Extremophiles (2007) 11:211–216
DOI 10.1007/s00792-006-0050-1

REVIEW

Life at low temperatures: is disorder the driving force?

Georges Feller

Received: 17 August 2006 / Accepted: 13 November 2006 / Published online: 8 December 2006

Abstract The thermodynamic characterization of various biological systems from psychrophiles points to a larger entropic contribution when compared to the corresponding mesophilic or (hyper) thermophilic counterparts, either at the level of the macromolecules (thermodynamic and kinetic stabilities) or of their function (ligand binding, catalytic activity). It is suggested here that in an environment characterized by a low heat content (enthalpy) and at temperatures that strongly slowdown molecular motions, the cold-adapted biological systems rely on a larger disorder to maintain macromolecular dynamics and function. Such pre-eminent involvement of entropy is observed in the experimental results and, from a macroscopic point of view, is also reflected for instance by the steric hindrances introduced by *cis*-unsaturated and branched lipids to maintain membrane fluidity, by the loose conformation of psychrophilic proteins or by the local destabilization of tRNA by dihydrouridine in psychrophilic bacteria.

Keywords Psychrophiles · Proteins · Stability · Ligand binding · Entropy

Communicated by D. A. Cowan.

G. Feller (✉)
Laboratory of Biochemistry, University of Liège,
Institute of Chemistry B6a, 4000 Liège-Sart,
Tilman, Belgium
e-mail: gfeller@ulg.ac.be

Introduction

Although the title may appear provocative, there is in fact, an array of recent evidence that suggests that more attention should be paid to the entropic contribution in biological systems governing the development of organisms at low temperatures. Researchers were previously reluctant, and rightly so, to overinterpret entropic data for two main reasons. The first is the fact that entropy is not an experimental parameter. Referring to the classical Gibbs–Helmholtz equation

$$\Delta G = \Delta H - T\Delta S, \tag{1}$$

one should note that free energy values ΔG can be calculated from equilibrium constants for instance, whereas the enthalpic contribution ΔH is obtained from the co-operativeness of a system or more directly from microcalorimetric measurements. By contrast, the entropic term $T\Delta S$ is derived from the knowledge of both ΔG and ΔH, and therefore, reliable estimates of entropy strongly depend on the accuracy of the experimental parameters. The second main reason to look at entropic data with caution is the fact that a macroscopic interpretation is frequently elusive, if not illusive. If indeed the entropic variation reflects the variation of disorder of a system, accessory events such as the removal of water molecules from a binding site during ligand capture can strongly modify the magnitude of the entropic term and even change its sign therefore impairing interpretations at the molecular level. Nevertheless, the convergence of recent results suggests a central role of entropy and possibly opens new prospects for the study of macromolecules and supramolecular assemblies in temperature adaptation.

All data were gained from proteins or enzymes and, although similar data are currently lacking for other macromolecules, the essential function of enzymes in the cell cycle makes them a paradigm for similar future approaches.

Thermodynamic stability of proteins

The thermodynamic stability of a protein that unfolds reversibly according to a two-state mechanism $N \rightleftharpoons U$ is described by its stability curve (Becktel and Schellman 1987), i.e. the free energy of unfolding as a function of temperature (Fig. 1). In other words, this is the work required to disrupt the native state at any given temperature (Privalov 1979) and is also referred to as the conformational stability. By definition, this stability is nil at the melting point (equilibrium constant $K = [U]/[N] = 1$ and $\Delta G = -RT\ln K$). At temperatures below the melting point, the stability increases, as expected, but perhaps surprisingly for the non-specialist, the stability reaches a maximum close to room temperature, then it decreases at lower temperatures (Fig. 1). In fact, this function predicts a temperature of cold-unfolding, which is generally not observed because it occurs below 0°C. Nevertheless, cold-unfolding has been well demonstrated under specific conditions (Privalov 1990). Increasing the stability of a protein is essentially obtained by lifting the curve towards higher free energy values, also resulting in an increased melting point (Kumar et al. 2001, 2002, 2003; Kumar and Nussinov 2004). As far as extremophiles are concerned, one of the most puzzling observations of the last decade is that most proteins obey this pattern, i.e. whatever the microbial source, either from deep vents or from polar ice, the maximal stability of their proteins is clustered around room temperature (for more details see Kumar and Nussinov 2004). Accordingly, the environmental temperatures for mesophiles and (hyper)thermophiles lie on the right limb of the bell-shaped stability curve, and obviously, the thermal dissipative force is used to promote molecular motions in these molecules. By contrast, the environmental temperatures for psychrophiles lie on the left limb of the stability curve. It follows that molecular motions in proteins at low temperatures are gained from the factors ultimately leading to cold-unfolding (Feller et al. 1999), i.e. the hydration of polar and non-polar groups (Makhatadze and Privalov 1995). This is far from anecdotal.

Indeed, a plot of the enthalpic and entropic contributions to the free energy of unfolding (Fig. 2) has the shape of two slightly curved functions that cross each other at both low- and high-temperature melting points (out of scale in Fig. 2). Interestingly, at temperatures below the maximal protein stability, in the environmental range of temperatures for psychrophiles, both the enthalpy and the entropy change their sign (Fig. 2). Referring to Eq. 1, it follows that in this region of the stability curve, the enthalpic term is a destabilizing factor, whereas the entropic contribution becomes the stabilizing factor of the native state. No macroscopic interpretation is needed to explain this surprising observation. We will simply conclude from these well-

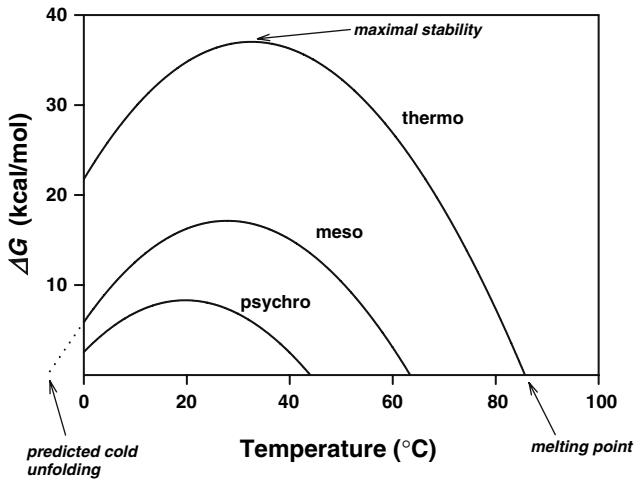

Fig. 1 Representative stability curves of homologous proteins from psychrophilic, mesophilic and thermophilic organisms (adapted from D'Amico et al. 2003)

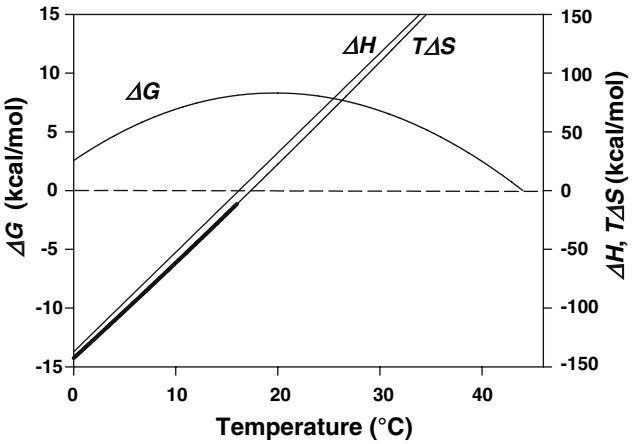

Fig. 2 Plot of the conformational stability ΔG for a psychrophilic enzyme, and of the enthalpic (ΔH) and entropic ($T\Delta S$) contributions. Note the different scales used: the stability of a protein is marginal as it is only a small difference between two large and opposite contributions. At temperatures below the maximal stability, entropy becomes the stabilizing contribution (*heavy line*) in the environmental thermal range of psychrophiles

established thermodynamic functions that at low temperatures, maintenance of the proper conformation of proteins from psychrophiles, and therefore, of their biological functions, is entropy driven.

Kinetic stability

The kinetic stability refers to studies based on the rate at which a macromolecule unfolds irreversibly according to the scheme $N \rightarrow U$. Such studies have been performed on various psychrophilic proteins (see Siddiqui and Cavicchioli 2006 for a review). Comparison of psychrophilic, mesophilic and (hyper)thermophilic proteins can be complex as they unfold in a narrow range of temperatures but separated by large temperature intervals (i.e. it is virtually impossible to record unfolding of an unstable psychrophilic protein at the unfolding temperature of a thermophilic protein). This has been overcome by comparing these extremozymes at the temperature at which they unfold at the same rate and to analyze the results in the formalism of the transition state theory (Collins et al. 2003; D'Amico et al. 2003; Georlette et al. 2003). Table 1 reports an example of the thermodynamic characteristics for the irreversible unfolding (loss of structure) and for inactivation (loss of activity). The free energy of activation $\Delta G^{\#}$ represents the energy barrier that has to be mastered before protein denaturation (unfolding is not spontaneous). The lower the $\Delta G^{\#}$ value, the lower the kinetic stability. Although the three extremozymes are compared at an identical denaturation rate, the lower $\Delta G^{\#}$ value for the psychrophilic protein arises from the low temperature at which this rate is reached. At this point, it is worth mentioning that the low kinetic stability of the psychrophilic protein is the result of the largest enthalpic and entropic contributions and, by contrast, the high kinetic stability of the thermophilic protein arises from much smaller contributions. The activation enthalpy $\Delta H^{\#}$ essentially reflects the temperature dependence of the system: the high value for the psychrophilic protein translates the fact that for a given increase of temperature, the increase of the denaturation rate will be much higher for the cold-adapted protein. Hence, assuming no entropic contribution in Eq. 1, the kinetic stability of the psychrophilic protein would be extremely high. This is not the case because of the large unfavorable entropic contribution and, interestingly, the same observation is made when considering a structural parameter (unfolding) or a functional parameter (inactivation). One can propose that when jumping the $\Delta G^{\#}$ barrier, the transition state of the psychrophilic protein is already very disordered before denaturation, whereas the transition state of the thermophilic protein resists the disorder before denaturation (D'Amico et al. 2003). But here again, no macroscopic interpretation is required to conclude that the low kinetic stability of psychrophilic proteins is entropy driven.

Ligand binding

The recent thermodynamic characterization of ligand binding to a psychrophilic protein (D'Amico et al. 2006a) is certainly the most demonstrative example of the involvement of entropy in a cold-adapted biological system for two reasons. First, the binding free energies were calculated from high-precision determination of the binding constants and the binding enthalpies were recorded experimentally by isothermal titration microcalorimetry, thereby ensuring reliable calculation of the binding entropies. Second, no assumption has to be made to interpret the thermodynamic parameters as they relate to a simple system at equilibrium between the free state and the bound state. This will be helpful to provide a macroscopic interpretation. The thermodynamic parameters of chloride (an allosteric effector) binding to psychrophilic and mesophilic α-amylases are shown in Table 2.

The first important observation is that, with the exception of the sign (binding is spontaneous), the three parameters follow exactly the same trend as in

Table 1 Thermodynamic parameters for the irreversible heat inactivation of activity and for irreversible unfolding in α-amylases (adapted from D'Amico et al. 2003)

	Inactivation			Unfolding		
	$\Delta G^{\#}$ (kcal mol^{-1})	$\Delta H^{\#}$ (kcal mol^{-1})	$T\Delta S^{\#}$ (kcal mol^{-1})	$\Delta G^{\#}$ (kcal mol^{-1})	$\Delta H^{\#}$ (kcal mol^{-1})	$T\Delta S^{\#}$ (kcal mol^{-1})
Psychrophile	20.5	172.3	151.8	20.2	109.7	89.2
Mesophile	21.5	153.0	131.5	21.5	84.7	63.2
Thermophile	22.9	58.6	35.7	22.9	74.2	51.3

Table 2 Standard thermodynamic parameters of chloride binding to psychrophilic and mesophilic α-amylases (D'Amico et al. 2006a)

	K_a (M^{-1})	$\Delta G°$ (kcal mol^{-1})	$\Delta H°$ (kcal mol^{-1})	$T\Delta S°$ (kcal mol^{-1})
Psychrophile	164	–3.02	–13.53	–10.51
Mesophile	3448	–4.82	–8.30	–3.48

Table 3 Activation parameters of the amylolytic reaction at 10°C (D'Amico et al. 2003)

	k_{cat} (s^{-1})	$\Delta G^{\#}$ (kcal mol^{-1})	$\Delta H^{\#}$ (kcal mol^{-1})	$T\Delta S^{\#}$ (kcal mol^{-1})
Psychrophile	294	13.8	8.3	–5.5
Mesophile	97	14.0	11.1	–2.9
Thermophile	14	15.0	16.8	1.8

Table 1. Indeed, the low affinity of the psychrophilic protein for its ligand (low $\Delta G°$) arises from the large and opposite (refer to Eq. 1) enthalpic and entropic contributions. Here also, in the absence of the entropic contribution, the affinity of the psychrophilic protein would be very large, but again the entropic term counteracts this effect. Accordingly, the low affinity of the psychrophilic protein for its ligand is also entropically driven. In this simple system at equilibrium, this strongly suggests a large reduction of the disorder between the relatively loose unbound protein and the more organized bound state. It has therefore been suggested that the large structural changes between the free and bound states of the psychrophilic protein may account for the higher number of enthalpy-driven interactions formed (larger negative $\Delta H°$) and the higher reduction of the apparent disorder (larger negative $\Delta S°$) when compared with its mesophilic homolog (D'Amico et al. 2006a).

Although similar thermodynamic parameters have not been recorded for other ligands, it should be noted that the low affinity of the psychrophilic α-amylase for all ligands investigated to date (the structural calcium ion, a large proteinaceous inhibitor and the macromolecular or synthetic substrates) suggests that entropy may be involved in this generally observed weak binding of ligands by cold-adapted proteins.

Activation parameters

Activation parameters, obtained from measurement of the activity temperature dependence, have been recorded for a large number of psychrophilic enzymes. The significance of these parameters and their implications for cold adaptation have been extensively discussed (Fields and Somero 1998; Lonhienne et al. 2000; Feller and Gerday 2003a, b; Siddiqui and Cavicchioli 2006) and do not need to be presented here in detail. A typical example is given in Table 3. In the formalism of the transition-state theory of enzyme activity, the free energy of activation $\Delta G^{\#}$ represents the energy barrier between the ground state [the enzyme–substrate complex (ES)] and the activated state $ES^{\#}$, the latter eventually breaking down into the enzyme and the product. Therefore, the lower this energy barrier, the higher the activity, as illustrated in Table 3. Here also, the enthalpy of activation $\Delta H^{\#}$ basically depicts the temperature dependence of the activity and the low value found for almost all psychrophilic enzymes highlights one of the main strategies used by these enzymes to maintain a high activity in the cold. Indeed, by keeping a low temperature dependence of the activity, their reaction rate is less reduced than for other enzymes when the temperature is lowered. Table 3 also shows that the entropic contribution for the cold-active enzyme is larger and negative. This has been interpreted as a large reduction of the apparent disorder between the ground state, presumably keeping a relatively loose conformation, and the well-organized and compact transition state (Lonhienne et al. 2000). It should be noted that this assumption has received strong experimental support by using microcalorimetry to compare the stabilities of free extremophilic enzymes with the same enzymes trapped in the transition state conformation by a non-hydrolyzable substrate analog (D'Amico et al. 2003). The larger increase in stability for the psychrophilic enzyme in the transition state conformation demonstrated larger conformational changes between the free and bound states when compared to mesophilic and thermophilic homologs. It is worth mentioning that the same conclusion has been reached for ligand binding to a psychrophilic protein (see above) using a different experimental approach. Accordingly, these large structural changes seem to be the macroscopic origin of the entropic penalty in psychrophilic proteins.

Conclusions

Considering the various results on psychrophilic proteins summarized above, it seems that the weak structural stability, the weak active site stability and the weak ligand or substrate binding are linked by a common molecular and thermodynamic factor, the latter involving a substantial entropic contribution. Leaving the experimental data aside, we can possibly rationalize,

at least intuitively, this pre-eminent involvement of the disorder in cold-adapted biological systems. The immediate consequences of the low temperatures characterizing cold environments are a low heat-content (enthalpy) and a reduction in the amplitudes and frequencies of atomic motions as well as of molecular motions (Tehei et al. 2004). However, macromolecules require an appropriate conformation dynamic to perform their biological functions: to counteract the reduction of atomic motions by low temperatures, the cold-adapted biological systems seem to rely on an improved intrinsic disorder (in a broad sense) to maintain this optimal conformation dynamic.

Two well-established adaptations of psychrophiles illustrate this relationship between an improved disorder and maintenance of the biological function at low temperatures. Psychrophilic microorganisms modify the lipid composition of their membranes to maintain the proper fluidity of the lipid bilayer. This involves the introduction of steric constraints, by reducing the packing order of acyl chains in the membrane. Such steric constraints destabilize the membrane and reduce lipid viscosity. This is achieved for instance by synthesizing lipids with *cis*-unsaturated double bonds that induce a kink in the acyl chain, or by incorporation of branched lipids or of short fatty-acyl chains that reduce the contacts between adjacent chains and perturb the organization of the membrane bilayer (Russell 1997, 2003; Russell and Hamamoto 1998; Margesin et al. 2002). These adaptations can be regarded as the maintenance of membrane function based on the disorder introduced into the lipid bilayer. As a second example, the adjustment of the conformational flexibility of proteins at the environmental temperatures is a well-documented adaptation of extremophiles (Zavodszky et al. 1998; Kohen et al. 1999; Tehei et al. 2004; Shlyk-Kerner et al. 2006). In the case of cold-adapted proteins, this is achieved by reducing the number or strength of all known weak interactions stabilizing the folded and biologically active conformation. Furthermore, some psychrophilic proteins reduce the hydrophobicity of the core clusters or expose a larger hydrophobic surface to the solvent: both induce an entropy-driven destabilization by weakening the hydrophobic effect on folding (Smalås et al. 2000; Feller and Gerday 2003a, b; Siddiqui and Cavicchioli 2006; D'Amico et al. 2006b). These properties result in a loose structure characterized by a low conformational stability and a marked heat-lability of the activity in enzyme catalysts. It has been also suggested that some psychrophilic proteins cannot be less stable than this loose state reached during natural evolution, therefore imposing a lower limit to protein adaptation to the cold (D'Amico et al. 2001). Here again, the maintenance of the protein function can be regarded as a consequence of the improved disorder introduced into the cold-adapted polypeptides by the absence of specific stabilizing weak interactions. Finally, a third example concerning nucleic acids also fits this view. Unprecedented high levels of posttranscriptional modification of tRNA by dihydrouridine have been reported in three psychrophilic bacteria (Dalluge et al. 1997). The specific chemical properties of dihydrouridine perturb the stacking that usually stabilizes RNA molecules, and it has been proposed that dihydrouridine confer local structural flexibility to RNA molecules. In other words, the disorder introduced by this posttranscriptional modification seems to contribute to the maintenance of the tRNA function at low temperatures. Furthermore, the prevalence of A:U base-pairing in rRNA of psychrophilic prokaryotes, that is less stable than the triple-hydrogen-bonded G:C base pair, can also be regarded as the involvement of the disorder in the function of the folded ribosome in cold-adapted microorganisms (Galtier and Lobry 1997; Khachane et al. 2005).

The specialist reader may have the impression that we have substituted here the terms ''flexibility'' or ''mobility'', frequently used when referring to cold adaptation, by a new word ''disorder''. This is only partly true. First, this disorder can affect a system without the involvement of ''flexible'' macromolecules. In the case of psychrophilic membranes, the lipids creating steric hindrances are not more flexible than in mesophiles but they promote membrane fluidity. Dihydrouridine-modified tRNA molecules in psychrophiles have similar melting temperatures to their counterparts in *E. coli*, but the modification promotes greater dynamic motion of a functional region of the molecule. Second, reference to the disorder suggests a clear link between the thermodynamic experimental data and the biological observations on cold-adapted organisms. It is hoped that such view will stimulate other approaches in this field, which will also determine to what extent the reverse conclusions could be valid for thermophiles.

References

Becktel WJ, Schellman JA (1987) Protein stability curves. Biopolymers 26:1859–1877

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

Dalluge JJ, Hamamoto T, Horikoshi K, Morita RY, Stetter KO, McCloskey JA (1997) Posttranscriptional modification of tRNA in psychrophilic bacteria. J Bacteriol 179:1918–1923

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, Sohier JS, Feller G (2006a) 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

D'Amico S, Collins T, Marx JC, Feller G, Gerday C (2006b) Psychrophilic microorganisms: challenges for life. EMBO Rep 7:385–389

Feller G, Gerday C (2003a) Catalysis and low temperatures: molecular adaptations. In: Gerday C, Glansdorff N (eds) Extremophiles (Life under extreme environmental Condition), Encyclopedia of Life Support Systems (EOLSS) Developed under the Auspices of the UNESCO. Eolss Publishers, Oxford, UK. http://www.eolss.net

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

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

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

Galtier N, Lobry JR (1997) Relationships between genomic G + C content, RNA secondary structures, and optimal growth temperature in prokaryotes. J Mol Evol 44:632–636

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

Khachane AN, Timmis KN, dos Santos VA (2005) Uracil content of 16S rRNA of thermophilic and psychrophilic prokaryotes correlates inversely with their optimal growth temperatures. Nucleic Acids Res 33:4016–4022

Kohen A, Cannio R, Bartolucci S, Klinman JP (1999) Enzyme dynamics and hydrogen tunnelling in a thermophilic alcohol dehydrogenase. Nature 399:496–499

Kumar S, Nussinov R (2004) Experiment-guided thermodynamic simulations on reversible two-state proteins: implications for protein thermostability. Biophys Chem 111:235–246

Kumar S, Tsai CJ, Nussinov R (2001) Thermodynamic differences among homologous thermophilic and mesophilic proteins. Biochemistry 40:14152–14165

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

Kumar S, Tsai CJ, Nussinov R (2003) Temperature range of thermodynamic stability for the native state of reversible two-state proteins. Biochemistry 42:4864–4873

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

Makhatadze GI, Privalov PL (1995) Energetics of protein structure. Adv Protein Chem 47:307–425

Margesin R, Feller G, Gerday C, Russell NJ (2002) Cold-adapted microorganisms: adaptation strategies and biotechnological potential. In: Bitton G (eds) Encyclopedia of Environmental Microbiology (2). Wiley, New York, pp 871–885

Privalov PL (1979) Stability of proteins: small globular proteins. Adv Protein Chem 33:167–241

Privalov PL (1990) Cold denaturation of proteins. Crit Rev Biochem Mol Biol 25:281–305

Russell NJ (1997) Psychrophilic bacteria: molecular adaptations of membrane lipids. Comp Biochem Physiol A 118:489–493

Russell NJ (2003) Psychrophily and resistance to low temperature. In: Gerday C, Glansdorff N (eds) Extremophiles (Life under extreme environmental Condition), Encyclopedia of Life Support Systems (EOLSS) Developed under the Auspices of the UNESCO. Eolss Publishers, Oxford, UK. http://www.eolss.net

Russell NJ, Hamamoto T (1998) Psychrophiles. In: Horikoshi K, Grant D (eds) Extremophiles: microbial life in extreme environments. Wiley-Liss, New York, pp 25–45

Shlyk-Kerner O, Samish I, Kaftan D, Holland N, Maruthi Sai PS, Kless H, Scherz A (2006) Protein flexibility acclimatizes photosynthetic energy conversion to the ambient temperature. Nature 442:827–830

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

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

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

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