doi:10.1006/jmbi.2001.4774 available online at http://www.idealibrary.com on IDEAL® *J. Mol. Biol.* (2001) **310**, 291–297

JMB

COMMUNICATION

Modular Structure, Local Flexibility and Cold-activity of a Novel Chitobiase from a Psychrophilic Antarctic Bacterium

Thierry Lonhienne[1,2], Jérôme Zoidakis[1,2], Constantinos E. Vorgias[3] Georges Feller[1]*, Charles Gerday[1] and Vassilis Bouriotis[2]

[1]*Laboratory of Biochemistry Institute of Chemistry B6 University of Liege B-4000 Liege, Belgium*

[2]*Enzyme Technology Division Institute of Molecular Biology and Biotechnology, P.O. Box 1515, 71110 Heraklion, Crete Greece*

[3]*Department of Biochemistry and Molecular Biology University of Athens Panepistimiopolis-Zographou 15784 Athens, Greece*

**Corresponding author*

The gene *archb* encoding for the cell-bound chitobiase from the Antarctic Gram-positive bacterium *Arthrobacter* sp. TAD20 was cloned and expressed in *Escherichia coli* in a soluble form. The mature chitobiase *Ar*Chb possesses four functionally independent domains: a catalytic domain stabilized by Ca^{2+}, a galactose-binding domain and an immunoglobulin-like domain followed by a cell-wall anchorage signal, typical of cell-surface proteins from Gram-positive bacteria. Binding of saccharides was analyzed by differential scanning calorimetry, allowing to distinguish unequivocally the catalytic domain from the galactose-binding domain and to study binding specificities. The results suggest that *Ar*Chb could play a role in bacterium attachment to natural hosts. Kinetic parameters of *Ar*Chb demonstrate perfect adaptation to catalysis at low temperatures, as shown by a low activation energy associated with unusually low K_m and high k_{cat} values. Thermodependence of these parameters indicates that discrete amino acid substitutions in the catalytic center have optimized the thermodynamic properties of weak interactions involved in substrate binding at low temperatures. Microcalorimetry also reveals that heat-lability, a general trait of psychrophilic enzymes, only affects the active site domain of *Ar*Chb.

Keywords: chitobiase; glycosyl hydrolase; microcalorimetry; psychrophile; extremophile

Chitin, one of the most abundant organic compounds in nature, is a structural polysaccharide composed of *N*-acetylglucosamine (GlcNAc) residues. The polysaccharide, sometime referred to as ''animal cellulose'', is a major component of the crustacean exoskeleton corresponding to an annual production of billions of tons, resulting in a continuous deposition of chitin on the ocean floor. However, marine sediments contain relatively little chitin thanks to a wide distribution of marine bacteria that degrade and catabolize chitinous particles, allowing carbon and nitrogen to return to the ecosystem.[1,2] Complete chitin hydrolysis proceeds *via* two probable major pathways,[3] the most common one being a three-enzyme system: a chitinase first hydrolyzes chitin to chitobiose (*N*,*N*′-diacetyl chitobiose), a chitobiase hydrolyzes chitobiose to GlcNAc which is further converted to glucosamine by a deacetylase. Chitobiases are found in bacteria, fungi, and eukaryotes.[4] These enzymes, classified under family 20 of glycosyl hydrolases,[5] may have a broad substrate specificity according to their origin but share the ability to hydrolyze chitobiose and its synthetic derivatives. Chitiobiases that act on chitotriose (*N*,*N*′,*N*″-triacetyl chitotriose) or longer oligomers are termed β-*N*-acetylglucosaminidases. Such enzymes from higher organisms exhibit a broad specificity and are unable to distinguish between β-(1,4)-linked *N*-acetylglucosaminyl and β-(1,4)-linked *N*-acetyl-

Abbreviations used: GlcNAc, *N*-acetylglucosamine; GalNAc, *N*-acetylgalactosamine; *Ar*Chb, chitobiase from *Arthrobacter* sp. TAD20; *Sm*Chb, chitobiase from *Serratia marcescens*; *Mv*Sial, sialidase from *Micromonospora viridifaciens*; GBD, galactose-binding domain; ILD, immunoglobulin-like domain; DSC, differential scanning calorimetry.

E-mail address of the corresponding author: gfeller@ulg.ac.be

0022-2836/01/020291–7 $35.00/0

galactosaminyl residues. They are therefore also referred to as *N*-acetylhexosaminidases. Human *N*-acetylhexosaminidases have a specific activity on GM2 gangliosides and deficiency in such enzymes causes the Tay-Sachs and Sandhoff disease.[6]

The marine psychrophile *Arthrobacter* sp. TAD20 is a chitinolytic Gram-positive bacterium collected along the Antarctic ice-shell.[7,8] Its cell-bound chitobiase *Ar*Chb is involved in chitin hydrolysis at sub-zero temperatures. The common property of enzymes from cold-evolving organisms is their high specific activity, which compensates for the slow chemical reaction rates at low temperatures.[9–11] The structural properties of *Ar*Chb provide new insights into the mechanisms of cold-adaptation of enzymes.

Modular structure of chitobiase *Ar*Chb

The structural gene of *Ar*Chb was cloned from a genomic library of the psychrophile *Arthrobacter* sp. TAD20. In order to circumvent thermal denaturation of the cloned products, *E. coli* transformants were grown at 18 °C. Amongst 2000 transformants screened by 4-methylumbelliferyl *N*-acetyl-β-D-glucosaminide spray, one clone carrying a 16 kb insert was detected by the appearance of a fluorescent halo when exposed to UV light. After subcloning, a plasmid containing a 6.1 kb fragment and conferring chitobiase activity to transformed *E. coli* cells was isolated and sequenced on both strands. These sequence data have been submitted to the DDBJ/EMBL/GenBank databases under accession number AJ250587. The nucleotide sequence revealed an open reading frame of 4494 bp designated *archb*, corresponding to a protein of 1498 amino acid residues. The determined NH_2-terminal amino acid sequence of the purified recombinant *Ar*Chb (see below) allowed us to locate the signal peptidase cleavage site, releasing a leader peptide of 26 amino acid residues. Therefore, the primary structure of the mature recombinant *Ar*Chb is composed of 1472 amino acid residues with a calculated $M_r = 153{,}954$ in good agreement with electrophoretic estimation. Sequence analysis[12,"13] of *Ar*Chb primary structure revealed four distinct functional regions. From NH_2 to COOH termini:

(1) Near the N terminus is a region (residues 346-900) containing the catalytic domain homologous to that of chitobiases. The *Ar*Chb catalytic domain shows 36 % and 32 % residue identity with chitobiase from *Streptomyces plicatus*[14] and domain III of the crystallized chitobiase from *Serratia marcescens*[6] (*Sm*Chb), respectively. Amino acid residues Asp676 and Glu677 of *Ar*Chb are the conserved catalytic residues.[6,15] The residue Arg498 in a conserved region is possibly involved in substrate binding. The two disulfide linkages Cys400-Cys408 and Cys505-Cys578 in *Sm*Chb are, however, lacking in *Ar*Chb. No significant similarity was found in the first 345 residues of *Ar*Chb.

(2) The second region (residues 1020-1243) contains a galactose-binding domain (GBD) showing 35 % identity with that of the crystallized *Micromonospora viridifaciens* sialidase[16] (*Mv*Sial). His1137, Trp1140 and Arg1169 of *Ar*Chb are three conserved residues amongst the five responsible for galactose-binding.[16] Ser575 and Glu578 of *Mv*Sial also involved in galactose binding are missing in *Ar*Chb. In addition, the two sequential glycine residues Gly402-403 in *Mv*Sial correspond to Gly1041-1042 or to Gly1068-1069 in *Ar*Chb.

(3) Near the C terminus, a region (residues 1358-1430) containing an immunoglobulin-like domain (ILD) shows 34 % identity with domain D from the crystallized *Bacillus circulans* cyclodextrinase which adopts an immunoglobulin folding pattern.[17] Analysis of the sequence also reveals a Pro/Thr-rich region of 105 residues between the GBD and the ILD. This sequence contains three consecutive repeats (DPTTDPTTDPTVTASPSVEPTATT) that show homology with the motif found in the Pro/Thr-rich region of some endoglucanases.[18]

(4) Hydrophilicity analysis also revealed a sequence motif at the carboxyl terminus showing typical features of transmembrane regions responsible for anchorage of cell-surface proteins in Gram-positive bacteria,[19] such as a partially conserved LPXTGX motif followed by a stretch of 18 hydrophobic residues and a short charged tail.

In comparison, the X-ray structure of *Sm*Chb (98,548 Da) displays four compact domains.[6] Domain I (residues 28-181) is similar to the cellulose binding domain of cellulases, domain II (residues 214-335) has no identified function, domain III (residues 336-818) is a $(\beta/\alpha)_8$-barrel containing the active site and domain IV (residues 819-885) adopts an immunoglobulin β-sandwich fold.

Expression and purification of the recombinant chitobiases *Ar*Chb and *Sm*Chb

E. coli BL21 (DE3) harboring the recombinant plasmid p*Ar*ChbT (derived from pET22b) was grown in five litres of medium containing 10 g/l bactotryptone, 5 g/l yeast extract, 0.5 g/l NaCl (pH 7.3), supplemented with 100 μg ampicillin/ml. The culture broth was equally distributed in ten hermetically sealed glass bottles of one litre capacity. The bottles were incubated at 15 °C for five days and shaken at 150 rpm. Such unusual culture conditions improved 800-folds the basal production. Cells were harvested by centrifugation and the periplasmic fraction was obtained by the osmotic shock procedure.[20] This fraction (0.5 liter) was loaded on a Biogel HTP hydroxyapatite column (2.5 cm × 20 cm) equilibrated with 1 mM sodium phosphate buffer (pH 6.8) and eluted with a phosphate linear gradient (1 mM-50 mM, 100-100 ml). Fractions containing chitobiase activity were applied on a QFF-Sepharose column (2.5 cm × 15 cm) equilibrated with 20 mM Tris (pH 7.5) and eluted with a NaCl linear gradient (1 mM-300 mM, 150-150 ml). The active fractions

corresponding to pure chitobiase (±16 mg) were concentrated to 5 mg/ml and conditioned in 50 mM Bis-Tris, 1 mM $CaCl_2$, 50% (v/v) glycerol (pH 6.3) and kept at −20 °C. For further comparative studies, the mesophilic chitobiase *Sm*Chb was obtained as described.[21]

Differential scanning calorimetry

The denaturation curve, or thermogram, of *Ar*Chb in the presence of 1 mM $CaCl_2$ shows a multistep pattern characterized by two calorimetric transitions termed 1 and 2 (Figure 1(a)). Deconvolution of the thermogram according to a non-two-state denaturation process for the two separate domains gives a melting temperature t_m value of 45.1 °C and of 60 °C for transitions 1 and 2, respectively. A ratio $\Delta H_{cal}/\Delta H_{eff}$ around 4 for transition 1 indicates that denaturation of several calorimetric units contributes to this transition.[22] The effect of ligands on t_m was used to evaluate the binding capacities of *Ar*Chb.[23] The thermogram of *Ar*Chb in the presence of 10 mM EDTA demonstrated the stabilizing effect of Ca^{2+}. Indeed, Ca^{2+} removal strongly destabilizes *Ar*Chb by shifting both t_m and ΔH_{cal} (area of the transition) to lower values (Figure 1(b)), whereas the enzyme remained fully active. Thermograms of *Ar*Chb in the presence of 100 mM GlcNAc or 100 mM galactose display opposite results. GlcNAc, the product of chitobiose hydrolysis, is an usual inhibitor of chitobiases and its binding to *Ar*Chb clearly stabilizes transition 1 ($t_m + 6$ °C) while it has no effect on transition 2 (Figure 1(c)). By contrast, as shown in Figure 1(d), galactose stabilizes transition 2 ($t_m + 4.5$ °C) but has only a weak effect on transition 1 ($t_m + 0.6$ °C). This allows to unequivocally assign transition 1 to the catalytic domain and transition 2 to the GBD. Transition 1 of *Ar*Chb is moderately stabilized by glucose ($t_m + 2.2$ °C; Figure 1(c)) and *N*-acetylgalactosamine (GalNAc, $t_m + 2.4$ °C), whereas the latter also stabilizes transition 2 ($t_m + 2.3$ °C; Figure 1(d)). Strong aggregation of the mesophilic *Sm*Chb precluded similar reliable analysis. Nevertheless, thermograms of *Sm*Chb displayed a single transition with an apparent t_m value around 60 °C, followed by a sharp decrease of C_p due to exothermic aggregation. In order to evaluate the function of the C-terminal region of *Ar*Chb, the ILD domain and the Pro/Thr-rich region of the *archb* gene were deleted by PCR, leaving an open reading frame of 3768 bp (130,081 Da). The characterization of the purified mutant enzyme showed identical kinetic and thermodynamic parameters compared to *Ar*Chb (data not shown). The DSC thermograms of this ILD-deleted mutant showed that transitions 1 and 2 were nearly unaffected when compared to that of *Ar*Chb (Figure 1(a)) but that aggregation occurred at 65 °C.

In conclusion, the four domains of *Ar*Chb are functionally and structurally independent. Indeed, the deletion of the immunoglobulin-like domain (ILD) and of the cell-wall anchorage signal did not

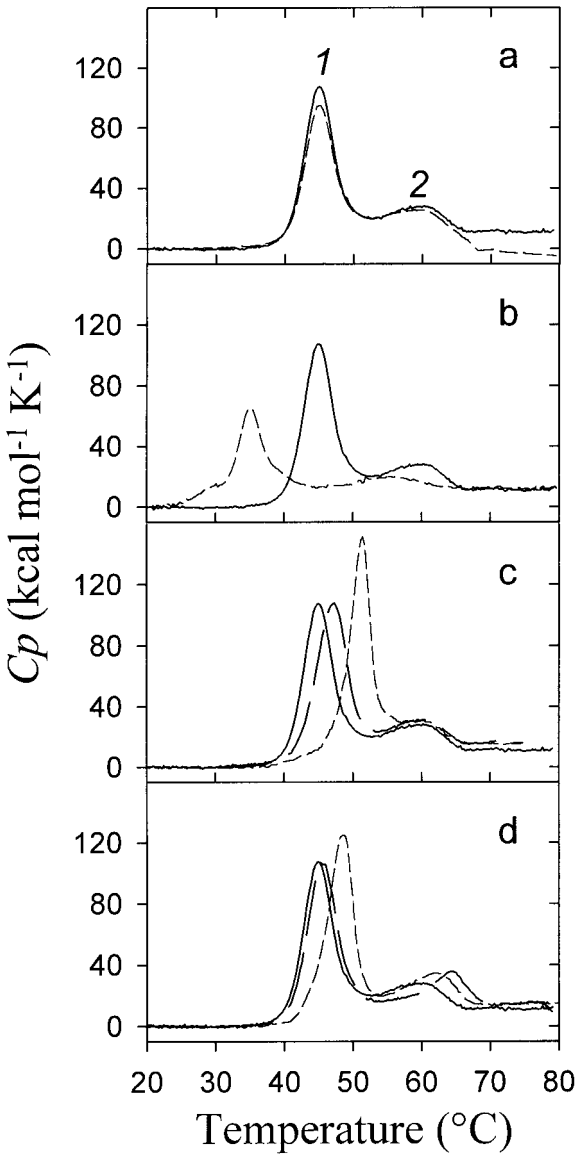

Figure 1. Differential scanning calorimetry of *Ar*Chb. The thermogram of the native form *Ar*Chb is presented in all panels (continuous line), showing two main transitions (1 and 2). (a) Thermogram of the ILD-deleted mutant (short dashed). (b) Thermogram of *Ar*Chb in 10 mM EDTA (short dashed). (c) Thermogram of *Ar*Chb in 100 mM glucose (long dashed) or in 100 mM GlcNAc (short dashed). (d) Thermogram of *Ar*Chb in 100 mM galactose (long dashed) or in 100 mM GalNAc (short dashed). All buffers contained 20 mM Bis-Tris (pH 6.3) and 1 mM $CaCl_2$ (except with EDTA). DSC data have been baseline subtracted and normalized for protein concentration. DSC measurements were performed using a MicroCal MCS-DSC instrument at a scan rate of 60 K h^{-1} and under a nitrogen pressure of 2 atm. Samples were dialyzed overnight against the appropriate buffer, the latter being used in the reference cell and for base line determination. Protein concentration after dialysis was ~4 mg/ml. Calcium removal was carried out by dialysis against 20 mM Bis-Tris, 10 mM EDTA (pH 6.3). Thermograms were analyzed using the MicroCal Origin software (version 2.9).

affect the catalytic properties, confirming their specific roles. The structural independence of ILD is supported by the occurrence of a Pro/Thr-rich region between the galactose-binding domain (GBD) and ILD that typically acts as a flexible hinge that joins discrete domains in some endoglucanases and xylanases.[18] The GBD is separated from the catalytic domain by two glycine doublets that probably play a role of pivot, ensuring relative movements between the two connected domains, as observed in *Mv*Sial.[16] The DSC thermograms of *Ar*Chb in the presence of GlcNAc and galactose confirm the structural independence of these domains, since GlcNAc only stabilizes the catalytic domain while galactose only stabilizes the GBD (Figure 1). One should note that the epicuticle of decapod crustaceans is composed of chitinous microfibers associated to a proteic matrix rich in glycans ending with neuraminic acid, galactose and GalNAc. Since the GBD of *Ar*Chb specifically binds to galactose and GalNAc, one can propose that this domain is involved in the cell-host attachment process. Accordingly, the ILD domain of *Ar*Chb could also bind to specific sugar or protein motifs of the exoskeleton. Such a multiple binding potential has been observed in bacterial cell-surface proteins involved in the infection mechanisms of human diseases.[24]

Cold adaptation: kinetic and thermodynamic activation parameters

The activity of *Ar*Chb is markedly less thermostable than that of its mesophilic counterpart. In optimal conditions (50 mM Bis-Tris, 1 mM $CaCl_2$ (pH 6.3) and 50 mM Tris, 100 mM KCl (pH 7.8) for *Ar*Chb and *Sm*Chb, respectively) the half-life of activity for *Ar*Chb at 40 °C is about 15 minutes, whereas *Sm*Chb remains stable for hours at this temperature. The psychrophilic *Ar*Chb is also more urea-sensitive than *Sm*Chb. For instance, addition of 1 M urea in the reaction medium induces 83 % and 23 % decrease of the activity of *Ar*Chb and *Sm*Chb, respectively. However, the psychrophilic enzyme *Ar*Chb displays an exceptional adaptation to catalysis at low temperatures. The specific activity of *Ar*Chb towards *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide (pNP-GlcNAc) is higher at all temperatures tested (Figure 2(a)) and is about ninefold that of *Sm*Chb at 3 °C (40 s^{-1} *versus* 4.5 s^{-1}). It should be noted that *Sm*Chb was previously considered as one of the most active microbial chitobiase.[6] The K_m values of *Ar*Chb and *Sm*Chb have been plotted as a function of temperature (Figure 2(b)). Two aspects are worth mentioning: the K_m value of both enzymes tends to minimum values at their respective environmental temperatures and sharply increases outside this range, giving rise to a cross-shaped plot. Moreover, the K_m value of *Ar*Chb (23 μM at 7 °C, 33 μM at 20 °C) is ten times lower than that of *Sm*Chb at 20 °C and is also the lowest value for chitobiase found in the literature..[21,25-30] As a result, the catalytic efficiency or

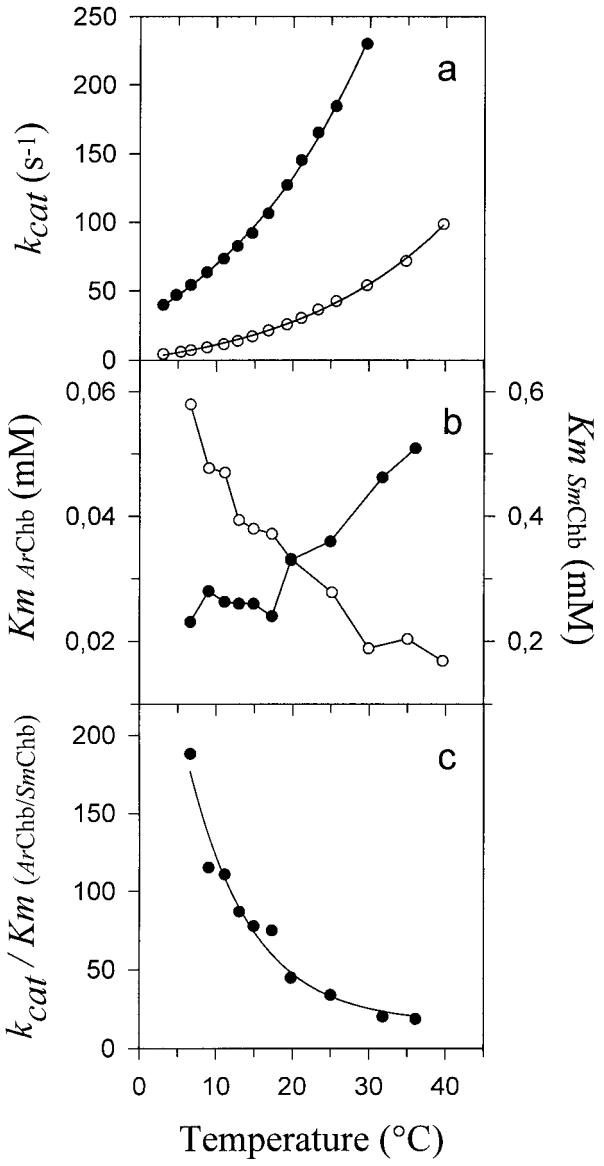

Figure 2. Temperature dependence of the kinetic parameters for *Ar*Chb (●) and *Sm*Chb (○) using pNP-GlcNAc as substrate. Data for the catalytic rate constant k_{cat} (a), K_m ((b), note the different scales used) and the relative catalytic efficiency k_{cat}/K_m (*Ar*Chb/*Sm*Chb) (c). Optimal buffers for activity were 50 mM NaH_2PO_4, 100 mM NaCl (pH 7.3) and 50 mM Tris, 100 mM KCl (pH 9) for *Ar*Chb and *Sm*Chb, respectively. Activities were recorded in a thermostated Uvicon 860 Spectrophotometer (Kontron) and calculated on the basis of extinction coefficients at 405 nm for *p*-nitrophenol of 14,700 and 16,200 M^{-1} cm^{-1} at pH 7.3 and pH 9, respectively. Kinetic parameters K_m and k_{cat} were determined from initial reaction rates at substrate concentrations ranging from 0.1 to 10 K_m and by non-linear regression on the Michaelis-Menten equation. Protein concentrations were measured using the bicinchoninic acid protein assay reagent (Pierce) and checked using the calculated extinction coefficient[34] for *Ar*Chb $\varepsilon_{280\ nm} = 150{,}600\ M^{-1}\ cm^{-1}$.

specificity constant k_{cat}/K_m for the psychrophilic enzyme exceed that of the mesohilic chitobiase. The ratio of this constant for both enzymes is plotted *versus* the reaction temperature in Figure 2(c), showing that the cold-adapted *Ar*Chb is nearly 200 times more efficient for substrate hydrolysis at 7 °C. The thermodependence curves of k_{cat} for *Ar*Chb and *Sm*Chb (Figure 2(a)) were used to construct Arrhenius plots and to calculate the thermodynamic activation parameters of the enzymatic reactions (Table 1). The lower free energy of activation ($\Delta G^{\#}$) of *Ar*Chb when compared to *Sm*Chb correlates with its higher specific activity. However, the contribution of the enthalpy term ($\Delta H^{\#}$) and of the entropy term ($T\Delta S^{\#}$) to $\Delta G^{\#}$ differs markedly in both enzymes. The lower activation enthalpy $\Delta H^{\#}$ indicates that the reaction rate is less affected by a decrease of the temperature, whereas the larger variation of the entropy term $T\Delta S^{\#}$ has been tentatively related to a broader distribution of conformational states for the enzyme-substrate complex in cold-active enzymes as a result of their improved flexibility.[31,32]

Comparison of the protein sequences indicates that all amino acid residues involved in substrate binding in *Sm*Chb are conserved in *Ar*Chb, except Trp616 and Trp639 which perform hydrophobic interactions with the planar *N*-acetyl group of the inner non-reducing GlcNAc residue of chitobiose or with the synthetic derivative pNP-GlcNAc.[6] As no other aromatic residue in the *Ar*Chb sequence could replace these two tryptophane residues, it may be suggested that hydrophobic interactions with the *N*-acetyl group have been replaced by polar interactions. This hypothesis is supported by the DSC thermograms of *Ar*Chb obtained in the presence of glucose and GlcNAc (Figure 1(c)). Indeed, the catalytic domain (transition 1) is stabilized by glucose ($t_m = 47.3$ °C) and GlcNAc ($t_m = 51.5$ °C), both binding to the catalytic pocket. The large difference between the apparent melting temperatures indicates that the *N*-acetyl group of GlcNAc undergoes strong electrostatic interactions with the catalytic domain. Thermograms obtained with galactose ($t_m = 45.7$ °C) and GalNAc ($t_m = 48.5$ °C) also demonstrates the stabilizing effect of the *N*-acetyl group. Hydrophobic interactions form endothermically and are weakened by a decrease in temperature. By contrast, electrostatic interactions form exothermically and are therefore stabilized at low temperatures.[10] It follows that the replacement of hydrophobic interactions by electrostatic interactions in the substrate binding site of *Ar*Chb may explain the cross-shaped plot of K_m (Figure 2(b)), revealing a new adaptation strategy that takes advantage of the thermodynamic properties of the bonds involved.

According to the current hypothesis, optimization of the k_{cat} parameter probably involves the highly flexible structure of psychrophilic enzymes, providing enhanced abilities to undergo conformational changes during catalysis at low temperatures. Such adaptation can be achieved through various structural changes, each enzyme adopting its own strategy, but invariably leading to a weak stability.[9–11] The heat-lability of *Ar*Chb activity, its sensitivity to urea and the DSC thermograms also demonstrate the low stability of the cold-active chitobiase. Some structural features of *Ar*Chb can account for its lability. Arginine can perform as many as five electrostatic interactions with surrounding groups and is abundant in thermophilic proteins. Comparison of the catalytic domain of *Ar*Chb (arginine content = 16) and *Sm*Chb (arginine content = 30) suggests that Arg-avoidance can improve the protein flexibility. The lack of any disulfide linkage in the catalytic domain of *Ar*Chb, whereas *Sm*Chb possesses two bridges, is another factor that can generate more conformational plasticity. Three glycine doublets (Gly674-675, Gly713-714 and Gly718-Gly719) near the active site of *Ar*Chb, instead of one in *Sm*Chb, can also provide an increased degree of freedom to the polypeptide backbone bearing the catalytic residues.[11] The isoelectric point of *Ar*Chb and of *Sm*Chb was found at pH 4.6 and pH 7.1, respectively. Such differences in *p*I values, frequently observed for cold-adapted enzymes, have been tentatively correlated with improved interactions with the solvent therefore reducing the compactness of the molecular surface.[11]

The DSC thermograms of *Ar*Chb (Figure 1) display a heat-labile domain ($t_m = 45.1$ °C) and a heat-stable domain ($t_m = 60$ °C) corresponding to the catalytic domain and to the GBD domain, respectively, as demonstrated by saccharide binding experiments. By contrast, the mesophilic *Sm*Chb only displays one heat-stable calorimetric domain ($t_m \sim 60$ °C) as also indicated by circular dichroism.[21] It has been shown that a cold-active α-amylase has evolved towards the lowest possible stability of the native state.[33] Thermograms of *Ar*Chb clearly demonstrate that heat-lability only affects the calorimetric domain containing the active site while leaving stability of the non-

Table 1. Kinetic and thermodynamic activation parameters at 15 °C for *Ar*Chb from the Antarctic strain *Arthrobacter* sp. TAD20 and for *Sm*Chb from the mesophile *Serratia marcescens*

Parameter	*Ar*Chb	*Sm*Chb
k_{cat} (s^{-1})	98 ± 2	18 ± 1
E_a (kJ mol^{-1})[a]	47.1 ± 1.3	73.9 ± 1.2
$\Delta G^{\#}$ (kJ mol^{-1})	59.5 ± 0.1	63.5 ± 0.2
$\Delta H^{\#}$ (kJ mol^{-1})	44.7 ± 1.3	71.5 ± 1.2
$T\Delta S^{\#}$ (kJ mol^{-1})	−14.8 ± 1.4	8.0 ± 1.4

The activation energy was calculated from the slope ($-E_a/R$) of Arrhenius plots and the thermodynamic activation parameters of the chitobiase reaction were calculated as previously described.[32]

[a] Experimental energy of activation below 15 °C.

catalytic GBD unchanged. It follows that heat-lability is not simply the result of the lack of selective pressure for stable protein in a cold environment (as demonstrated by the stable GBD) but seems to be the consequence of the improved plasticity required around the active site for efficient activity at low temperatures.

Acknowledgments

We acknowledge the Expeditions Polaires Françaises for support and facilities offered at the Dumont d'Urville Antarctic station. This work was supported by the European Union under the form of a TMR contract CT970131, a Biotech contract COLDZYME BIO4-CT96-0051 and by the Fonds National de la Recherche Scientifique, contract FRFC C.2.4523.97 to Ch. G.

References

1. Zobell, C. E. & Rittenberg, S. C. (1937). The occurrence and characteristics of chitinoclastic bacteria in the sea. *J. Bacteriol.* **35**, 275-287.
2. Yu, C., Lee, A. M., Bassler, B. L. & Roseman, S. (1991). Chitin utilization by marine bacteria: a physiologycal function for bacteria adhesion to immobilized carbohydrates. *J. Biol. Chem.* **266**, 24260-24267.
3. Muzzarelli, R. A. (1999). Analytical biochemistry and clinical significance of *N*-acetyl-beta-D-glucosaminidase and related enzymes. *EXS.* **87**, 235-247.
4. Cannon, R. D., Niimi, K., Jenkinson, H. F. & Shepherd, M. G. (1994). Molecular cloning and expression of the *Candida albicans* β-*N*-acetylglucosaminidase (HEX1) gene. *J. Bacteriol.* **176**, 2640-2647.
5. Henrissat, B. & Bairoch, A. (1993). New families in the classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochem. J.* **293**, 781-788.
6. Tews, I., Perrakis, A., Oppenheim, A., Dauter, Z., Wilson, K. S. & Vorgias, C. E. (1996). Bacterial chitobiase structure provides insight into catalytic mechanism and the basis of Tay-Sachs disease. *Nature Struc. Biol.* **3**, 638-648.
7. Lonhienne, T., Mavromatis, K., Vorgias, C. E., Buchon, L., Gerday, C. & Bouriotis, V. (2001). Cloning, sequences, and characterization of two chitinase genes from the Antarctic *Arthrobacter* sp. strain TAD20: isolation and partial characterization of the enzymes. *J. Bacteriol.* **183**, 1773-1779.
8. Lonhienne, T., Baise, E., Feller, G., Bouriotis, V. & Gerday, C. (2001). Enzyme activity determination on macromolecular substrates by isothermal titration calorimetry: application to mesophilic and psychrophilic chitinases. *Biochim. Biophys. Acta,* **1545**, 349-356.
9. Hochachka, P. W. & Somero, G. N. (1984). *Biochemical Adaptations*, Princeton University Press, Princeton, NJ.
10. Somero, G. N. (1995). Proteins and temperature. *Annu. Rev. Physiol.* **57**, 43-68.
11. Feller, G. & Gerday, C. (1997). Psychrophilic enzymes: molecular basis of cold adaptation. *Cell. Mol. Life Sci.* **53**, 830-841.
12. Altschul, S. F., Gish, W., Miller, E., Myers, E. W. & Lipman, D. J. (1990). Basic local alignment search tool. *J. Mol. Biol.* **215**, 403-410.
13. Pearson, W. R. (1990). Rapid and sensitive sequence comparison with FASTP and FASTA. *Methods Enzymol.* **183**, 63-98.
14. Mark, B. L., Wasney, G. A., Salo, T. J. S., Khan, A. R., Cao, Z. & Robbins, P. W. *et al*. (1998). Structural and functional characterization of *Streptomyces plicatus* β-*N*-acetylhexosaminidase by comparative molecular modeling and site-directed mutagenesis. *J. Biol. Chem.* **273**, 19618-19624.
15. Prag., G., Papanikolau, Y., Tavlas, G., Vorgias, C. E., Petratos, K. & Oppenheim, A. B. (2000). Structures of chitobiase mutants complexed with the substrate di-*N*-acetyl-D-glucosamine: the catalytic role of the conserved acidic pair, aspartate 539 and glutamate 540. *J. Mol. Biol.* **300**, 611-617.
16. Gaskell, A., Crennell, S. & Taylor, G. (1995). The three domains of a bacterial sialidase: a beta-propeller, an immunoglobulin module and a galactose-binding jelly-roll. *Structure,* **3**, 1197-1205.
17. Klein, C. & Schulz, G. E. (1991). Structure of cyclodextrin glycosyltransferase. *J. Mol. Biol.* **217**, 737-750.
18. Gilkes, N. R., Henrissat, B., Kilburn, D. G., Miller, R. C. & Warren, R. A. J. (1991). Domains in microbial β-1,4-glycanases: sequence conservation, function, and enzyme families. *Microbiol. Rev.* **55**, 303-315.
19. Kehoe, M. A. (1994). Cell-wall-associated proteins in Gram-positive bacteria. In *Bacterial Cell Wall* (Ghuysen, J. M. & Hakenbeck, R., eds), pp. 217-261, Elsevier Science BV, Amsterdam.
20. Suzuki, H., Kumagai, H. & Tochikura, T. (1986). gamma-Glutamyltranspeptidase from *Escherichia coli* K-12: formation and localization. *J. Bacteriol.* **168**, 1332-1335.
21. Tews, I., Vincentelli, R. & Vorgias, C. E. (1996). *N*-Acetylglucosaminidase (chitobiase) from *Serratia marcescens*, gene sequence, protein production and purification in *Escherchia coli*. *Gene,* **170**, 63-67.
22. Privalov, P. L. (1982). Stability of proteins: proteins which do not present a single cooperative system. *Advan. Protein Chem.* **35**, 1-104.
23. Haynie, D. T. (1998). Quantitative analysis of differential scanning calorimetry data. In *Biocalorimetry, Applications of Calorimetry in the Biological Sciences* (Ladbury, J. E. & Chowdhry, B. Z., eds), pp. 183-205, Wiley, Chichester, UK.
24. Goward, C. R., Scawen, M. D., Murphy, J. P. & Atkinson, T. (1993). Molecular evolution of bacterial cell-surface proteins. *Trends Biochem. Sci.* **18**, 136-140.
25. Li, S. C. & Li, Y. T. (1970). Study of the glycosidases of jack bean meal. *J. Biol. Chem.* **245**, 5153-5160.
26. Jones, C. S. & Kosman, D. J. (1980). Purification, properties, kinetics, and mechanism of β-*N*-acetylglucosamidase from *Aspergillus niger*. *J. Biol. Chem.* **255**, 11861-11869.
27. Bhal, O. P. & Agrawal, K. M. L. (1968). Glycosidases of *Aspergillus niger*. *J. Biol. Chem.* **144**, 2970-2978.
28. Bedi, G. H., Shah, R. H. & Bahl, O. P. (1984). Study of *Turbatix aceti* β-*N*-acetylglucosaminidase. *Arch. Biochem. Biophys.* **233**, 237-250.
29. Zhu, B. C., Lo, J. Y., Li, Y. T., Li, S. C., Jaynes, J. M. & Gildemeister, O. S. *et al*. (1992). Thermostable, salt tolerant, wide pH range novel chitobiase from *Vibrio parahemolyticus*: isolation, characterization, molecular cloning and expression. *J. Biochem. (Tokyo),* **11**, 163-167.

30. Keyhani, N. O. & Roseman, S. (1996). The chitin catabolic cascade in the marine bacterium *Vibrio furnissii*. *J. Biol. Chem.* **271**, 33425-33432.
31. Fields, P. A. & Somero, G. N. (1998). Hot spots in cold adaptation: localized increases in conformational flexibility in lactate dehydrogenase A4 orthologs of Antarctic notothenioid fishes. *Proc. Natl Acad. Sci. USA,* **95**, 11476-11481.
32. 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.
33. 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.
34. Gill, S. C. & von Hippel, P. H. (1989). Calculation of protein extinction coefficients from amino acid sequence data. *Anal. Biochem.* **182**, 319-326.

Edited by I. B. Holland

(Received 21 December 2000; received in revised form 6 May 2001; accepted 14 May 2001)