extreme thermal environments. J Mol Biol 374:547–562

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, Gerday C, Feller G (2002) Dual effects of an extra disulfide bond on the activity and stability of a cold-adapted α-amylase. J Biol Chem 48:46110–46115

D'Amico S, Marx J-C, Gerday C, Feller G (2003) Activity-stability relationship in extremophilic enzymes. J Biol Chem 278:7891–7896

D'Amico S, Sohier J-S, Feller G (2006) Kinetics and energetics of ligand binding determined by microcalorimetry: insight 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* TA 41. J Biol Chem 269:17448–17453

Demchenko AP, Rusyn OI, Saburova EA (1989) Kinetics of the lactate dehydrogenase reaction in high-viscosity media. Biochim Biophys Acta 998:196–203

Dias CL, Ala-Nissila T, Wong-E kkabut J, Vattulainen I, Grant M, Karttunen M (2010) The hydrophobic effect and its role in cold denaturation. Cryobiology 60:91–99

Eyring H (1935) The activated complex in chemical reactions. J Chem Phys 3:107–115

Fedoy A-E, Yang N, Martinez A, Leiros H-K, Stee H (2007) Structural and functional properties of isocitrate dehydrogenase from the psychrophilic bacterium *Desulfotalea psychrophila* reveal a cold-active enzyme with an unusual high thermal stability. J Mol Biol 372:130–149

Feller G (2003) Molecular adaptations to cold in psychrophilic enzymes. Cell Mol Life Sci 60:648–662

Feller G (2010) Protein stability and enzyme activity at extreme biological temperatures. J Phys Condens 22:32101–32118

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

Feller G, Gerday C (2003) Psychrophilic enzymes: hot topics in cold adaptation. Nature Rev Microbiol 1:200–208

Feller G, Narinx E, Arpigny JL, Zekhnini Z, Swings J, Gerday C (1994) Temperature dependence of growth, enzyme secretion and activity of psychrophilic Antarctic bacteria. Appl Microbiol Biotechnol 41:477–479

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

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

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

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

Garsoux G, Lamotte-Brasseur J, Gerday C, Feller G (2004) Kinetic and structural optimisation to catalysis at low temperatures in a psychrophilic cellulase from the Antarctic bacterium *Pseudoalteromonas haloplanktis*. Biochem J 384:247–253

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

Georlette D, Damien B, Blaise V, Depiereux E, Uversky VN, Gerday C, Feller G (2003) Structural and functional adaptations to extreme temperatures i 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 J-C, Sonan G, Feller G, Gerday C (2004) Some like it cold: biocatalysis at low temperatures. FEMS Microbiol Rev 28:25–42

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

Giver L, Gershenson A, Freskgard P-A, Arnold FH (1998) Directed evolution of a thermostable esterase. Proc Natl Acad Sci USA 95(4):12809–12813

Gorfe AA, Bransdal BO, Leiros HK, Helland R, Smalas AO (2000) Electrostatics of mesophilic and psychrophilic trypsin isoenzymes: qualitative evaluation of electrostatic differences at the substrate binding site. Proteins 40:207–217

Gudmundsdottir A (2002) Cold-adapted and mesophilic brachyurins. Biol Chem 383:1125–1131

Heidarsson PO, Sigurdsson ST, Asgeirsson B (2009) Structural features and dynamics of a cold-adapted alkaline phosphatase studied by EPR spectroscopy. FEBS J 276:2725–2735

Hess E (1934) Effects of low temperatures on the growth of marine bacteria. Contribs Can Biol Fisheries Ser C 8:491–505

Hochachka PW, Somero GN (1973) Strategies of biochemical adaptation. WB Saunders, Philadelphia

Hochachka PW, Somero GN (2002) Biochemical adaptation. Oxford University Press, New York

Homchaudhuri L, Sarma N, Swaminathan R (2006) Effect of crowding by dextrans and ficols on the rate of alkaline phosphatase-catalysed hydrolysis: a size-dependent investigation. Biopolymers 83:477–486

Hoyoux A, Blaise V, Collins T, D'Amico S, Gratia E, Huston AL, Marx J-C, Sonan G, Zeng Y, Feller G, Gerday C (2004) Extreme catalysts from low-temperature environments. J Biosci Bioeng 98:317–330

Huston AL, Haeggström JZ, Feller G (2008) Cold adaptation of enzymes: structural, kinetic and microcalorimetric characterizations of an aminopeptidase from the Arctic psychrophile *Colwellia psychrerythraea* and of human leukotriene A (4) hydrolase. Biochim Biophys Acta 1784:1865–1872

Ingraham JL, Stokes JL (1959) Psychrophilic bacteria. Bacteriol Rev 23:97–108

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

Kobori H, Sullivan CW, Shizuya H (1984) Heat-labile alkaline phosphatase from Antarctic bacteria: rapid 5′ end-labelling of nucleic acid. Proc Natl Acad Sci USA 81:6691–6695

Koutsiolis D, Wang E, Tzanodaskalaki M, Nikiforaki D, Deli A, Feller G, Heikinheimo P, Bouriotis V (2008) Directed evolution on the colddapted properties of TAB 5 alkaline phosphatase. Protein Eng 21:319–327

Kumar S, Tsai CJ, Nussinov R (2002) Maximal stabilities of reversible two-state proteins. Biochemistry 41:5359–5374

Leiros HK, Willassen NP, Smalas AO (2000) Structural comparisonof psychrophilic and mesophilic trypsins. Elucidating the molecular basis of cold-adaptation. Eur J Biochem 267:1039–1049

Leiros I, Moe E, Lanes O, Smalas AO, Willassen NP (2003) The structure of uracil-DNA glycosylase from Atlantic cod (*Gadus mohua*) reveals cold-adaptation features. Acta Crystallogr D Biol Crystallogr 59:1357–1365

Lonhienne T, Baise E, Feller G, Bouriotis V, Gerday C (2001a) Enzyme activity determination on macromolecular substrates by isothermal calorimetry: application to mesophilic and psychrophilic chitinases. Biochim Biophys Acta 1545:349–356

Lonhienne T, Zoidakis J, Vorgias E, Feller G, Gerday C, Bouriotis V (2001b) Modular structure, local flexibility and cold-activity of a novel chitobiase from a psychrophilic Antarctic bacterium. J Mol Biol 310:291–297

Margesin R (2009) Effect of temperature on growth parameters of psychrophilic bacteria and yeasts. Extremophiles 13:257–262

Marshall CJ (1997) Cold-adapted enzymes. TIBTECH 15:359–363

Marx J-C, Collins T, D'Amico S, Feller G, Gerday C (2007) Cold-adapted enzymes from marine Antarctic microorganisms. Mar Biotechnol 9:293–304

Matsuura A, Yao M, Aizawa T, Koganesawa N, Masaki K, Miyazawa M, Demura M, Tanaka I, Kawano K, Nitta K (2002) Structural analysis of an insect lysozyme exhibiting catalytic efficiency at low temperature. Biochemistry 41:12086–12092

Merlino A, Russo Kraus I, Castellano I, De venditis E, Rossi B, Conte M, Vergara A, Sica F (2010) Structure and flexibility in cold-adapted iron superoxide dismutases: the case of the enzyme isolated from Pseudoalteromonas haloplanktis. J Struct Biol 172:343–352

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

Moe E, Leiros I, Rijse EK, Olufsen M, Lanes O, Smalas A, Willassen NP (2004) Optimisation of the surface electrostatics as a strategy for cold adaptation of uracil-DNA N-glycosylase. J Mol Biol 343:1221–1230

Narinx E, Baise E, Gerday C (1997) Subtilisin from antarctic bacteria: characterisation and site-directed mutagenesis of residues possibly involved in the adaptation to cold. Prot Engineer 10:1271–1279

Pace CN, Laurents DV (1989) A new method for determining the heat capacity change for protein folding. Biochemistry 28:2520–2525

Papaleo E, Olufsen M, de Gioia L, Bransdal BO (2007) Optimization of electrostatics as a strategy for cold-adaptation: a case study of cold- and warm-active elastases. J Mol Graph Model 26:93–103

Papaleo E, Pasi M, Tiberti M, de Gioia L (2011) Molecular dynamics of mesophilic-like mutants of a cold-adapted enzyme: insight into distal effects induced by the mutations. PLoS ONE 6:e24214

Paredes DI, Watters K, Pitman DJ, Bystrff C, Dordick JS (2011) Comparative void-volume analysis of psychrophilic and mesophilic enzymes: Structural bioinformatics of psychrophilic enzymes reveals sources of core flexibility. BMC Struct Biol 11:42

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

Radestock S, Gohlke H (2011) Protein rigidity and thermophilic adaptation. Proteins 79: 1089–1108

Ramli AN, Mahadi NM, Shamsir MS, Rabu A, Joyce-Tan KH, Murad AM, Illias RM (2012) Structural prediction of a novel chitinase from the psychrophilic *Glaciozyma antarctica* PI12 and an analysis of its structural properties and function. J Comput Aided Mol Des 26:947–961

Roman EA, Faraj SE, Cousido-Siah A, Mitscler A, Podjarny A, Santos J (2013) Frataxin from *Psychromonas ingrahmii* as a model to study stability modulation within the CYaY protein family. Biochim Biophys Acta 1834:1168–1180

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

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

Santarossa G, Gatti-Lafranconi P, Alquati C, DeGioia L, Alberghina L, Fantucci P, Lotti M (2005) Mutations in the «lid» region affect chain length specificity and thermostability of a *Pseudomonas fragi* lipase. FEBS Lett 579:2383–2386

Sato Y, Watanabe S, Yamaoka N, Takada Y (2008) Gene cloning of cold-adapted isocitrate lyase from a psychrophilic bacterium, *Colwellia psychrerythraea*, and analysis of amino acid residues involved in cold adaptation of this enzyme. Extremophiles 12:107–117

Schmidt-Nielsen S (1902) Ueber einige psychrophile Mikrooganismen und ihr Vorkommen. Centr Bakteriol Parasitenk Abt II 9:145–147

Serrano L, Fersht AR (1989) Capping and alpha-helix stability. Nature 342:296–299

Siddiqui KS, Cavicchioli R (2006) Cold-adapted enzymes. Ann Rev Biochem 75:403–433

Smalas AO, Leiros HK, OS V, Willassen NP (2000) Cold-adapted enzymes. Biotechnol Annu Rev 6:1–57

Somero GN (1977) Temperature as a selective factor in protein evolution: the adaptational strategy of compromise. J Exp Zool 194:175–188

Somero GN, Low PS (1976) Temperature: a «shaping force» in protein evolution. Biochem Soc Symp 41:33–42

Sonan GK, Receveur-Brechot V, Duez C, Aghajari N, Czjzek M, Haser R, Gerday C (2007) The linker region plays a key role in the adaptation to cold of the cellulase from an Antarctic bacterium. Biochem J 407:293–302

Sotelo-Mundo RR, Lopez-Zavala AA, garcia-Orozco KD, Arvizu AA, Velazquez-Contreras EF, Valenzuela-Soto EM, Rojo-Dominguez A, Kanost MR (2007) The lysozyme from insect (manduca sexta) is a cold-adapted enzyme. Protein Pept Lett 14:774–778

Spiwok V, Lipovova P, Skalova T, Duskova J, Dohnalek J, Hasek J, Russell N, Kralova B (2007) Cold-active enzymes studied by comparative molecular dynamics simulation. J Mod Model 13:485–497

Stadler AM, Garvey CJ, Bocahut A, Sacquin-Mora S, Digel I, Schneider GJ, Natali F, Artmann GM, Zaccai G (2012) Thermal fluctuations of haemoglobin from different species: adaptation to temperature via conformational dynamics. J R Soc Interface 7:2845–2855

Sun-Yong K, Kwang-Yeon H, Sung-Hou K, Ha-Chin S, Ye-Sun H, Yunge C (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

Suzuki T, Yamamoto K, Tada H, Uda K (2012) Cold-adapted features of arginine kinase from the deep-sea *Calyptogena kaikoi*. Mar Biotechnol 14:294–303

Svingor A, Kardos J, Hajdju 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

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

Taguchi S, Ozaki A, Nonaka T, Momose H (1999) A cold-adapted protease engineered by experimental evolution system. J Biochem 126:689–693

Talla-Singh D, Stites WE (2008) Refinement of noncalorimetric determination of the change in heat capacity, ΔC_p, of protein unfolding and validation across a wide temperature range. Proteins 71:1607–1616

Tiberti M, Papaleo E (2011) Dynamic properties of extremophilic subtilisin-like serine-proteases. J Struct Biol 174:69–83

Tindbaek N, Svendsen A, Oestezrgaard PR, Draborg H (2004) Engineering a substrate-specific cold-adapted subtilisin. Protei Eng Des Sel 17:149–156

Tsigos I, Velonia K, Smonou I, Bouriotis V (1998) Purification and characterization of an alcohol dehydrogenase from the Antarctic psychrophile *Moraxella* sp. TAE123. Eur J Biochem 254:356–362

Vieille C, Zeikus G (2001) Hyperthermophilic enzymes: sources, uses and molecular mechanisms for thermostability. Microbiol Mol Biol Rev 65:1–43

Watanabe S, Yasutake Y, Tanaka I, Takada Y (2005) Elucidation of stability determinants of cold-adapted monomeric isocitrate dehydrogenase from a psychrophilic bacterium, *Colwellia maris*, by construction of chimeric enzymes. Microbiology 151:1083–1094

Wintrode PL, Miyazaki K, Arnold FH (2001) Patterns of adaptation in a laboratory evolved thermophilic enzyme. Biochim Biophys Acta 1549:1–8

Wolf-Watz M, Thai V, Henzler-Wildman K, Hadjipavlou G, Eisemesser EZ, Kern D (2004) Linkage between dynamics and catalysis in a thermophilic-mesophilic enzyme pair. Nat Struct Mol Biol 11:945–949

Xie BB, Bian F, Chen XL, He HL, Guo J, Gao X, Zeng YX, Chen B, Zhou BC, Zhang YZ (2009) Cold adaptation of zinc metalloprotease in the thermolysin family from deep sea and arctic sea ice bacteria revealed by catalytic and structural properties and molecular dynamics: new insights into relationship between conformational flexibility and hydrogen bonding. J Biol Chem 284:9257–9269

Xu Y, Feller G, Gerday C, Glansdorff N (2003) Metabolic enzymes from psychrophilic bacteria: challenge of adaptation to low temperatures in ornithine carbamoyltransferase from *Moritella abyssi*. J Bacteriol 185:2161–2168

Zhong CQ, Song S, Fang N, Liang X, Zhu H, Tang XF, Tang B (2009) Improvement of low-temperature caseinolytic activity of a thermophilic subtilase by directed evolution and site directed mutagenesis. Biotechnol Bioeng 104:862–870