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Molecular adaptation to cold of an Antarctic bacterial lipase

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Abstract

The lipase from the Antarctic psychrophilic bacterium *Psychrobacter immobilis* B10 shows a very limited thermal stability when compared to the lipase from a mesophilic strain of *Pseudomonas aeruginosa*. The thermal dependence of its activity is shifted by at least 30°C towards low temperatures and its activation energy is reduced by a factor of 2.

The three-dimensional model of the *P. immobilis* lipase reveals several features typical of cold-adapted enzymes: a very low proportion of arginine residues as compared to lysines, a low content in proline residues, a small hydrophobic core, a very small number of salt bridges and of aromatic–aromatic interactions. All these properties should confer on the enzyme a more flexible structure, in accord with its low activation energy and its low thermal stability.

Keywords: Lipase; Molecular modeling; Antarctic bacterium; Psychrophile; Adaptation to cold

1. Introduction

The rate of chemical reactions, and of enzyme-catalysed reactions in particular, is strongly influenced by the temperature. A decrease of the temperature by 10°C can readily reduce the activity of an enzyme by a factor of 2 or 3. Ectothermic organisms living in permanently cold environments such as the polar regions must therefore compensate for the loss of enzyme activity in order to maintain their metabolism at a level compatible with life [1,2]. To restore the desirable level of activity in their tissues they could produce more enzyme but this would be very expensive in terms of metabolic costs. Another strategy consists in producing truly adapted enzymes displaying a high catalytic efficiency at temperatures close to 0°C.

Several enzymes belonging to the families of trypsin, subtilisin, α-amylase, etc. have now been isolated from Antarctic fish and bacteria [3,4]. They all have in common a high specific activity in the temperature range 0–30°C and a marked sensitivity to thermal denaturation when compared to homologous enzymes from mesophilic organisms. A hypothesis was formulated to explain this behaviour [1]. Cold-adapted enzymes are supposed to have a more flexible structure that would allow them to undergo the conformational changes necessary for catalysis with a lower energy demand. Their structure is believed to be less compact, less hydrophobic and maintained by a relatively small number of

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intramolecular weak bonds. This hypothesis would at the same time explain the enhanced lability of cold-adapted enzymes.

The nucleotide sequence of the gene encoding a lipase from the Antarctic psychrophilic bacterium *Psychrobacter immobilis* B10 has already been published [5,6]. The present paper reports enzymatic experiments showing the adaptation to cold of this enzyme. A molecular model of the lipase has been built to try and explain the molecular features responsible for this adaptation.

2. Material and methods

2.1. Strains and cultures

Psychrobacter immobilis B10 was isolated at the French Antarctic Station Dumont d'Urville (Terre Adélie: 66° 40′ S – 140° 01′ E). *Pseudomonas aeruginosa* 45377 was supplied by the Laboratory of Microbiology at the University Hospital of Liège. Both strains were cultivated in Luria Broth in 6 l flasks equipped with an air diffuser. *P. immobilis* was grown at 4°C and *P. aeruginosa* at 37°C.

2.2. Concentration and dialysis of culture supernatants

The culture supernatants of *P. immobilis* and *P. aeruginosa* were concentrated 20 times and dialysed against 5 volumes of 50 mM Tris–HCl buffer at pH 8.0 using a Minitan ultrafiltration device (Millipore) equipped with four PTGC membranes with a 10 kDa cut-off.

2.3. Detection of lipase activity

The strains were grown on agar plates containing an emulsion of tributyrin (0.5% v/v) or Lipase Reagent (1% v/v) made from cotton seed oil (DIFCO). The secreted lipolytic activity produced a clear halo around the colonies. Lipolytic activities were assayed spectrophotometrically using 1 mM 4-nitrophenylbutyrate dissolved in a 100 mM phosphate buffer at pH 7.25 containing 100 mM NaCl.

2.4. Molecular modeling of the P. immobilis lipase

The program BLAST [7] was used to screen the NRL-3D Data Bank for a protein of known crystallographic structure showing a maximum sequence identity with the lipase from *P. immobilis* B10. The model was based on the fold of the haloalkane dehalogenase from *Xanthobacter autotrophicus* GJ10 [8] and was constructed with the program COMPOSER [9,10]. Structurally conserved regions (SCR) were determined from a sequence alignment obtained with the program BESTFIT of the GCG package [11]. Within the SCR's, all backbone and conserved amino acid residues side chains were given the same atomic coordinates as in the original structure. The side chain conformation of non-conserved residues was selected following rules based on the nature of the residue and on the secondary structure in which it appeared. Loops and insertions were modeled whenever possible from similar sequence stretches found in a local database of over 200 protein structures at a minimum resolution of 2 Å. Short contacts between side-chains were relieved and optimal conformations were obtained by energy minimisation of the model calculated with the molecular dynamics program AMBER [12]. The molecular structures were visualized and manipulated with the program Insight II 95.0 (Biosym/MSI).

3. Results and discussion

3.1. Growth and lipase production of P. immobilis B10

The highest lipase secretion was obtained when the cells were cultivated at 4°C. At this temperature their doubling time is approxi-

mately 4 h. At higher temperatures, as usually observed with Antarctic psychrophiles [13], the cells grow more rapidly but the enzyme secretion is deeply altered and a large part of the secreted activity is heat-inactivated during the cultivation time. The lipase produced by *P. immobilis* B10 is presumably a lipoprotein and is secreted into the culture supernatant in the form of large aggregates containing lipopolysaccharides [6]. These aggregates are resistant to mild detergents, to moderate sonication, to non-denaturing concentrations of urea or guanidinium chloride, etc. We were not able therefore to isolate the lipase in a pure and active form.

3.2. Thermal dependence and stability of the P. immobilis lipase.

The activity of the dialysed supernatant was measured as a function of temperature and compared to the lipolytic activity secreted by the mesophilic bacterium *Pseudomonas aeruginosa* 45377. Fig. 1 shows the large shift of the activity towards the low temperatures observed with the Antarctic lipase. The activity of the *P. aeruginosa* lipase still increases above 60°C and tends to zero below 20°C. By contrast, the *P. immobilis* lipase presents an apparent optimal activity around 35°C and retains approximately 20% of its activity at temperatures close to 0°C. The activation energies of the reactions catalysed by the two enzymes were calculated from the Arrhenius plots derived from the activity curves presented in Fig. 1. The value obtained for the Antarctic enzyme (63 kJ/mol) is almost twice as low as in the case of the mesophilic enzyme (110 kJ/mol). This property can be directly related to the particular ability of the Antarctic lipase to perform catalysis efficiently in a low temperature environment.

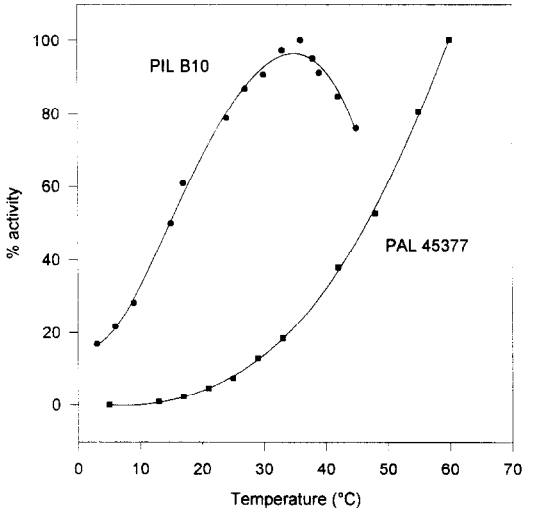


Fig. 1. Thermal dependence of lipase activity produced by the psychrophilic strain *Psychrobacter immobilis* B10 and by the mesophilic strain *Pseudomonas aeruginosa* 45377 determined with 4-nitrophenylbutyrate as the substrate.

The *P. immobilis* lipase is also characterized by a high sensitivity to thermal denaturation. Its half-life at 60°C is reduced by two orders of magnitude when compared to the mesophilic enzyme (2 min *versus* 120 min). This marked lability of the *P. immobilis* lipase together with its high catalytic efficiency near 0°C clearly denote the adaptation to cold of this enzyme.

3.3. Molecular modeling of the P. immobilis lipase

Among the proteins of known crystallographic structure, the haloalkane dehalogenase from *Xanthobacter autotrophicus* GJ10 (XAH) [8] shows maximum sequence identity (21%) to the *P. immobilis* lipase (PIL) and served as a template for modeling the lipase structure. We also found it important to compare PIL to the crystallographic structure of the lipase from *Pseudomonas glumae* (PGL) [14], since this enzyme is, up to now, the only bacterial lipase with a known three-dimensional structure. The two lipase sequences are 11% identical. Both *X. autotrophicus* GJ10 and *P. glumae* are mesophilic bacteria. In contrast to cold-adapted enzymes, the dehalogenase is characterized by a low catalytic efficiency resulting from a high Michaelis constant and a low turnover number [8]. Unfortunately, to our knowledge, no data are available concerning the thermal depen-

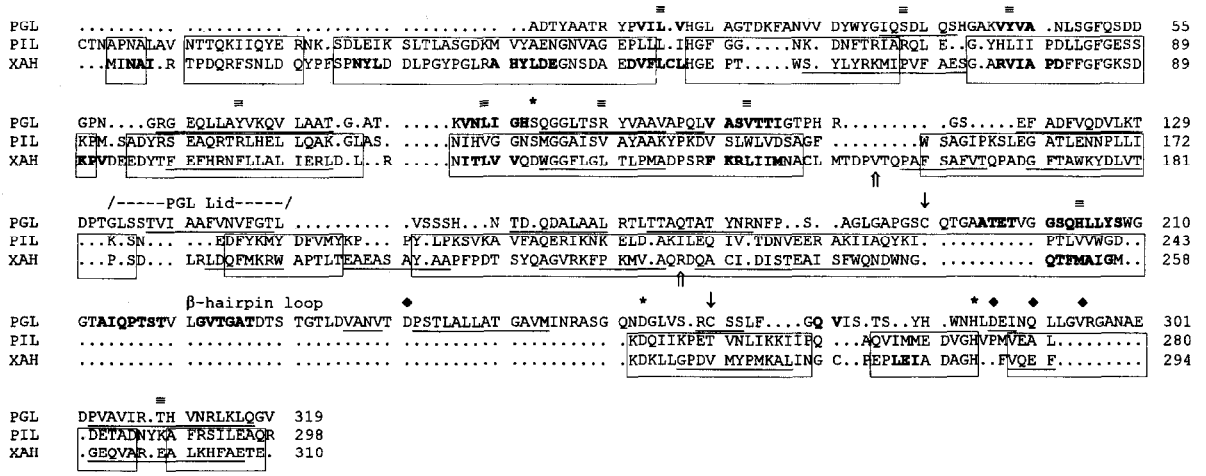


Fig. 2. Sequence alignment of the *Psychrobacter immobilis* B10 lipase (PIL) and of two reference proteins, the haloalkane dehalogenase from *Xanthobacter autotrophicus* GJ10 (XAH) and the lipase from *Pseudomonas glumae* PG1 (PGL). The structurally conserved regions (SCR) of PIL and of XAH are boxed. Helical regions are underlined; β-strands are in boldface. Meaning of symbols: ≡, secondary structure elements conserved in the crystallographic structures; *, catalytic triad residues; ◆, calcium ligands in PGL; ↓, Cys forming the disulfide bridge in PGL; ⇑, limits of domain II. The lid and the β-hairpin loop in PGL are also indicated.

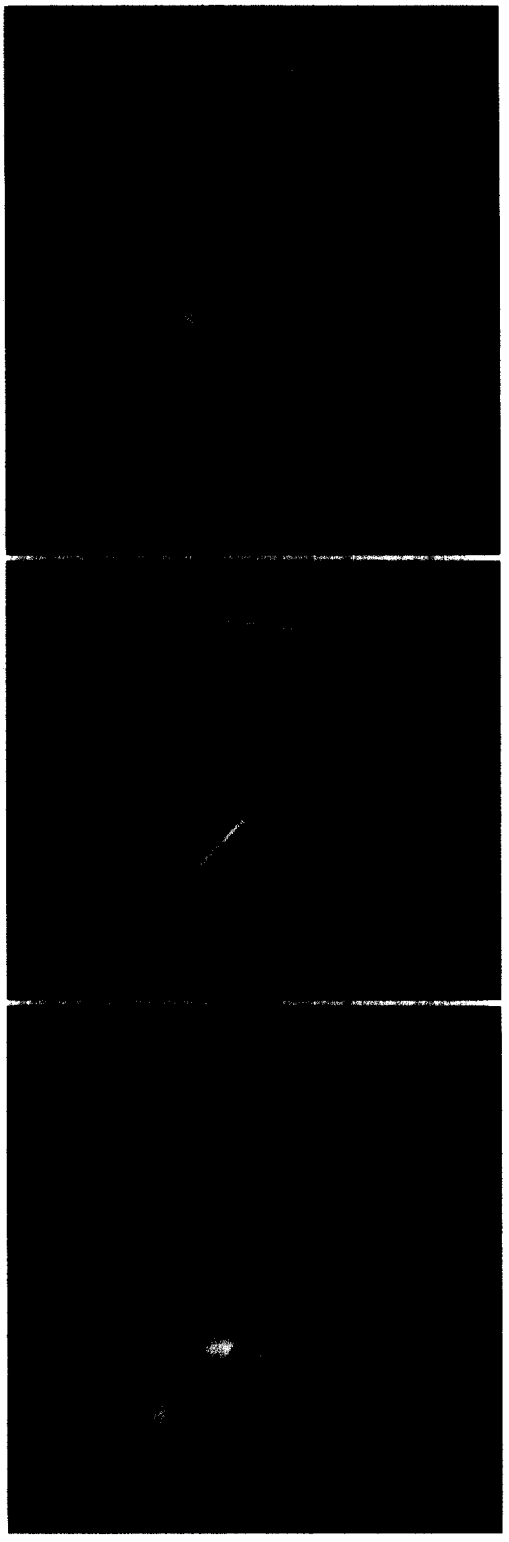
dence of the PGL's activity. However, PGL was initially selected for use in detergents [15]. We can therefore reasonably assume that PGL is a physically and chemically resistant enzyme much better adapted than PIL to relatively high temperatures and other denaturing agents. The sequence alignment of the two lipases and of the dehalogenase (Fig. 2) shows the structurally conserved regions (SCR), the helical regions and the β-strands. The PGL is characterized by a long insertion forming a hairpin loop outside the molecule, and a helical lid covering the catalytic cavity. The two reference structures belong to the α/β-hydrolase fold described by Ollis et al. [16] and therefore have a large part of their structure in common. Four helices and five β-strands coincide remarkably in space and contribute to the first domain (I) of the molecules (Figs. 2 and 3). The second domain (II) is inserted into the sequence of domain I (Fig. 2). Its structure is variable and contains the helical lid of the lipase. In both cases the catalytic cavity is located at the junction between the two domains. The first domain of PIL is very well conserved as compared to the two reference structures. The structure of the second domain is more uncertain because of a poor sequence identity with the dehalogenase or any other structure available. Nevertheless, a careful examination of the three molecules reveals striking differences summarized in Table 1. PIL has small hydrophobic clusters involving only 19 residues in four groups. This might decrease the hydrophobic interactions in the core of the molecule and therefore destabilize its structure. The very high number of Phe-residues in the dehalogenase would have the opposite effect.

40% of Pro residues in XAH, most of them located in loops, are substituted in PIL, giving

Table 1
Structural features related to the stability of the *P. immobilis* lipase (PIL) as compared to the reference structures of XAH and PGL

	XAH	PIL	PGL
hydrophobic clusters[a]	5c/32r	4c/19r	4c/40r
Phe	23	8	8
Pro	23	14	10
Arg/(Lys + Arg)	56%	29%	65%
% charged residues	23	24	12
aromatic–aromatic interactions[b]	14i/16r	2i/3r	5i/8r
salt bridges[c]	11 (+3)	3	2 (+1)
disulfide bridges	–	–	1
calcium sites	–	?	1

[a] Number of clusters/number of residues involved.
[b] Number of interactions/number of residues involved.
[c] Less stabilizing salt bridges are mentioned in brackets.



rise to a more flexible backbone in these regions of the molecule.

PIL is also characterized by a very small number of electrostatic interactions between aromatic rings which can significantly contribute to the stability of proteins [17]. Moreover, Arg residues are considered more stabilizing when compared to Lys because of their larger hydrophilic head and their ability to establish as many as five polarized interactions [18]. Interestingly, the proportion of Arg is drastically reduced in PIL (Table 1); this could further contribute to destabilize the enzyme.

The structure of XAH is rigidified by 11 salt bridges (Table 2). Three other ion pairs are present but have a weaker stabilizing effect since they involve residues which are close to each other in the primary structure. The two domains of the molecule are linked together by four salt bridges. Moreover two other salt bridges (Arg 64–Glu 293 and Lys 261–Asp 170) stabilize the direct environment of His 289 and Asp 260 belonging to the catalytic triad and restrain the mobility of these two important residues.

PGL and PIL do not differ much with respect to their number of salt bridges and of aromatic–aromatic interactions (Tables 1 and 2). However, PGL is marked by a strong hydrophobicity, as deduced from the large size of its hydrophobic core and from its very low content in charged residues (Table 1), especially at the surface of the molecule. These features significantly differentiate PGL from PIL and could strongly favour the stability of PGL. Like in XAH, the catalytic triad of PGL seems to be particularly stabilized: a disulfide bridge and a calcium ion, which is essential for the activity [14], are probably crucial for maintaining the local structure around the catalytic cavity. One

Fig. 3. Schematic view of the three-dimensional structure of XAH (A), PIL (B), and PGL (C). The histidine of the active site is shown in each model. The disulfide bridge and the calcium ion in PGL are depicted in yellow.

Table 2
List of ion pairs in PIL as compared to the two reference molecules XAH and PGL. I and II indicate the domains of the molecule involved in the salt bridge. Less stabilizing salt bridges are shown in brackets

XAH		PIL		PGL	
R6–D89	I–I	K38–E27	I–I	(K128–D125)	II–II
R64–E202	I–II	R104–E203	I–II	R257–D125	I–II
R64–E293	I–I	K188–E20	II–I	R298–D36	I–I
R76–D40	I–I				
R112–D27	I–I				
(R140–D137)	I–I				
R140–D114	I–I				
K142–D48	I–I				
(R186–D184)	II–II				
R193–D178	II–II				
R220–E94	II–I				
K224–E94	II–I				
K261–D170	I–II				
(R300–E296)	I–I				

of the 2 Cys residues of the disulfide bridge is located only 6 positions apart from Asp 263 of the active site. The calcium ion is coordinated by Asp 241 in helix α8 and by 3 other ligands (OD1–Asp 287, O–Gln 291, O–Val 295) very close in the sequence to His 285 of the active site [14].

PIL displays a very small number of salt bridges when compared to the dehalogenase (Table 1) because the ionic residues concerned are substituted in PIL. Only two salt bridges subsist between the two domains instead of four in XAH. The presence of a calcium ion in PIL cannot be ruled out but no calcium site equivalent to the one found in PGL could be identified in PIL. Moreover, no particular structural element maintains the catalytic triad of the Antarctic enzyme. Its catalytic cavity would therefore possess more conformational freedom and, consequently, it would be energetically easier for the enzyme to accommodate the substrate, to form the activated complex and to release the products. This feature in particular might be related to the low activation energy of the Antarctic enzyme and would favour its adaptation to cold.

4. Conclusion

Apparently PIL, like other enzymes from psychrophiles studied so far, is characterized by a relatively unstable structure mainly due to a very small hydrophobic core and to a reduced number of internal electrostatic bonds. The model of PIL tends therefore to confirm the hypothesis that the high catalytic efficiency of this enzyme at low temperature and its low thermal stability are related to a high flexibility of the molecule's structure.

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References

[1] P. Hochachka and G. Somero, Biochemical Adaptation, Princeton University Press, Princeton, NJ, 1984, pp. 355–449.
[2] R. Margesin and F. Schinner, J. Biotechnol., 33 (1994) 1–14.
[3] J.L. Arpigny, G. Feller, S. Davail, S. Genicot, E. Narinx, Z. Zekhnini and Ch. Gerday, Adv. Comp. Environ. Physiol., 20 (1994) 270–295.
[4] G. Feller, E. Narinx, J.L. Arpigny, M. Aittaleb, E. Baise, S. Genicot and Ch. Gerday, FEMS Microbiol. Rev., 18 (1996) 189–202.
[5] J.L. Arpigny, G. Feller and Ch. Gerday, Biochim. Biophys. Acta, 1171 (1993) 331–333.
[6] J.L. Arpigny, G. Feller and Ch. Gerday, Biochim. Biophys. Acta, 1263 (1995) 103.
[7] S.F. Altschul, W. Gish, W. Miller, E.W. Myers and D.J. Lipman, J. Mol. Biol., 215 (1990) 403–410.
[8] K. Verschueren, S. Franken, H. Rozeboom, K. Kalk and B. Dijkstra, J. Mol. Biol., 232 (1993) 856–872.
[9] M. Sutcliffe, I. Haneef, D. Carney and T. Blundell, Protein Eng., 1 (1987) 377–385.

[10] M. Sutcliffe, F. Hayes and T. Blundell, Protein Eng., 1 (1987) 385–392.
[11] J. Devereux, P. Haeberli and O. Smithies, Nucl. Acid Res., 12 (1984) 387–393.
[12] S. Weiner, P. Kollman, D. Nguyen and D. Case, J. Comput. Chem., 7 (1986) 230–236.
[13] G. Feller, E. Narinx, J.L. Arpigny, Z. Zekhnini, J. Swings and C. Gerday, Appl. Microbiol. Biotechnol., 41 (1994) 477–479.
[14] M. Noble, A. Cleasby, L. Johnson, M. Egmond and L. Frenken, FEBS Lett., 331 (1993) 123–128.
[15] L. Frenken, J. Bos, C. Visser, W. Müller, J. Tommassen and C. Verrips, Mol. Microbiol., 9 (1993) 579–589.
[16] D.L. Ollis, E. Cheah, M. Cygler, B. Dijkstra, F. Frolow, S. Franken, M. Harel, S. Remington, I. Silman, J. Shrag, J. Sussman, K. Verschueren and A. Goldman, Protein Eng., 5 (1992) 197–211.
[17] S. Burley and G. Petsko, Science, 229 (1985) 23–28.
[18] C.L. Borders, J.A. Broadwater, P.A. Bekeny, J.E. Salmon, A.S. Lee, A.M. Eldridge and V.B. Pett, Protein Sci., 3 (1994) 541–548.