Extremophiles (2000) 4:137–144

ORIGINAL PAPER

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Xylanase from the psychrophilic yeast *Cryptococcus adeliae*

Received: April 20, 1999 / Accepted: January 13, 2000

Abstract A xylanase belonging to family 10 is produced by *Cryptococcus adeliae*, an Antarctic yeast that exhibits optimal growth at low temperature. The mature glycosylated xylanase secreted by *C. adeliae* is composed of 338 amino acid residues and 26 ± 3 osidic residues, and shares 84% identity with its mesophilic counterpart from *C. albidus*. The xylanase from *C. adeliae* is less thermostable than its mesophilic homologue when the residual activities are compared, and this difference was confirmed by differential scanning calorimetry experiments. In the range 0°–20°C, the cold-adapted xylanase displays a lower activation energy and a higher catalytic efficiency. All these observations suggest a less compact, more flexible molecular structure. Analysis of computerized molecular models built up for both psychrophilic and mesophilic xylanases indicates that the adaptation to cold consists of discrete changes in the tridimensional structure: of 53 substitutions, 22 are presumably involved in the adaptation process. These changes lead mainly to a less compact hydrophobic packing, to the loss of one salt bridge, and to a destabilization of the macrodipoles of the helices.

Communicated by K. Horikoshi

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The nucleotide sequence reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number Y15434

Key words Xylanases · Psychrophile · Yeast · Molecular adaptation · Molecular modeling

Introduction

Temperature is the main selective factor leading to the biochemical adaptation of living organisms to their environment. The study of the molecular adaptations of enzymes from organisms adapted to extreme temperatures should provide consistent elements enabling a better understanding of structure–stability relationships. Many studies of enzymes from thermophilic microorganisms have demonstrated, for example, that the strengthening of their noncovalent intramolecular interactions leads to a thermostable structure, often detrimental to the specific activities (Jaenicke 1991).

On the other hand, psychrophilic microorganisms, to display appropriate metabolic flows at the environmental temperature, have to adapt to exponential reduction of reaction rates at often subzero temperatures. Enzymes from these microorganisms are thought to have evolved to a more flexibile structure when compared to their mesophilic and thermophilic counterparts. This characteristic probably originates from a weakening of intramolecular interactions and is supposed to be responsible for the increased catalytic efficiency and the low thermal stability of psychrophilic enzymes in general (Hochachka and Somero 1984). Recent crystallographic data related to cold-active enzymes such as metalloprotease from *Pseudomonas aeruginosa* (Villeret et al. 1997), α-amylase from *Alteromonas haloplanctis* (Aghajari et al. 1996, 1998a,b), triose-phosphate isomerase from *Vibrio marinus* (Alvarez et al. 1998), citrate synthase (Russell et al. 1998) and malate dehydrogenase from *Aquaspirillium articum* (Kim et al. 1999) support this hypothesis. The analysis of molecular models of several other enzymes originating from cold-adapted organisms and compared to the refined structures of mesophilic counterparts equally converge to the idea that cold-adapted enzymes are less rigid than their mesophilic homologues (Feller et al. 1996).

Xylan is, after cellulose, the more abundant polysaccharide on earth. Xylan-degrading enzymes, called xylanases, have been extensively studied, and the many crystallographic data available on mesophilic enzymes make them very good reference models for studying the molecular adaptations and stability–activity relationship in a psychrophilic xylanase. This usefulness is the reason why the xylanase from the Antarctic yeast *Cryptococcus adeliae* has been characterized. The encoding gene has been sequenced, and a tridimensional model has been built and compared to the modeled structure of the homologue enzyme secreted by the mesophile *C. albidus*. To our knowledge, this is the first report on a xylanase secreted by a cold-adapted yeast.

Material and methods

Strains and culture conditions

The mesophilic strain *Cryptococcus albidus* (ATCC 34633) was kindly provided by Dr. Rolf Morosoli, Institut Armand-Frappier, Québec, Canada.

The psychrophilic strain *Cryptococcus adeliae* (ATCC 201412) was isolated from decayed algae in the icepack at the Antarctic station Dumont d'Urville (60°40′ S;40°01′ E) by selection at 4°C on the solid minimal medium described here. This strain was fully characterized by Prof. J.F. Fell, University of Miami (J.F. Fell and G. Scorzetti, personal communication.). Cells were grown in a medium containing (per liter) 3 g NaH_2PO_4, 7 g K_2HPO_4, 2 g $(NH_4)_2SO_4$, 0.005 g $CaCl_2$, 0.0125 g $MgSO_4$, 0.5 ml trace metal solution (Morosoli et al. 1986), 50 mg ampicilline, 0.4 g yeast extract, and 10 g oat spelt xylan (Fluka). The temperature dependency of growth was evaluated in this medium for both mesophilic and psychrophilic strains at 4°, 12°, 20°, and 28°C. Samples were taken at regular intervals and after appropriate dilution plated on Potatoe Dextrose Agar (Oxoid). The cfu (colony-forming units) was determined after incubation at 18°C.

Purification of xylanases

The strains of *C. adeliae* and *C. albidus* were grown at 4°C and 18°C, respectively, during 7 and 3 days, respectively. After 20 min centrifugation at 27 000 g and 4°C, the supernatants were concentrated tenfold by ultrafiltration and then diafiltered against 20 mM Tris/HCl pH 8 using a Minitan tangential ultrafiltration unit (Millipore) fitted with PTGC membranes (10 000-Da cutoff). The resulting samples were loaded on a Q-Sepharose Fast Flow (Pharmacia) column (2.5 × 19 cm) equilibrated in the aforementioned buffer and eluted with a linear NaCl gradient (200–200 ml, 0–0.2 M NaCl). Two peaks of activity were eluted successively and correspond to xylanases X_A and X_B. The second peak of xylanase X_B, representing the major fraction of the eluted xylanolytic activity, was studied thereafter. The fractions containing the xylanase X_B were concentrated on a Amicon system fitted with PTGC membranes.

Enzyme assays

Xylanase activity for X_B was assayed at 25°C by measuring the amount of reducing sugars liberated from oat spelt xylan (Fluka) in 500 µl 50 mM sodium acetate buffer, pH 5.4, containing 11.2 mg ml^{-1} substrate. The samples were stirred to avoid xylan sedimentation. The reaction was stopped by the addition of 1 ml alkaline dinitrosalicylic acid reagent (Rick and Stegbauer 1986). After 10 min boiling, the samples were ice-chilled and submitted to centrifugation to eliminate residual xylan. The absorbance was measured at 546 nm. One unit of activity is defined as the amount of xylanase needed to liberate 1 µmol D-xylose/min under the assay conditions.

For comparative studies, V_{max} was measured at 5°, 10°, 15°, 20°, 25°, and 30° using 21.6 mg ml^{-1} oat spelt xylan. The activation energy, E_a, was determined from the slope ($-E_a$/R) of Arrhenius plots. The thermodynamic parameters of xylan hydrolysis were calculated according to the following equations:

$$\Delta G^* = \Delta H^* - T\Delta S^*$$

$$\Delta H^* = E_a - RT$$

$$\Delta S^* = 2.303R(\log k_{cat} - 10.753 + E_a/2.303RT)$$

Analytical procedures

Analytical 12% SDS-PAGE was run essentially as described by the instrument supplier (Hoefer, San Francisco, CA, USA). The NH_2-terminal amino acid sequence of the xylanases X_B was carried out from samples blotted on a Problott membrane using a pulsed liquid-phase protein sequencer (Procise Applied Biosystems 492). Differential scanning calorimetry experiments were carried out on a MicroCal apparatus (Microcal, Northhampton, MA, USA) using the Origin standard software package for data acquisition, analysis, and deconvolution. Xylanases were dissolved and dialyzed overnight against a 50 mM sodium acetate buffer, pH 5.4, at 4°C. The dialysate was used in the reference cell.

For the determination of sugar composition, X_B xylanases were further purified by RP-HPLC (Kontron, Zurich, Switzerland) on a Vydac C8 column. A 60-min acetonitrile gradient at a flow of 1 ml min^{-1} was used (A, 0.1% TFA; B, 0.1% TFA, 30% acetonitrile). The fractions containing the xylanase were lyophilized and then hydrolyzed at 100°C during 4 h in 4 N trifluoroacetic acid. The composition of released sugars was determined on a Dionex PA10 column. Schiff staining of proteins after 12% SDS-PAGE analysis was performed as previously described (Glossmann and Neville 1971). The total amount of sugar linked to the xylanase was determined by a micromethod of the phenol sulfuric assay (Fox and Robyt 1990) using mannose as a reference. The protein concentration was measured by the bicinchoninic acid method using the BCA

protein assay kit (Pierce, Rockford, IL, USA) using bovine serum albumin (Sigma) as standard.

Cloning the xyl_B gene encoding for xylanase X_B

Total RNA was extracted from cells grown on the minimal medium by the heat-freeze procedure using SDS and phenol (Domdey et al. 1984) and then purified with an RNEasy kit (Qiagen). The mRNA was purified with the PolyA Quick mRNA Isolation kit (Stratagene, La Jolla, CA, USA). The synthesis of the first cDNA strand was carried out using Superscript II reverse transcriptase (Gibco BRL) starting from a 5′-*Not*I-T_{15}-3′ oligonucleotide primer (Pharmacia).

The gene was obtained by PCR using the first strand of cDNA as matrix. The two following oligonucleotide primers were employed: 5′-*Not*I-T_{15}-3′ and that derived from the NH_2-terminal amino acid sequence corresponding to the following nucleotide sequence, 5′-AGGCCT-GCCGACAAGGATTCG-3′. *Pfu* DNA polymerase (Stratagene) was used in five different PCR experiments to check, by nucleotide sequencing, that no errors were introduced by the polymerase. After 3 min initial denaturation at 94°C, 40 cycles of amplification were performed using a Progene apparatus (Techne, Cambridge, UK). Each cycle includes denaturation at 94°C 45 s, hybridization at 60°C 1 min, and elongation at 72°C 1.5 min.

The five fragments obtained were cloned into a PCR-Script SK(+) (Stratagene) vector, as recommended by the supplier, and used to transform Epicurian Coli XL1-Blue MRF' Kan cells (Stratagene). Blue–white selection allows us to select white colonies carrying the fragments obtained from the PCR. Plasmid preparations (Qiagen Plasmid Midi kit) were sequenced on an automatic sequencer (ALFexpress DNA sequencer; Pharmacia Biotech) until no uncertainty remained concerning the nucleotide sequence. The orientation of the inserted fragments was determined by sequencing with universal/reverse primers. The sequencing of the inserted fragments was carried out using the following oligonucleotides: 5′-AGGCCTGCCGACA AGGATTCGCTC-3′, 5′-CCACATTCAGGGAGTCATC GGTCG-3′,5′-CACTGCATCGGTCTGGAATCTCA CTTC-3′, 5′-GAAAGACACCGGGGATCCACGAAG-3′, 5′-AAGTGAGATTCCAGACCGATGCAGTGC-3′, and 5′-CCGTTCTCGTTGATAGGTTCGTTAA-3′.

Molecular modeling

Atomic coordinates were obtained from the Brookhaven Protein Data Base for Cex (p07986.sw-2EXO) from *Cellulomonas fimi* (White et al. 1994) and XynZ (p10478.sw-1XYZ) from *Clostridium thermocellum* (Dominguez et al. 1995). These enzymes belong to family 10 of glycosyl hydrolases and exhibit a common $(\alpha/\beta)_8$ barrel fold. The primary amino acid sequences of the xylanases from *C. albidus* (js0734.pir) and *C. adeliae* (Y15434) were aligned on these glycosyl hydrolases using the Pileup program (Devereux et al. 1983), and the structurally conserved regions (SCR) were determined. Three-dimensional models were built using the Composer program (Blundell et al. 1988). The backbone coordinates of the SCRs and the coordinates of the conserved side chains were taken from the known structures. The other side chains were exchanged according to the sequence and their conformation was selected following a set of rules (Blundell et al. 1988), depending on the amino acid type and its secondary structure location. Loops were modeled from similar sequences found in a local database of about 200 protein structures solved at a minimal resolution of 2 Å (Centre d'Ingénierie des Protéines, Liège, Belgium). The molecular structures were manipulated with the program Insight II 95 (Biosym/MSI, San Diego, CA, USA).

Results

Effect of temperature on growth of *C. albidus* and *C. adeliae*

A maximum cell density of 12×10^7 cfu ml^{-1} was reached by *C. adeliae* after 260 h culture at 4°C in the minimal medium (Fig. 1). This result exceeds by sixfold the cell density reached by *C. albidus* grown at 4°C. Similar data were obtained at 12°C after 60 h culture. At 20°C the opposite result was recorded, with the cell density of the mesophilic strain being 2.5 times as large as that of the psychrophilic strain. At 28°C, only *C. albidus* still grew.

Purification and characterization of xylanases X_B from both strains

The purification protocol used to purify the xylanases from the supernatants made use of only three steps: supernatant concentration, diafiltration, and anion-exchange chromatography on Q-Sepharose (Pharmacia). Gradient elution from the anion exchanger showed a weak peak of activity, corresponding to xylanase X_A, and a major peak called xylanase X_B. Major peak of xylanases X_B, studied in detail thereafter, eluted at 40 mM and 50 mM of NaCl concentration for the psychrophile and mesophile, respectively. This protocol leads to 48% and 18% recovery, respectively, of the total xylanolytic activity found in the culture supernatants of *C. adeliae* and *C. albidus*. The purity of the two proteins was judged by SDS-gel electrophoresis, partial NH_2-terminal amino acid sequencing, and RP-HPLC. The apparent molecular weights estimated from SDS gels were 45 and 43 kDa for the mesophilic and cold-adapted xylanase, respectively. The bands were diffused and stained strongly with Schiff's reagent (not shown), suggesting protein glycosylation. Maldi-TOF experiments allowed us to determine a molecular mass of 41.4 kDa for both enzymes. This value was used for further calculations because important errors in the molecular masses of glycoproteins are usual when they are estimated by SDS-PAGE (Glossmann and Neville 1971).

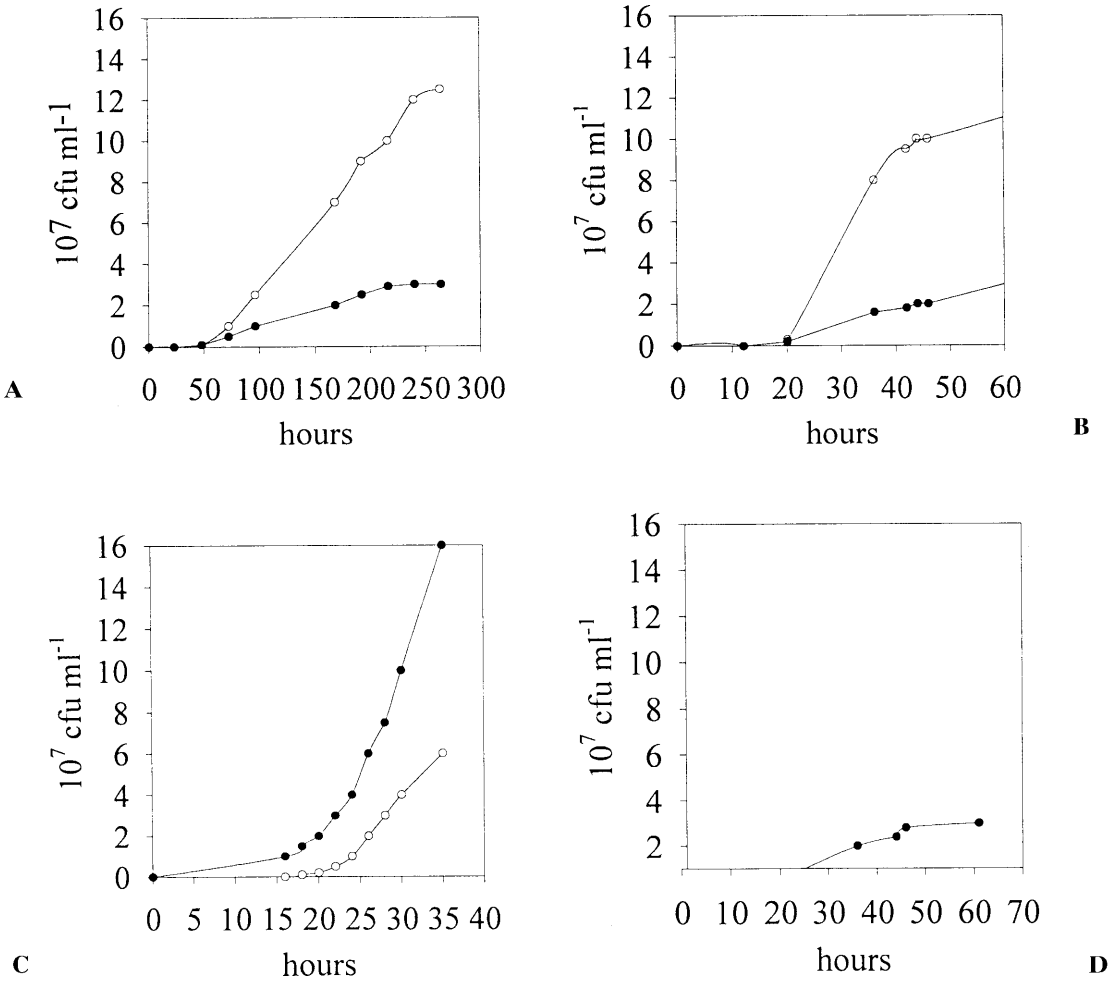

Fig. 1A–D. Comparison of the growth of *Cryptococcus albidus* (*solid circles*) and *C. adeliae* (*open circles*) at different temperatures as a function of time by counting cfu (colony-forming units) from timed aliquots of cultures at 4°C (**A**), 12°C (**B**), 20°C (**C**), and 28°C (**D**)

Table 1. Kinetic and thermodynamic activation parameters for X_B xylanases at 5°C using oat spelt as substrate

Parameter	Cold-adapted X_B	Mesophilic X_B
k_{cat} (s^{-1})	14.8 ± 2.1	4.9 ± 0.4
ΔH^* ($J mol^{-1}$)	45390 ± 860	49890 ± 200
ΔS^* ($J mol^{-1} K^{-1}$)	−25 ± 5	−17 ± 1.8
ΔG^* ($J mol^{-1}$)	52340 ± 1200	54620 ± 200

Sugar composition of glycosidic moieties

The amount of total hexoses estimated by the phenol-sulfuric acid method is 25 ± 5 μmole·μmole^{-1} for both xylanases. The detailed sugar composition of the glycosidic part indicates that, in both xylanases, the hexose content is mannose 20 ± 1, glucose 4 ± 1, and glucosamine and galactose 2 ± 1.

Kinetic parameters and thermostability

At 5°C (Table 1), the specific activity of the psychrophilic enzyme is three times as great as that of the mesophilic xylanase. This difference extends, in fact, over the range of 0° to 20°C. The activation energies, E_a, of the psychrophilic and mesophilic enzymes are 47.7 kJ and 52.2 kJ mol^{-1}, respectively, in good agreement with what is already known about psychrophilic enzymes (Hochachka and Somero 1984). The stability of the cold xylanase also appears to be much lower than that of its mesophilic counterpart, because at 30°C the half-life of the cold enzyme is 60 min whereas the mesophilic enzyme is quite stable at this temperature (Fig. 2A). The differential scanning calorimetry (DSC) experiment also clearly illustrates the higher thermosensitivity of the cold xylanase. Indeed, the melting point was found to be 48°C and 62°C for psychrophilic and mesophilic enzymes, respectively (Fig. 2B).

Sequence and homology modeling

The primary structure of the xylanase X_B from *C. adeliae*, deduced from the nucleotide sequence of xyl_B, the cDNA encoding for the mature xylanase, is composed of 338 amino acids with a predicted molecular mass of 37426 Da (Fig. 3), close to that of the xylanase from *C. albidus* (37074 Da). These two xylanases exhibit a high degree of isology (84%) and are also close to representatives of family 10 of glycosyl hydrolases, such as Cex of *Cellulomonas fimi* (49.4%) (White et al. 1994) and XynZ of *Clostridium thermocellum*

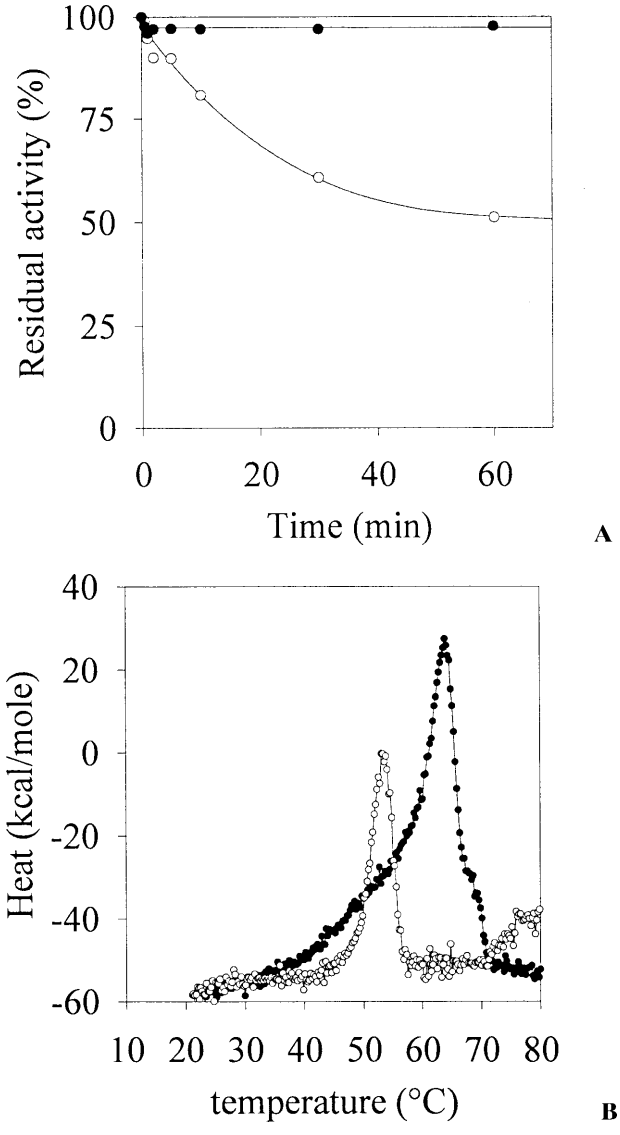

Fig. 2A,B. Thermal stability of the xylanases X_B from *C. albidus* (*solid circles*) and *C. adeliae* (*open circles*). **A** Enzymes were incubated at 30°C in 50 mM sodium acetate, pH 5.4. Residual activities were measured on oat spelt xylan in standard conditions. **B** Absorption of heat as measured by differential scanning calorimetry (DSC) experiments resulting from unfolding of the psychrophilic (*open circles*) and mesophilic (*solid circles*) xylanases at concentration of 1.5 mg ml^{-1} in acetate buffer. Temperature gradient, 1°C min^{-1}

(48.9%) (Dominguez et al. 1995), of known tridimensional structure. These reference structures were used to build tridimensional models of the mesophilic and psychrophilic xylanases from *Cryptococcus* species (Fig. 4). The topology of these modeled structures is very similar and exhibits a common $(\alpha/\beta)_8$ fold typical of family 10 of glycosyl hydrolases. The xylanase from *C. adeliae* differs at 53 positions from the more stable xylanase from *C. albidus*. Analysis of these substitutions indicated that 22 substitutions could account for the adaptation to cold (Table 2).

Discussion

According to previous studies (Van Uden 1984), psychrophilic yeasts display an upper limit of growth at 25°C, which exceeds by 5°C the commonly accepted value for psychrophilic bacteria (Morita 1975). These limits are of course somewhat arbitrary and, in the genus *Cryptococcus*, for example, growth occurs in a continuous spectrum of temperature (Vishniac 1987). In the case of *C. adeliae*, failure to grow at 28°C together with the better growth recorded in the range 4°–12°C when compared to *C. albidus* indicate that the Antarctic strain is physiologically and biochemically adapted to cold.

The molecular mass of the polypeptide as deduced from the nucleotide sequence, together with the mass of the glycosidic moiety, is in good agreement with the molecular mass determined by mass spectrometry. The similar sugar compositions mentioned are not necessarily of identical structure, and the possible different organization of the sugar residues can have a crucial effect on the respective protein stabilities. Glycosylation has been shown to play a role in protein stabilization, possibly through the lower entropy of the unfolded form of the glycoprotein (Jentoft 1990). The role of glycosylation in adaptation to cold, however, requires further studies.

The xylanase X_B secreted by *C. adeliae* displays a higher specific activity at low temperatures and a weak thermal stability, which are common features of cold-adapted enzymes. The commonly accepted hypothesis is that they display an increased structural flexibility, allowing easier conformational changes during catalysis at low tempera-

Table 2. Structural analysis of the 22 amino acids substitutions that could be involved in the adaptation to cold of the xylanase from *Cryptococcus adeliae*

Substitutions and location in the secondary structure in the sense mesophile → psychrophile	Type of effect in terms of destabilization
P27Y (H2), N117G (H6), P252A (loop)	Entropic effect
G32D (loop), Q37K (H3), A127E (loop), Q187K (H9), A194R (H9), G195K (H9), Q309E (loop)	Increase of the surface charge, interaction with the solvent
V57I (H4), V73I (H5), V192I (H9), V257I (H11), V269I (H11) T67S (H5), V262A (H11), V318S (H13), V319T (H13)	Geometry factors, density of packing
D116Q (H6)	Loss of a salt bridge with K112
A188S (H9)	S is a poor α-helix former
E260T (H11)	Loss of a charge–dipole interaction

Comparison is made with the mesophilic xylanase from *C. albidus*

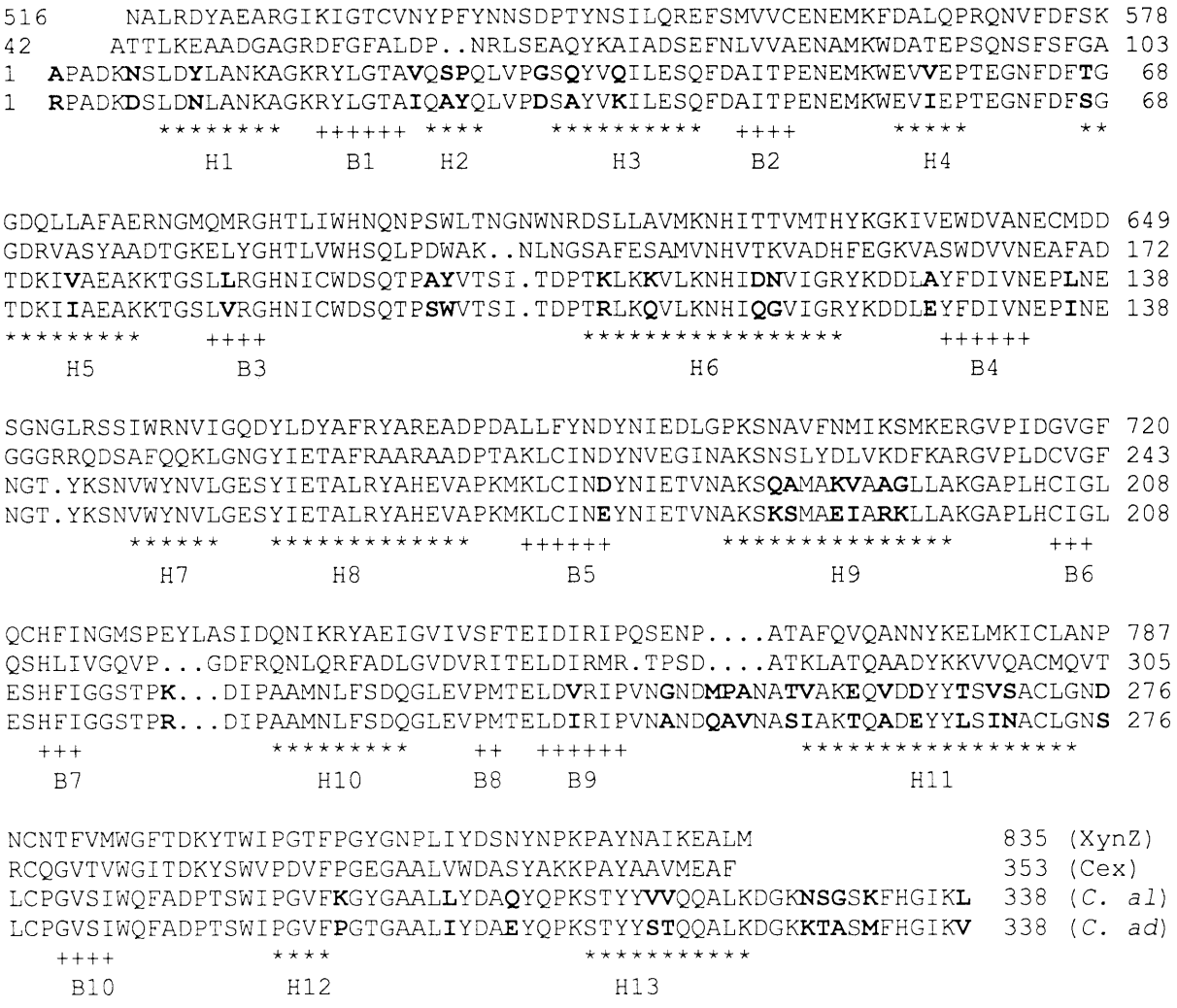

Fig. 3. Multiple amino acid sequence alignment of xylanase XynZ from *C. thermocellum* (*XynZ*), Cex from *C. fimi* (*Cex*), and xylanases from *C. albidus* (*C. al*) and *C. adeliae* (*C. ad*). Amino acid substitutions between cold-adapted xylanase (*C. ad*) and its mesophilic homologue (*C. al*) are shown in ***bold***. *Numbers* flanking the amino acid sequences are those found in the databank. The secondary structure as α-helices (*H*) and β-sheets (*B*) are represented by *asterisks* and *pluses*, respectively, under the multiple alignment. The number attributed to H or B refers to the corresponding secondary structure number

tures (Hochachka and Somero 1984), so that the transition state of the enzyme–substrate complex is reached using a lower activation energy (see Table 1). The thermal stability of both psychrophilic and mesophilic enzymes together with the DSC experiments support this hypothesis. Lower melting temperatures of psychrophilic enzymes when compared to their mesophilic counterparts, corresponding to the transition N→U, have also been observed in other enzymes from Antarctic strains such as the metalloprotease from *Pseudomonas aeruginosa* (J.P. Chessa, J.M. François, I. Petrescu, J. van Beeumen, and C. Gerday, submitted for publication), the chitobiase from A*rthrobacter* sp. TAD20 (J. Zoidiakis, personal communication), the α-amylase from *Alteromonas haloplanctis* (Feller et al. 1999), and the β-lactamase from *Psychrobacter immobilis* A8 (G. Feller, personal communication).

The high percentage of identity between the amino acid sequences of the psychrophilic and mesophilic xylanase points to the importance of discrete changes of the tertiary structure in the adaptation process, enabling catalysis at low temperatures. Among the 53 amino acid substitutions, 22 could contribute to the increased flexibility of the cold enzyme. Their possible effects are described in Table 2. Of particular importance are the following modifications:

D116 is replaced in the cold enzyme by a glutamine: this substitution eliminates the salt bridge formed with K112 within helix H6 (see Fig. 4). The influence of a single salt bridge on protein stability is well documented. For example, in the case of T4 lysozyme, an electrostatic interaction can contribute to an increase in the energy of stabilization by 3–5 kcal mol^{-1} (Anderson et al. 1990). This effect strongly depends on the environment of the salt bridge, however.

In helix H11, E260 is replaced by a threonine in the psychrophile: the negative charge of E260, located near

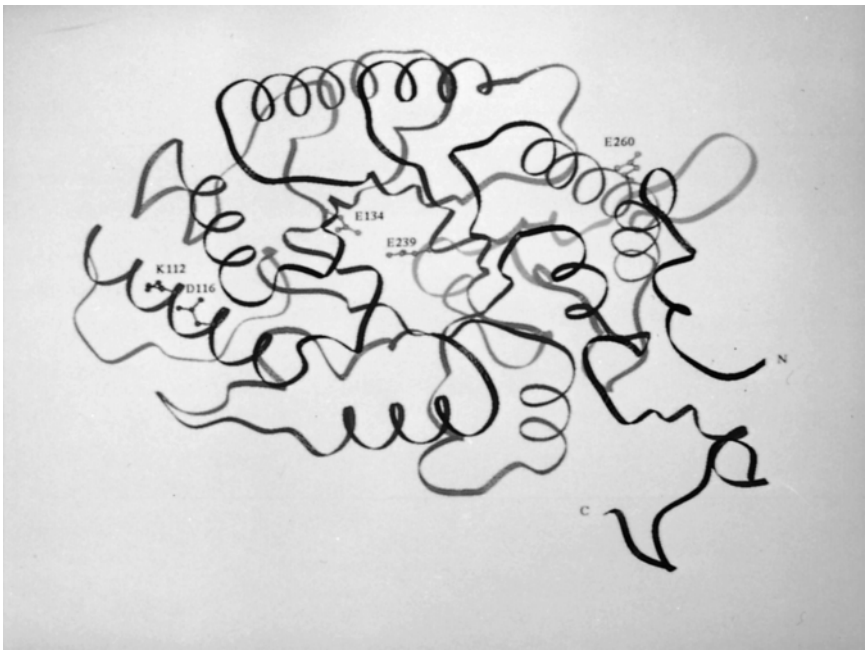

Fig. 4. Diagram of the common α-carbon tracing of the xylanases from *C. albidus* and *C. adeliae*. On the common $(\alpha/\beta)_8$ topology, two stabilizing substitutions are shown: the salt bridge occurring in the mesophile between D116 and K112 and the charge–macrodipole stabilizing interaction attributed to E260. The two catalytic glutamates (134 and 239) are shown in the core of the $(\alpha/\beta)_8$ barrel

the NH_2-terminus of the α-helix, can interact with the helix dipole and therefore stabilize the mesophilic xylanase compared to the cold-adapted one (Fig. 4) (Eijsink et al. 1992; Nicholson et al. 1991).

The hydrophobic packing near tyrosines 265 and 266 is less compact in the case of the psychrophile in which A262, S318, and T319 replace the corresponding valines. A similar negative effect is expected at position 67 where a threonine is replaced by a serine. Also, T67 exerts a stabilizing effect by decreasing the entropy of the mesophilic unfolded form (Alber et al. 1987).

The presence of a P or G at certain positions of the polypeptide chain can stabilize or destabilize native proteins by decreasing or increasing, respectively, the entropy of the unfolded state of the protein (Matthews et al. 1987; Hardy et al. 1993). Three substitutions of this type occur in the cold xylanases P27Y, N117G, and P252A, and possibly contribute to the destabilization of the cold-adapted xylanase.

More controversial is the replacement of valine residues in the mesophilic enzyme by isoleucine in the cold enzyme; this replacement occurs five times in α-helices H4, H5, H9, and H11. Isoleucine is a better α-helix former than valine (Blaber et al. 1993), and the transition of valine to isoleucine has been frequently observed from thermophilic to mesophilic proteins (Argos 1989). Isoleucine probably does not provide an ideal arrangement of the hydrophobic core, so that dense packing cannot be achieved.

Another important factor is the relative abundance of charged residues at the surface of the protein, which, by increasing interaction with the solvent, can contribute to protein destabilization (Schiffer and Dötsch 1996). This strategy has been possibly adopted by some psychrophilic enzymes such as subtilisin (Davail et al. 1994; Narinx et al. 1997) and is apparently also used by the psychrophilic xylanase; indeed, the substitutions G32D, Q37K, A127E, Q187K, A194R, G195K, and Q309E occur at the surface of the molecular edifice.

The ratio R/(R + K) is usually high in thermophilic enzymes because of the number of interactions, as many as five (Borders et al. 1994), that can be established between arginine residues and their environment. Surprisingly, this ratio is not lower in the case of the psychrophilic xylanase; on the contrary, it is higher (27%) than that (16.1%) found in the mesophilic enzyme. This situation has also been encountered in other psychrophilic enzymes such as subtilisin (Davail et al. 1994, Narinx et al. 1997) and a Ca^{2+}–Zn^{2+} metalloprotease (J.P. Chessa, J.M. François, I. Petrescu, J. van Beeumen, and C. Gerday, submitted for publication). The implication of arginine residues in the stability of a protein of course depends on the location of this residue in the molecular edifice.

In conclusion, the cold-adapted xylanase from *C. adeliae* also appears to have evolved toward a molecular structure characterized by a high plasticity that is reflected by its thermosensitivity. Several structural parameters could be involved in the the adaptation of the enzyme to low temperature. All are, however, hypothetical and must be corroborated by site-directed mutagenesis experiments.

Acknowledgments We are grateful to the Institut Français pour la Recherche et la Technologie Polaires for the support and facilities offered at the Antarctic Station J S. Dumont d'Urville. This work has been supported by a concerted action ARC 93-98/170 (Belgium) and by the European Union contracts CT97-013, CT95-0017, and CT96-0051. We also thank the Fonds National de la Recherche Scientifique for their continous support (contract n°2.4523.97 to Charles Gerday). The expert technical assistance of Iris Thamm and Nicole Gérardin-Otthiers is also greatly appreciated.

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