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Nucleotide Sequence of the Lipase Gene *lip2* from the Antarctic Psychrotroph *Moraxella* TA144 and Site-Specific Mutagenesis of the Conserved Serine and Histidine Residues

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ABSTRACT

The *lip2* gene from the antarctic psychrotroph *Moraxella* TA144 was sequenced. The primary structure of the Lip2 preprotein deduced from the nucleotide sequence is composed of 433 amino acids with a predicted M_r of 47,222. This enzyme contains a Ser-centered consensus sequence and a conserved His-Gly dipeptide found in most lipase amino-terminal domains. These sequences are involved in the lipase active site conformation since substitution of the conserved Ser or His residues by Ala and Gln, respectively, results in the loss of both lipase and esterase activities. Structural factors that would allow proper enzyme flexibility at low temperatures are discussed. It is suggested that only subtle changes in the primary structure of these psychrotrophic enzymes can account for their ability to catalyze lipolysis at temperatures close to 0°C.

INTRODUCTION

THE COMPARISON between properties of extremophilic proteins and of their mesophilic counterparts is commonly used to gain further insights in the structure–function and structure–stability relationships of enzymes (Mozhaev *et al.*, 1988). However, enzymes from psychrotrophic bacteria have received little attention when compared to numerous studies of enzymes of thermophilic species, despite the fact that they raise considerable interest in both fundamental and applied fields. These enzymes are characterized by higher k_{cat} and physiological efficiency (k_{cat}/K_m) and by a lower and rather constant K_m at temperatures close to 0°C. In addition, psychrotrophic enzymes display an apparent optimal activity shifted towards lower temperatures and manifest pronounced heat lability (Hug and Hunter, 1974; Morita, 1975; Somero, 1977). These unique properties potentially confer great economical value to cold-active enzymes as enzymatically driven reactions can be carried out in a temperature range of 0–20°C at which homologous mesophilic enzymes are essentially inactive (Dambmann and Aunstrup, 1981; Genicot *et al.*, 1988). One can also use the heat lability of these enzymes to inactivate them selectively in a complex medium, as proposed for an antarctic bacterial alkaline phosphatase (Kobori *et al.*, 1984).

The study of the structural and catalytic properties of these cold-active proteins is still fragmentary and the molecular basis of protein adaptation to cold remains unknown (Hochachka and Somero, 1984; Jaenicke, 1990). A more flexible conformation of these enzymes, responsible for their thermal sensitivity, may reduce the activation energy and increase catalytic efficiency at low temperatures. Elucidation of the three-dimensional structure of some psychrotrophic enzymes will contribute to a better understanding of protein folding and dynamics.

We recently isolated and characterized antarctic *Moraxella* strains excreting lipases (triacylglycerol acyl hydrolase, EC 3.1.1.3) and showed that these thermolabile enzymes efficiently catalyze lipolysis at temperatures as low as 3°C (Feller *et al.*, 1990a). Cloning and expression in *Escherichia coli* of three lipases genes from the strain *Moraxella* TA144 was also described (Feller *et al.*, 1991b). The cloned gene products exhibited lipolytic activity, even at temperatures close to 0°C, and the level of lipase production was strongly temperature dependent due to heat instability of the recombinant enzymes. In this paper, the complete nucleotide sequence of the psychrotrophic lipase 2 gene (*lip2*) is presented, and the functional implication of the conserved Ser and His residues in lipolysis is analyzed by site-directed mutagenesis. The predicted primary struc-

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tures of the three *Moraxella* lipases are compared to those of other lipases and are discussed in relation to the adaptational strategy of enzymes to low temperatures.

MATERIALS AND METHODS

Strain

The origin and characterization of the antarctic psychrotroph *Moraxella* TA144 have been described previously (Feller *et al.*, 1990a) as well as the cloning of three lipase genes from this strain (Feller *et al.*, 1991b).

DNA sequence analysis of the lip2 *gene*

The DNA fragment containing the lipase gene from pULG332 was subcloned into the polylinker of pGEM 3Z f^+ and f^- (Promega). Single-strand DNA was obtained by infecting pGEM 3Zf-transformed *E. coli* JM109 cells with the helper phage M13K07, inducing the bacteriophage f1 replication (pGEM Single Strand System, Promega). The sequence was determined by the dideoxynucleotide chain-termination method using Sequenase (U.S. Biochemical Corp.). Synthetic oligonucleotides complementary to the plasmid SP6 or T7 promoters and internal sequences (provided by Eurogentec SA) were used as primers.

Site-directed mutagenesis

For the construction of mutant Lip2 clones, the *Pvu* II–*Pst* I fragment containing His-165 and Ser-239 was transferred to pSP73 (Promega). Recombinant circle polymerase chain reaction (RCPCR)-mediated site-specific mutagenesis was performed on this construction according to Jones *et al.* (1990). Mutating primers for PCR were designed by positioning 20 nucleotides 5′ to the mismatches and 11 nucleotides 3′ to the mutagenesis region. Nonmutating primers were 20 nucleotides in length. Mutants were generated by insertion of the mutated *Pvu* II–*Pst* I fragment in the original plasmid, in which the corresponding sites of the polylinker were deleted by *Xho* I–*Sal* I digestion and ligation. Purification of the restriction fragments from 1% agarose gel was carried out with Geneclean (Biol01).

Enzyme assays

Lipase activity was assessed by a sensitive solid-medium assay using Difco Lipase Reagent, whereas esterase activity was monitored spectrophotometrically on whole-cell suspensions using the chromogenic substrate *p*-nitrophenylbutyrate (Feller *et al.*, 1991b).

RESULTS

The DNA fragment containing the *lip2* gene from the antarctic psychrotroph *Moraxella* TA144, conferring lipolytic activity to *E. coli*, was sequenced on both strands (Fig. 1). The nucleotide sequence, 2,134 bp in length, is shown in Fig. 2 along with the region coding for the cold-active lipase Lip2 and the flanking sequences. Upstream from the ATG codon are short sequences that may function as Shine–Dalgarno translational initiation sites (Stormo *et al.*, 1982) and −35, −10 promoters (Staden, 1984). The open reading frame is terminated by a stop codon and two inverted repeat sequences that may function as transcriptional termination signals. The deduced primary structure of the preprotein is 433 amino acids with a predicted M_r of 47,222. Boxed in Fig. 2 are two hydrophobic peptides containing a conserved His–Gly dipeptide (Van Oort *et al.*, 1989) and the Gly–X_1–Ser–X_2–Gly consensus sequence around the potential active Ser residue (Persson *et al.*, 1989). In Fig. 3, these peptides have been aligned with the corresponding regions of lipases Lip1 and Lip3 of the parent strain *Moraxella* TA144 and with the conserved sequences of various lipases and fatty acid related enzymes. Interestingly, Lip2 and rat hormone-sensitive lipase display an appreciable degree of homology at the level of these peptides; such a similarity is not frequent, being uniquely encountered in the small gene family of pancreatic, hepatic, and lipoprotein lipases. Hydropathy profiles of *Moraxella* lipases (Fig. 4) show that these conserved peptides are located on sharp positive peaks in the case of Lip2 and Lip3.

Mutant Lip2 clones were generated by recombinant circle PCR on the *Pvu* II–*Pst* I restriction fragment of *lip2* (Fig. 1) containing the conserved His-165 and Ser-239 residues. The mutated DNA was reinserted into the original plasmid pULG332. In control experiments, reinsertion of the unmutated fragment produces 97% lipolytic colonies. Mutant plasmids showing the expected size and restriction patterns were sequenced; pLIPS/A 21 and pLIPH/Q 41 contained only the desired mutation in the *lip2* gene. In pLIPS/A 21, Ser-239 was substituted by Ala (Table 1) because this residue has approximately the same size but lacks the hydroxyl group involved in the charge relay sys-

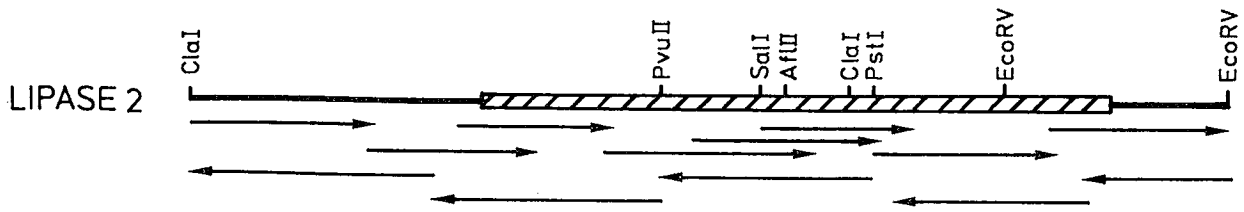

FIG. 1. Physical map of the *lip2* gene from *Moraxella* TA144 showing some restriction endonuclease cleavage sites and Lip2 coding region (dashed box). The sequencing strategy is indicated, arrows showing direction and extent of sequencing.

tem of Ser-active enzymes. *E. coli* cells carrying this mutant plasmid are devoid of both lipase and esterase activities. One clone containing a polymerase error at base 1,177 was found during the screening. This error, which suppresses the unique *Sal* I site and substitutes Val-195 for Ala, was introduced as a single mutation using the *Afl* II site (Table 1). Lipase and esterase activities of the mutant lipase coded by pLIV/A 21 are not affected by this amino acid replacement. Because the function of the His-Gly dipeptide is unknown, the His residue was substituted by a Gln residue of similar volume but lacking the titrable imidazole function. Clones carrying this mutation in pLIPH/Q 41 lost both enzyme activities. Attempts to purify wild or recombinant *Moraxella* lipases were unsuccessful (Feller *et al.*, 1991b) and therefore structural properties of mutant enzymes cannot be investigated.

DISCUSSION

Sequence analysis and functional residues

No significant degree of homology was found between the three lipases from the psychrotroph *Moraxella* TA144, nor with the other known lipase sequences, except at the level of two strongly conserved peptides (Fig. 3). A Gly-X_1-Ser-X_2-Gly consensus sequence around the putative essential serine has been found in all lipases investigated so far (Persson *et al.*, 1989). When extending the comparison to the three psychrotrophic lipases, to cutinase, which is a lipase specialized for polymeric substrates, and to other fatty acid-related enzymes, it can be seen that this pattern is found in all enzymes listed. This sequence does not seem specific to lipase but rather is common to numerous Ser-active hydrolases such as the trypsin-related enzymes which also display a Gly-Asp-Ser-Gly-Gly-Pro sequence at their active site (Dayhoff *et al.*, 1972; Lecroisey and Keil, 1983). Lipases differ however from proteases by a strong hydrophobic surrounding of the essential serine.

The X-ray structure of *M. miehei* and human pancreatic lipases have implicated the conserved Ser, along with Asp and His residues, in the formation of a catalytic triad structurally similar to that of serine proteases (Brady *et al.*, 1990; Winkler *et al.*, 1990). However, other data argue for the involvement of Ser in the adsorption of lipases at interfaces and the occurrence of a distinct esterolytic active site elsewhere in the protein (Chapus *et al.*, 1988). Substitution of Ser-239 by Ala in *Moraxella* Lip2 abolishes both lipase and esterase activities, strongly suggesting the involvement of Ser in a charge relay system and ruling out a distinct catalytic site for esterase activity. These results are in agreement with the recent work of Davis *et al.* (1990).

Van Oort *et al.* (1989) noticed a His-Gly sequence occurring in some lipases. We found that a peptide containing His-Gly located near the amino terminus and independent of the Ser-Asp-His catalytic triad is conserved in almost all lipases including *Moraxella* Lip2 and Lip3 (Fig. 3). Replacement of His-165 by Gln in Lip2 results in complete loss of lipase and esterase activities. This result indicates that the short His-Gly sequence could be an integral part of the active site. The involvement of a second His residue in a Ser-centered active site requires further demonstration but could be an important feature distinguishing the reaction mechanisms of lipases from proteases. Other lines of evidence also suggest its involvement in lipolysis. First, in human pancreatic lipase, the main-chain oxygen atom of His-75 (corresponding to His-165 in Lip2) collides with the α-carbon of Gly-154 in the Gly-X_1-Ser-X_2-Gly consensus sequence; in addition, Phe-77, located immediately downstream from the conserved His-Gly dipeptide, would be a hydrogen donor to the oxyanion (Winkler *et al.*, 1990). Second, when His-75 (conserved) and His-156 from porcine pancreatic lipase are ethoxyformylated, the activity toward tributyrylglycerol is inhibited (De Caro *et al.*, 1989). Finally, the reactive His in subtilisins (also Ser-enzymes) is also included in a conserved His-Gly dipeptide (Nedkov *et al.*, 1985).

Hydropathy analysis of the available lipase sequences reveals that both His- and Ser-centered peptides are located on sharp positive peaks of the hydropathy index, as illustrated in Fig. 4. It is reasonable to assume that this property facilitates lipase adsorption on micellar substrate and the interaction of the active site with the substrate hydrophobic core. By contrast, the location of the Ser active center on the hydropathy profile of *Moraxella* Lip1 is less clear. This lipase, as well as *M. miehei, Candida cylindracea* (Kawaguchi *et al.*, 1989), *Geotrichum* (Shimada *et al.*, 1989) lipases and other fatty acid-related enzymes do not display the His-Gly homology (Fig. 3). These enzymes, as well as cutinase, lysophospholipase, or LCATase, which also act on insoluble substrates, may belong to another class of lipases, differing by the architecture of their active site and their substrate specificity.

Clues of cold adaptation

To our knowledge, the three primary structures of *Moraxella* TA144 lipases are the first examples of enzyme sequences originating from organism adapted to extremely low environmental temperatures (−2° to 2°C). Somero (1977) and Hochachka and Somero (1984) have convincingly argued that in order to reduce the energy of activation and to perform catalysis at low temperature, these enzymes must possess an appropriate folding flexibility. Thermal instability is then regarded as the consequence of the highly flexible structure of cold-active enzymes that can be achieved in several ways. First, thermophilic microorganisms seem to rely on internal hydrophobic interactions to stabilize protein structure (Mozhaev *et al.*, 1988). Indeed, these weak bonds are formed endothermically and thus are stabilized by an increase in temperature. One would therefore expect a reduction of hydrophobic bonding in cold-active enzymes. This is apparently not reflected by the relative content of hydrophobic amino acid residues in the psychrotrophic lipases which fall within interspecies variations, neither by the comparison of hydropathy profile, nor by the relative hydrophobicity around the active centers. The second way to increase the flexibility of the peptide backbone would be to lower the number of covalent disulfide bridges. Even if the number of cysteine residues engaged in disulfide linkage is not known for most of

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                                                                ATC      3
GATGTCATTTTCGATGGTCTCATTAGCGTCAGTATCTGAATTATTTTTCGGATCAAACTCTTT    66
CATGGTTTCTTCTAAAATACTCTCAGCGTGCTCAACATTGTCATGTGACACGTTTTGGTCATG    129
AGATTCATGGTTGGTGTTCTGCTCGCTCATGGTAGCTCCTTAGGATTATTAATAATAATGGTT    192
GGTTTTAAGGCACAATAAACTCGCCAAACGCTGGTTATTGTGCTTTTATTAATGGCCTAACTG    255
TTTTTGTTCGTTCTTTTTAAAATGCGCTTCAATAGCTGCTTGATAAAGGTGGTGAGGATAAAT    318
TTCAAGCCCAACCTGAGGAATTTTTTGTTTTAGCAGGATAAATCATTGCAAAGCGTAAGGATT    381
ATTACTAATACCGCTATAAAAAGTGCTCAACATTAGGATAACTGGCTGGTAGAGACGATTACG    444
TTTTTGTCACCGATATGCGCTTAACTTTATCGTGACTAGCTTTTAAGATAGATTGGAATTTAA    507
TATAAATTTCACGATTAACGCTATATTGACAGTTGACTGATAAAGCCGACTGACTTTGTTTTA    570
                                  - 35
ACACCAATCACAAGAGAGGGTTT ATG CCT ATT CTA CCA GTA CCG GCG CTC AAT    623
-10             SD      Met Pro Ile Leu Pro Val Pro Ala Leu Asn     10

GCC TTG CTG ACA AAA ACG ATT AAA ACC ATT AAA ACT GGG GCC GCT AAA    671
Ala Leu Leu Thr Lys Thr Ile Lys Thr Ile Lys Thr Gly Ala Ala Lys     26

AAT GCT CAT CAG CAT CAT GTT TTG CAC CAT ACG CTA AAA GGA TTG GAC    719
Asn Ala His Gln His His Val Leu His His Thr Leu Lys Gly Leu Asp     42

AAT CTG CCA GCG CCG GTG CTC GAA CGT ATC AAT CGT CGA TTA AAG GCG    767
Asn Leu Pro Ala Pro Val Leu Glu Arg Ile Asn Arg Arg Leu Lys Ala     58

TCT ACA GCA GAG CAA TAT CCT CTA GCC GAT GCC CAT CTG CGC CTG ATA    815
Ser Thr Ala Glu Gln Tyr Pro Leu Ala Asp Ala His Leu Arg Leu Ile     74

CTC GCT ATT AGT AAT AAG CTC AAG CGA CCG TTA GCA ATA GAT AAG CTG    863
Leu Ala Ile Ser Asn Lys Leu Lys Arg Pro Leu Ala Ile Asp Lys Leu     90

CCC AAA CTA CGG CAG AAA TTT GGT ACT GAT GCG GTG TCT TTG CAA GCG    911
Pro Lys Leu Arg Gln Lys Phe Gly Thr Asp Ala Val Ser Leu Gln Ala    106

CCT AGC GTT TGG CAA CAA AAT GCG GAT GCT TCA GGC AGT ACA GAA AAT    959
Pro Ser Val Trp Gln Gln Asn Ala Asp Ala Ser Gly Ser Thr Glu Asn    122

GCC GTC AGC TGG CAA GAT AAA ACC ATT GCT AAT GCC GAT GGT GGT GAT   1007
Ala Val Ser Trp Gln Asp Lys Thr Ile Ala Asn Ala Asp Gly Gly Asp    138

ATG ACC GTG CGC TGC TAC CAG AAA TCG ACG CAA AAT AGT GAG AGA AAA   1055
Met Thr Val Arg Cys Tyr Gln Lys Ser Thr Gln Asn Ser Glu Arg Lys    154

AGT ACG GAT GAA GCC GCC ATG CTA TTT TTT CAT GGC GGC GGA TTT TGT   1103
Ser Thr Asp Glu Ala Ala Met Leu Phe Phe His Gly Gly Gly Phe Cys    170

ATC GGT GAC ATC GAC ACG CAC CAT GAG TTT TGT CAT ACG GTC TGT GCT   1151
Ile Gly Asp Ile Asp Thr His His Glu Phe Cys His Thr Val Cys Ala    186

CAA ACG GGC TGG GCG GTG GTA AGT GTC GAC TAT CGC ATG GCA CCT GAA   1199
Gln Thr Gly Trp Ala Val Val Ser Val Asp Tyr Arg Met Ala Pro Glu    202

TAT CCA GCG CCA ACT GCA CTT AAG GAC TGT CTG GCG GCT TAT GCT TGG   1247
Tyr Pro Ala Pro Thr Ala Leu Lys Asp Cys Leu Ala Ala Tyr Ala Trp    218

CTG GCT GAA CAT TCA CAG TCG CTT GGC GCA TCA CCA TCG CGT ATT GTA   1295
Leu Ala Glu His Ser Gln Ser Leu Gly Ala Ser Pro Ser Arg Ile Val    234

CTA TCT GGT GAT AGT GCG GGC GGT TGC TTG GCA GCA CTG GTT GCA CAG   1343
Leu Ser Gly Asp Ser Ala Gly Gly Cys Leu Ala Ala Leu Val Ala Gln    250

CAG GTT ATC AAA CCT ATC GAT GCA CTA TGG CAA GAT AAT AAT CAA GCT   1391
Gln Val Ile Lys Pro Ile Asp Ala Leu Trp Gln Asp Asn Asn Gln Ala    266

CCT GCA GCC GAT AAA AAA GTC AAT GAC ACT TTT AAA AAC TCA CTG GCT   1439
Pro Ala Ala Asp Lys Lys Val Asn Asp Thr Phe Lys Asn Ser Leu Ala    282

GAT TTA CCG CGA CCT TTA GCA CAA CTG CCG CTA TAT CCT GTC ACT GAT   1487
Asp Leu Pro Arg Pro Leu Ala Gln Leu Pro Leu Tyr Pro Val Thr Asp    298

TAT GAG GCT GAA TAT CCA AGC TGG GAG CTT TAT GGT GAA GGG TTG CTG   1535
Tyr Glu Ala Glu Tyr Pro Ser Trp Glu Leu Tyr Gly Glu Gly Leu Leu    314

CTC GAT CAC AAC GAT GCA GAA GTC TTT AAT TCT GCC TAC ACT CAG CAC   1583
Leu Asp His Asn Asp Ala Glu Val Phe Asn Ser Ala Tyr Thr Gln His    330

AGC GGT CTA CCA CAG TCC CAT CCA CTT ATC TCA GTC ATG CAT GGT GAC   1631
Ser Gly Leu Pro Gln Ser His Pro Leu Ile Ser Val Met His Gly Asp    346

AAT ACG CAA CTG TGT CCC AGC TAT ATT GTC GTT GCG GAA TTA GAT ATC   1679
Asn Thr Gln Leu Cys Pro Ser Tyr Ile Val Val Ala Glu Leu Asp Ile    362

TTA CGA GAT GAA GGA CTC GCC TAT GCC GAA CTT TTG CAA AAA GAG GGC   1727
Leu Arg Asp Glu Gly Leu Ala Tyr Ala Glu Leu Leu Gln Lys Glu Gly    378

GTT CAA GTA CAA ACT TAT ACA GTG CTA GGA GCG CCG CAC GGT TTT ATT   1775
Val Gln Val Gln Thr Tyr Thr Val Leu Gly Ala Pro His Gly Phe Ile    394

AAC TTA ATG AGT GTC CAT CAA GGT CTT GGC AAT CAG ACA ACT TAT ATT   1823
Asn Leu Met Ser Val His Gln Gly Leu Gly Asn Gln Thr Thr Tyr Ile    410

ATC AAT GAG TTT GCT TGT CTT GTG CAA AAC CTA CTG ACC AGT GAG GGT   1871
Ile Asn Glu Phe Ala Cys Leu Val Gln Asn Leu Leu Thr Ser Glu Gly    426

GAT AAG CCA AAT CTC AGA GCG TGA TCCCAAACGAAAATGGGATACCCCAAAAGCCG   1926
Asp Lys Pro Asn Leu Arg Ala ***

TAACCAGATGACACTACGAAGTGACTGTTATTGATGACCTCATAGCCATAACCTAAGCGGTTG   1989

ATGGCTTTGATGATAAAATTGGCAGGAATATTTGTCCATTATGGGTATGCCCTGAGAGCTGTA   2052

AATCAATAGGTAACTCACTGTGCTCGGTAATAGCGCTCGGTCTGTGATCATGAGTCGTCTGAA   2115
CTCTACCGTTGCCGATATC
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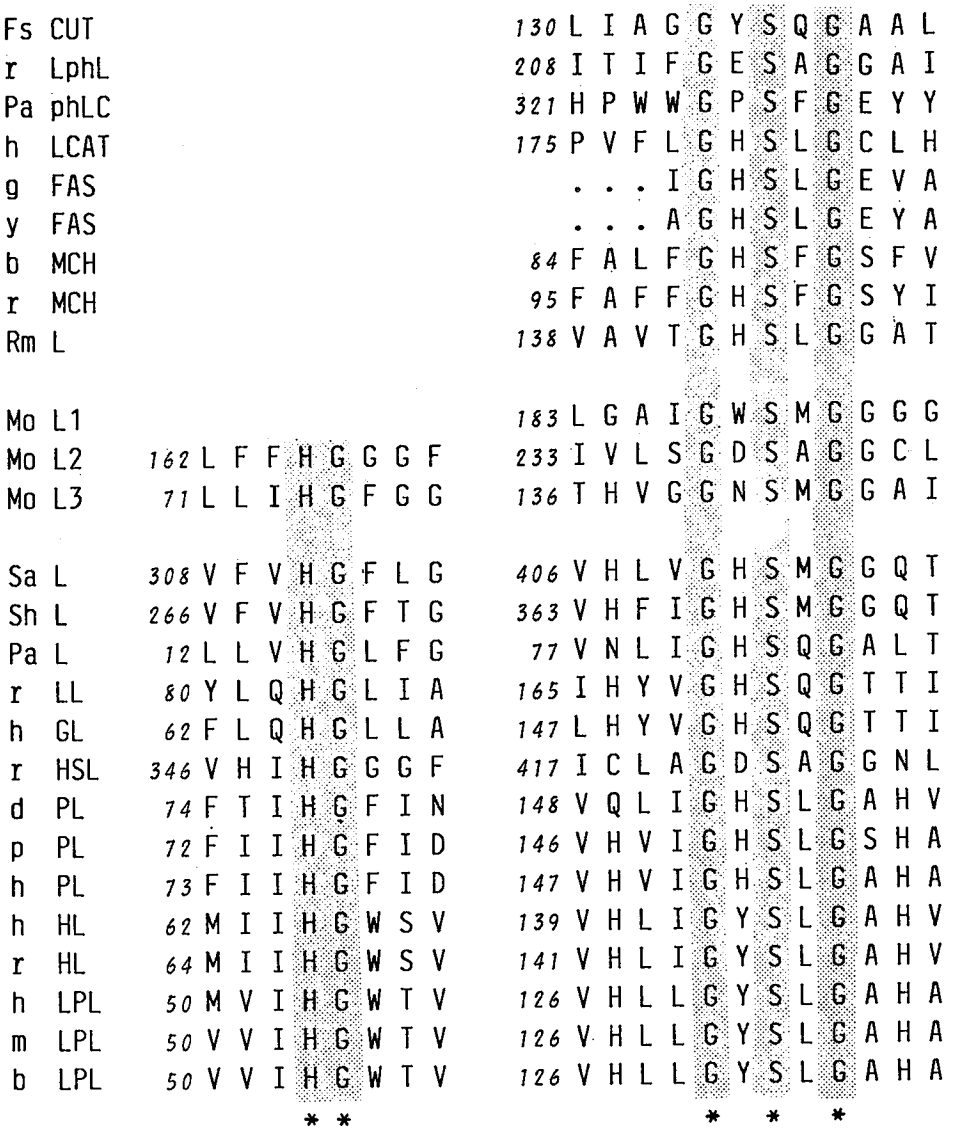

FIG. 3. Alignment of amino acid sequences containing the conserved His–Gly dipeptide and the Ser-centered consensus sequence in lipases and various fatty acid-related enzymes. Abbreviations used and sources: Fs CUT, *Fusarium solani* cutinase (Soliday *et al.*, 1984); r LphL, rat lysophospholipase (Han *et al.*, 1987); Pa phLC, *Pseudomonas aeruginosa* phospholipase C (Pritchard and Vasil, 1986); h LCAT, human lecithin-cholesterol acryltransferase; FAS, *S*-acyl fatty acid synthase thioesterase from g, goat (Mikkelsen *et al.*, 1985), and y, yeast (Engeser *et al.*, 1979); MCH, medium-chain *S*-acyl fatty acid synthase thio ester hydrolase from b, bird (Poulose *et al.*, 1985), and r, rat (Safford *et al.*, 1987); L, microbial lipases from Rm, *Rhizomucor miehei* (Boel *et al.*, 1988), Mo, *Moraxella* TA144 Lip1 (Feller *et al.*, 1990b), Lip2 (this study), Lip3 (Feller *et al.*, 1991a), Sa, *Staphylococcus aureus*, Sh, *Staphylococcus hyicus* (Götz *et al.*, 1985) and Pf, *Pseudomonas fragi* (Aoyama *et al.*, 1988); r LL, rat lingual lipase; h GL, human gastric lipase; r HSL, rat hormone-sensitive lipase (Holm *et al.*, 1988); PL, pancreatic lipases from d, dog, p, pig, and h, human (Lowe *et al.*, 1989); HL, hepatic lipases from h, human, and r, rat; LPL, lipoprotein lipases from h, human, m, mouse, and b, bovine. See Persson *et al.* (1989) for noncited sources.

FIG. 2. *Moraxella* TA144 Lip2 DNA sequence and predicted amino acid sequence. The potential promoter regions (−35, −10), the Shine–Dalgarno sequence (SD), the stop codon (***), and the inverted repeats (arrows) are indicated. Hydrophobic regions around the conserved His–Gly dipeptide and the active Ser residue are boxed.

the sequenced lipases, the lack of cysteine in *Staphylococcus aureus* lipase and the occurrence of only one residue in *Staphylococcus hyicus* and *Pseudomonas fragi* lipases rules out this possibility. The third way would be to increase the relative number of small-sized amino acids, such as glycine, involved in peptide chain β-turn structures. Comparison of glycine content and β-turns predicted by secondary structure analysis in *Moraxella* lipases and in other known lipase sequences does not show any significant differences. However, one common puzzling feature of the three psychrotrophic lipases is the stacking of glycine residues in the His-and Ser-containing conserved sequences (Fig. 3), although also noticeable in staphylococcal- and rat hormone-sensitive lipases. This peculiarity could indeed provide high active site flexibility.

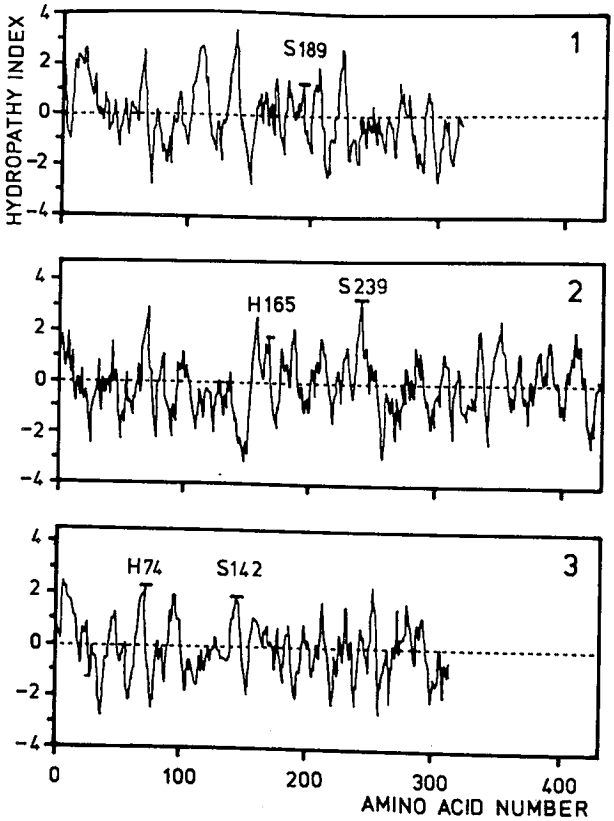

FIG. 4. Hydropathy profiles of the psychrotrophic *Moraxella* TA144 lipases. The conserved His- and Ser-centered hydrophobic peptides are located by horizontal bars. Subscripts refer to the preprotein position number. The method Kyte and Doolittle (1982) was used with an averaging window of the hydropathy indices from 6 amino acids.

Finally, one can expect that the active site primary structure of cold adapted enzymes have not been subjected to extensive modifications to preserve enzyme reaction mechanism. Indeed, the His-Gly pair, when present, and mainly the Gly-X_1-Ser-X_2-Gly sequence are conserved in *Moraxella* lipases. Interestingly, all the lipases investigated so far make use of a His, Tyr, or Glu residue at the X_1 position, suggesting an important function of these amino acids. At this position, *Moraxella* Lip1 and Lip3 include tryptophan and asparagine, which are not found in any other lipases nor in fatty acid-related enzymes (Fig. 3). However, the close homology at this level of *Moraxella* Lip2 and rat hormone-sensitive lipase, both having an aspartic acid at the X_1 position, suggest that variability at this position would be related to other factors, such as enzyme specificity, but not to cold adaptation.

From the analysis of the three psychrotrophic enzymes, it seems clear that the structural pecularities responsible for high catalytic efficiency at low temperature can hardly be deduced from the amino acid sequence. It is likely that, as in the case of thermophilic ferredoxin (Perutz and Raidt, 1975), only subtle changes of the primary structure are sufficient to alter the flexibility of the folded state, resulting in drastically divergent properties. Thus, a more detailed analysis of the structural parameters involved in the molecular adaptation of psychrotrophic enzymes to low temperatures would require the determination of the three-dimensional structure and comparison with closely homologous mesophilic proteins.

TABLE 1. SITE-DIRECTED MUTAGENESIS OF THE RECOMBINANT LIP2

	Mutant plasmid		
	pLIPS/A 21	*pLIPH/Q 41*	*pLIPV/A 21*
Original amino acid	Ser-239	His-165	Val-195
Original codon	AGT	CAT	GTC
Mutant codon	GCT	CAG	GCC
Resulting amino acid	Ala	Gln	Ala
Lipase activity	ND	ND	+
Esterase activity	ND	ND	+

ND, Not detectable.

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The sequence data in this paper have been submitted to the EMBL/GenBank Data Libraries under accession number X53868.

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