Influence of the conformational flexibility on the kinetics and dimerisation process of two *Candida rugosa* lipase isoenzymes

María A. Pernas[a], Cristina López[a], M. Luisa Rúa[a,*], Juan Hermoso[b]

[a] *Area de Bioquímica y Biología Molecular, Facultade de Ciencias de Ourense, Universidade de Vigo, As Lagoas s/n, 32004 Ourense, Spain*
[b] *Grupo de Cristalografía Macromolecular y Biología Estructural, Instituto 'Rocasolano' CSIC, Serrano 119, 28006 Madrid, Spain*

Received 13 April 2001; revised 18 June 2001; accepted 19 June 2001

First published online 29 June 2001

Edited by Judit Ovádi

Abstract We have investigated the interfacial activation process of two isoenzymes from *Candida rugosa* (Lip1 and Lip3) using triacetin as substrate. Kinetics were coupled to inhibition experiments in order to analyse the transition between the open and closed conformers. This process was slow, particularly for Lip1, in the absence of an interface provided by the substrate or a detergent. Dimers of Lip3 were also purified and their catalytic action was closer to that of a typical esterase. In spite of the high sequence homology between Lip1 and Lip3, small changes enhance hydrophobicity in the binding pocket of Lip3 and increase the flexibility of its flap. We postulated that these factors account for the higher tendency of Lip3 to dimerise fixing its open conformation. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Lipase; Kinetic; Interfacial activation; Inhibition; Dimerization; Structure; *Candida rugosa*

1. Introduction

Lipases (triacylglycerol lipase EC 3.1.1.3) catalyse the hydrolysis of triacylglycerides at the lipid–water interface. Their activity drastically increased upon binding to the interface, a phenomenon known as interfacial activation [1]. This process has been associated with a conformational change in which a flap consisting of at least one α-helix opens by rotating around its hinge regions. In the inactive closed conformation, the flap covers the active site avoiding its exposure to the aqueous solvent, but in the presence of a lipidic interface it opens (active conformation) making the active site accessible to the substrate [2,3].

A family of at least seven lipase genes was described in *Candida rugosa* (CRL) namely *lip1* to *lip7* [4–6]. Comparison of the predicted amino acid sequences revealed a close similarity among the five lipases whose genes were fully characterised (*lip1–lip5*), ranging between 77% and 88% identity for pairs of proteins. In all of them, the catalytic triad Ser/His/Glu is occluded by a polypeptide flap inaccessible from the solvent in the closed conformation. A simple chromatographic procedure allowed the separation of two major fractions from CRL commercial crude preparations: lipase A and lipase B (Lip3 and Lip1)[7], catalytically very different [8–12] also in terms of thermal and pH stability [8]. Fraction A was the product of the *lip3* gene whereas fraction B, representing 70% of the total lipase activity, could be further divided into four lipases by isoelectric focusing (IEF) [8]. The two major proteins of this pool were isolated and found very similar at the molecular and kinetic level [13]. Perhaps both are derived from *lip1* gene [12] but nonetheless their origin is unclear. The crystal structures of Lip1 [14–16] and a *C. rugosa* cholesterol esterase 100% identical in sequence to Lip3 [17,18] were solved. From now on in the text, we will use Lip3 to allude to the *C. rugosa* cholesterol esterase.

The aim of this work was to compare the kinetics of Lip1 and Lip3 isoenzymes, using triacetin (TA) as a substrate, focusing on the interfacial activation process. The kinetics were coupled with inhibition experiments using the serine specific inhibitor diethyl *p*-nitrophenyl phosphate (E600) in order to analyse the transition between the open and closed lipase conformers. For the E600 inhibition reaction to happen an exposed active site is required and thus is indicative of the opening of the flap [19,20]. Results will be discussed attending to the conformational flexibility of the Lip1 and Lip3 flaps deduced from their X-ray structures. The study was extended to homodimers of Lip3 that also were isolated from CRL commercial preparations.

2. Materials and methods

2.1. Materials

Lipase type VII from *C. rugosa* and E600 were from Sigma Chemicals Co. (St Louis, MO, USA), tributyrin and TA from Fluka (Deisenhofen, Germany) and sodium deoxycholate from Amresco (Solom, OH, USA).

2.2. Lipase purification

Dimeric Lip3 and Lip1 were purified as described previously [7] and monomeric Lip3 as in [8].

2.3. Determination of native molecular weight

Purified lipases were chromatographed in a Sephacryl S200 column (Pharmacia), equilibrated at 0.3 ml/min in 25 mM Tris–HCl buffer (pH 7.5) containing 0.15 M NaCl. The high molecular weight marker kit from Sigma was used for calibration.

2.4. Enzyme assays

During purification and inhibition experiments, the lipase activity was measured in a pH-stat (Methrom, Switzerland) at 30°C and pH 7.0 using tributyrin emulsions as described in [21].

2.5. Kinetic measurements

Kinetics were carried out following the initial hydrolysis rate of TA in a pH-stat. TA was used as substrate as this short chain triacyl-

*Corresponding author. Fax: (34)-988387001.
E-mail: mlrua@uvigo.es

Abbreviations: CRL, *Candida rugosa* lipase; TA, triacetin; E600, diethyl *p*-nitrophenyl phosphate

0014-5793/01/$20.00
PII: S0014-5793(01)02630-8

glyceride allowed to overcome the difficulties that accompany the poor water solubility of tributyrin, the substrate utilised during purification. The assays were performed in 5 mM Tris–HCl buffer (pH 7.0) containing 0.1 M $CaCl_2$ at 30°C and variable amounts of TA (from 35 mM to 1.06 M). All assays were done keeping the same stirring speed, while care was taken to avoid the formation of air bubbles in the reaction vessel. The reaction was started with the addition of the enzyme and at least triplicates of each assay were made. One activity unit was defined as the amount of enzyme that released 1 μmol of fatty acids per min. The solubility of TA in the reaction conditions was estimated measuring the turbidity as described in Ferrato et al. [22]. The lipase/esterase activity ratio (TA_{lip}/TA_{est}) for each lipase was determined by dividing the specific activity above (1.06 M) and below (0.18 M) the solubility limit of TA.

2.6. E600 inhibition

Experiments were performed in a large excess of inhibitor relative to enzyme concentration (1000, in molar units). Incubations were performed at 30°C in 25 mM Tris–HCl (pH 7.5) containing 150 mM NaCl and 4% (v/v) acetonitrile, in the presence or absence of micellar concentrations of sodium deoxycholate (3 mM). E600 was directly added from stock solutions prepared in acetonitrile. At different times, aliquots were withdrawn and activities were expressed as the percentage of initial activity determined with the tributyrin assay. In addition, the remaining activities of each enzyme were plotted on a semi-log scale as a function of time, from which the half-lives of inactivation ($t_{1/2}$) were determined. Control experiments in which the inhibitor was omitted were also performed.

2.7. Amino-terminal sequence analysis

Performed as previously described [23].

2.8. Electrophoresis

Proteins were analysed by denaturing electrophoresis (SDS–PAGE) in a mini Protean II cell (Hoefer). IEF was performed on a Multiphor Electrophoresis System (Pharmacia Biotech) following the manufacturer's instructions and using commercial gels in the pH range 3.5–9.5.

2.9. Other methods

Carbohydrate content was determined using the *N*-glycosidase F Deglycosylation and the DIG Glycan Detection kits (Boehringer Mannheim) following the manufacturer's instructions. Protein concentration was determined by the Lowry method using bovine serum albumin as a standard.

3. Results

3.1. Lipase purification and molecular characterisation

A chromatographic procedure allowed the separation of two lipases from CRL commercial preparations: Lip3 and Lip1 [7]. In our hands this procedure produced Lip3 consisting of two active isoforms separated by gel filtration chromatography, that however by SDS–PAGE showed the same M_r reported for Lip3, 60 kDa (not shown).

Rúa et al. [8] have reported a second method which included an ethanol precipitation in the first step. When we used this method, the main form of Lip3 was the low molecular weight enzyme. Aggregation of lipases is well documented in the literature and often attributed to hydrophobic interactions between monomers of the enzyme [24,25]. The positive effect of ethanol for the recovery of the low molecular weight enzyme supported this hypothesis. In addition, the existence of monomers and dimers of Lip3 in aqueous solution was previously demonstrated [17,18].

Further characterisation of the purified isoforms also indicated that they were monomeric and dimeric forms of Lip3: they shared the N-terminal sequence (APTAT), identical to that reported for Lip3 [26], and carbohydrate content (from 4% to 6%). A p*I* of 5.8 was found for putative monomeric Lip3, the same value reported for Lip3 [8]. Dimeric Lip3

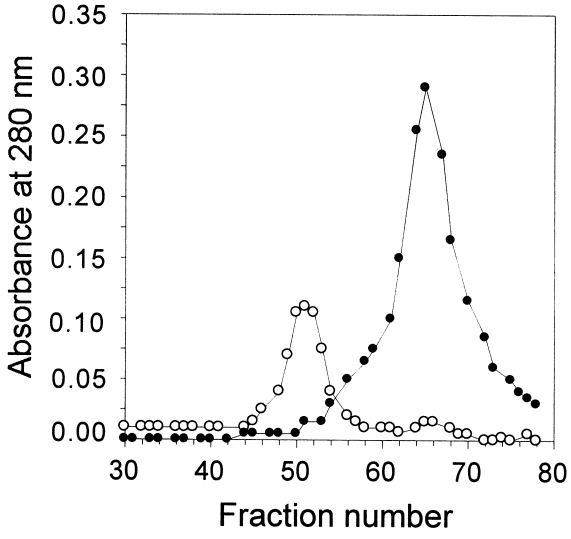

Fig. 1. Gel filtration chromatography on a Sephacryl S200 column. Samples of Lip3 monomer (●) and Lip3 dimer (○) were separately loaded on a Sephacryl S200 column (1.6×60 cm) equilibrated in 25 mM Tris–HCl (pH 7.5) containing 150 mM NaCl. Flow rate, 0.3 ml/min. Fractions, 1 ml

precipitated during electrophoresis, a phenomenon described for other aggregated lipases. By gel filtration chromatography (Fig. 1), an apparent molecular weight of 117 kDa was determined for putative Lip3 dimer and 43 kDa for the monomer; the later lower than that determined by SDS–PAGE. This abnormal M_r was also observed for Lip3 purified from post-incubates of *C. rugosa* [21]. Finally their specific activities, determined with tributyrin were very similar (1030 –

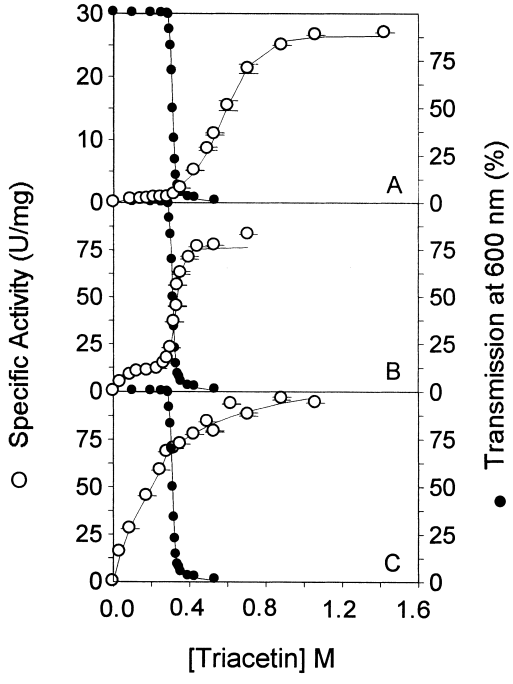

Fig. 2. Dependence of specific activity (○) on TA concentration (A) Lip1 (250 μM), (B) monomeric Lip3 (33 μM), (C) dimeric Lip3 (50 μM). (●) Transmission of the substrate solutions at 600 nm. The data points are the mean values with the standard deviations reflected by the error bars

Lip3 dimer and 910 U/mg – Lip3 monomer) and lower than that of pure Lip1 (1500 U/mg).

3.2. Kinetic measurements

The kinetics of the purified CRL are shown in Fig. 2. Dimeric Lip3 (Fig. 2C) showed a Michaelis–Menten-type kinetic behaviour whereas for the monomer (Fig. 2B) a clear jump at TA concentrations around 270 mM was observed, where the first large-size droplets started to form as indicated by the decrease in the turbidity of the solution. As result, dimeric Lip3 was more active than monomeric Lip3 particularly below the solubility limit of TA.

Lip1 displayed a low interfacial activation when compared to monomeric Lip3 (Fig. 2A). The activation started at higher TA concentrations (around 380 mM) and from this substrate concentration, the increase in activity was smoother than for monomeric Lip3. Additionally, Lip1 was less active than either monomeric or dimeric Lip3 over the whole substrate concentration range.

Significant differences in the TA_{lip}/TA_{est} ratio were observed: 2.1 – Lip3 dimer, 7.6 – Lip3 monomer and 43.7 – Lip1 – indicating that the capacity to hydrolyse soluble TA (relative to insoluble TA) decreased in this direction (Lip3 dimer > Lip3 monomer > Lip1).

3.3. Inhibition experiments

Inhibition of dimeric Lip3 by E600 was very fast as compared to either Lip1 or Lip3 monomer and was still slightly accelerated by the presence of deoxycholate micelles (Fig. 3A–C). As illustrated in Fig. 3D,E, the inactivation of the monomeric enzymes was consistent with a pseudo first-order process as shown by the linear relationship between the natural logarithm of the remaining activity and reaction time. From these plots, the estimated half-life for inactivation ($t_{1/2}$) indicated a faster inhibition of Lip3 (70.2 min vs 105.3 min). The same trend was observed in the presence of sodium deoxycholate micelles (Fig. 3D,E, ●), although $t_{1/2}$ values decreased significantly (4.3 min vs 19.7 min, for Lip3 and Lip1, respectively).

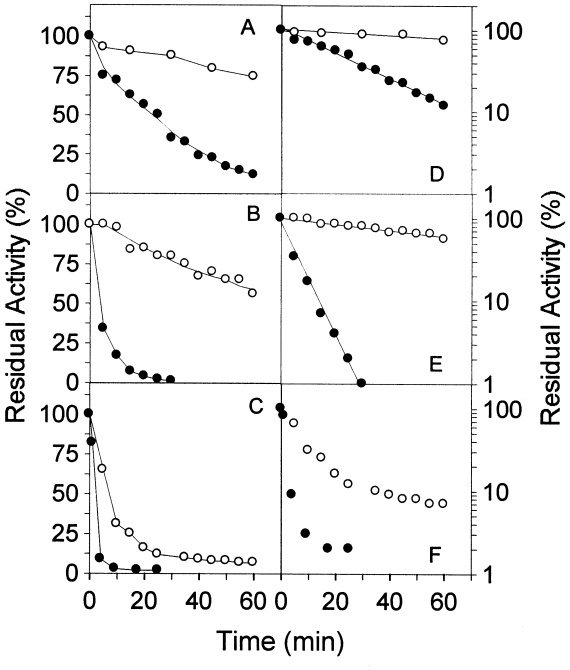

Fig. 3. Inhibition of CRL with E600. Fixed amounts of Lip1 (20 μM) (A, D), monomeric Lip3 (5 μM) (B, E) and dimeric Lip3 (5 μM) (C, F) were incubated with E600 in the presence (●) or absence (○) of sodium deoxycholate. A–C: Results expressed as residual activity as a function of time. D–F: Semi-log plots of the residual activity as a function of time. Other experimental conditions were described in the text. No inhibition was observed in the absence of E600 (not shown)

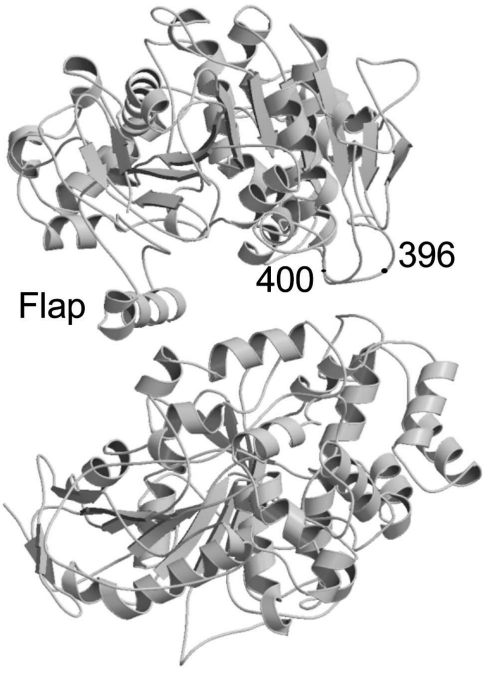

Fig. 4. A ribbon diagram of the Lip3 dimer produced with the program MOLSCRIPT [32].

On the other hand, inhibition of dimeric Lip3 did not follow pseudo first-order kinetics (Fig. 3F). A biphasic kinetic was observed with a substantial residual activity still detected after prolonged times of incubation particularly in the aqueous medium.

3.4. Lip3 vs Lip1 tertiary structure

The 3D structure of Lip3 consisted of a dimeric association of two monomers with their flaps open and the active-site gorges facing each other, thus shielding hydrophobic environment of the catalytic triads from the aqueous medium (Fig. 4) [18]. Since the structure of each Lip3 monomer was nearly identical to that of open Lip1 [14], we have compared them around those areas that are supposed to interact with substrate interfaces, the active-site gorge and flap structure. We found two stabilising salt bridges (Lys^{75}–Glu^{71} and Glu^{88}–Lys^{85}) within the flap of open Lip1. Nevertheless, only one could be possible in open Lip3 due to the substitution of Glu^{88} by Gln^{88} in this isoenzyme. Based on the reported closed Lip1 structure a closed conformation model can be extrapolated for Lip3. By doing that, we found that several interactions occurring between the flap (residues 60–97) and the remainder moiety of closed Lip1 could not exist in Lip3. That is the case of the Phe^{344}–Phe^{87} stacking interaction and Phe^{344}–Tyr^{69} T-type aromatic interaction, as position 344 in Lip3 is mutated into an Ile. In addition, Ser^{84}–Ser^{450} hydrogen

bond should be unique for Lip1 because in position 450 Lip3 has an Ala. From this analysis, it arises that the flap in Lip1 has a higher structural stability in both closed and open conformations whereas a higher flexibility is postulated for the flap in Lip3.

The reported structure of dimeric Lip3 showed that dimerisation generates four openings at the interface (two independent and two symmetry related). As we were interested on checking the accessibility of the openings to TA, we built a model for this triacylglyceride based on the trilaurin structure available in the Cambridge DataBase (code BTRILA). After energy minimisation using the program X-PLOR [27], the maximum dimension of this model was 8 Å, smaller than the dimensions of the openings (17×9 Å and 13×18 Å). The free access of TA through the openings was also confirmed using the O graphic program [28].

4. Discussion

The interfacial activation phenomenon of lipases was associated with conformational changes involving the movement of the flap. A direct confirmation of these rearrangements came from the determination of the *C. rugosa* Lip1 structure in both the closed and open conformations [16,14]. There is also indication of their existence in aqueous solution for a lipase from *C. rugosa* [29]. Thus, by analogy with allosteric enzymes, a free equilibrium between the two forms could exist lying over the closed in aqueous solution. The introduction of an interface could promote the adsorption of the lipase and could shift the overall equilibrium towards the open conformation [30,29]. For either Lip1 or Lip3 from *C. rugosa*, the transition between the two conformers could not be restricted to a single movement of the flap. On the contrary, some of its secondary structure refolded accompanied by a *cis*/*trans* isomerisation of a proline peptide bond that likely increased the energy required for the transition between the two stages [16,18]. Altogether, these factors should account for the low activity over soluble TA as well as for the low rate of inactivation determined for Lip1 and Lip3 monomer in aqueous solution. On the other hand, both the sodium deoxycholate micelles and the TA interface (above its solubility limit) were very efficient in activating the monomeric enzymes as deduced from the increased inhibition and hydrolysis rates detected in these conditions. At submicellar concentrations of the detergent, the inhibition rates were significantly lower indicating that the CRL open-flap conformations were better stabilised by micellar aggregates (not shown).

In general terms Lip1 and Lip3 monomer responded to the described model. Nevertheless, E600 inhibition experiments suggested that even in the absence of interfaces the open Lip3 conformation was slightly more favoured than that of Lip1 ($t_{1/2}$ significantly lower for Lip3). The higher relative capacity of Lip3 monomer to hydrolyse soluble TA in comparison to Lip1 reinforced this hypothesis. This fact was reflected in the lower TA_{lip}/TA_{est} ratio determined for Lip3 (7.6 vs 43.7).

In spite of the high sequence homology between these enzymes (89%), the different amino acids were not homogeneously distributed but concentrated within the flap and vicinity regions [31]. Thus, of the 55 residues that were different in Lip3, 23 were placed either near the active site, at the dimer interface or within the hydrophobic core. An exhaustive analysis of these areas indicated that several amino acid substitutions determined a superior flexibility of the flap in Lip3, a factor that could explain the higher stabilisation of open Lip3 in the aqueous solution. In addition to the flap flexibility, the higher hydrophobicity of its catalytic cavity [18] could also account for the high facility of Lip3 to self-associate. It is worthwhile to mention that dimerisation seemed to be an intrinsic property of Lip3 isoenzyme. Dimers of Lip3 have been purified in this work and previously by Kaiser et al. [17]. However, we did not detect dimers of Lip1 in CRL commercial preparations. Recently dimers of a *C. rugosa* lipase were isolated from the same commercial preparations [29]. Nevertheless, its ascription to a particular isoenzyme was not clear, as the N-terminal sequence was not given.

At the kinetic level, the most important consequence of Lip3 dimerisation was the increased capacity for the hydrolysis of soluble TA suggesting that the dimer had accessible active sites for this substrate in the absence of any interface. This hypothesis was confirmed by checking the sizes of the openings generated on its structure and the inhibition experiments from which a rapid and almost full inactivation was obtained with the small inhibitor E600 even in the absence of detergent. In this sense, the biphasic kinetic of inhibition obtained with the dimer might indicate a negative cooperativity in the interaction of E600 with the two active sites although other explanations can not be ruled out (work in progress).

In this study, we showed how minor differences in the primary structure of two CRL lipase isoenzymes had a profound influence on their kinetics. Furthermore, a functional significance for the dimerisation of Lip3, detected in crystallographic and biochemical studies, was provided. Altogether, our results suggested that the dynamics of the flap structure within the CRL family might be of mechanistic and kinetic importance.

Acknowledgements: We thank Dr. Lorenzo Pastrana for helpful discussions. Financial support was obtained from the CICYT. Spain. Project QUI97-0506-C03

References

[1] Sarda, L. and Desnuelle, P. (1958) Biochim. Biophys. Acta 30, 513–521.
[2] Brady, L., Brzozowski, A.M., Derewenda, Z.S., Dodson, G., Tolley, S., Turkenburg, J.P., Christiansen, L., Huge-Jensen, B., Norskov, L., Thim, L. and Menge, U. (1990) Nature 343, 767–770.
[3] Winkler, F.K., d'Arcy, A. and Hunziker, W. (1990) Nature 343, 767–770.
[4] Longhi, S., Fusetti, F., Grandori, R., Lotti, M., Vanoni, M. and Alberghina, L. (1992) Biochim. Biophys. Acta 1131, 227–232.
[5] Lotti, M., Grandori, R., Fusetti, F., Longhi, S., Brocca, S., Tramontano, A. and Alberghina, L. (1993) Gene 124, 45–55.
[6] Brocca, S., Grandori, R., Breviario, D. and Lotti, M. (1995) Curr. Genet. 28, 454–457.
[7] Rúa, M.L. and Ballesteros, A. (1994) Biotechnol. Tech. 8, 21–26.
[8] Rúa, M.L., Díaz-Mauriño, T., Fernández, V.M., Otero, C. and Ballesteros, A. (1993) Biochim. Biophys. Acta 1156, 181–189.
[9] Hernáiz, M.J., Sánchez-Montero, J.M. and Sinisterra, J.V. (1997) Biotechnol. Lett. 19, 303–306.
[10] Linko, Y. and Wu, X.Y. (1996) J. Chem. Technol. Biotechnol. 65, 163–170.
[11] Plou, F.J., Sogo, P., Calvo, M.V., Burguillo, F.J. and Ballesteros, A. (1997) Biocatal. Biotransform. 15, 75–89.
[12] Diczfalusy, M.A., Hellman, U. and Alexson, S.E.H. (1997) Arch. Biochem. Biophys. 348, 1–8.

[13] López, C., Guerra, N.P. and Rúa, M.L. (2000) Biotechnol. Lett. 22, 1291–1294.
[14] Grochulski, P., Li, Y., Schrag, J.D., Bouthillier, F., Smith, P., Harrison, D., Rubin, B. and Cygler, M. (1993) J. Biol. Chem. 268, 12843–12847.
[15] Grochulski, P., Li, Y., Schrag, J.D. and Cygler, M. (1994) Protein Sci. 3, 82–91.
[16] Cygler, M. and Schrag, J. (1999) Biochim. Biophys. Acta 1441, 204–214.
[17] Kaiser, R., Erman, M., Duax, W., Ghosh, D. and Jörnvall, H. (1994) FEBS Lett. 337, 123–127.
[18] Ghosh, D., Wawrzak, Z., Pletnev, V.Z., Li, N., Kaiser, R., Pangborn, W., Jornvall, H., Erman, M. and Duax, L. (1995) Structure 3, 279–288.
[19] Moreau, H., Moulin, A., Gargouri, Y., Nöel, J-P. and Verger, R. (1991) Biochemistry 30, 1037–1041.
[20] Patkar, S. and Björkling, F. (1994) in: Lipases: Their Structure, Biochemistry and Application (Woolley, P. and Petersen, S.B., Eds.), pp. 207–224, Cambridge University Press, Cambridge.
[21] Pernas, M.A., López, C., Pastrana, L. and Rúa, M.L. (2000) J. Biotechnol. 84, 163–174.
[22] Ferrato, F., Carriere, F., Sarda, L. and Verger, R. (1997) in: Methods in Enzymology (Rubin, B. and Dennis, E.A., Eds.), Lipases Vol. 286, part B, pp. 327–347, Academic Press, San Diego, CA.
[23] Sánchez, A., Ferrer, P., Serrano, A., Pernas, M.A., Valero, F., Rúa, M.L., Casas, C. and Solà, C. (1999) Enzyme Microb. Technol. 25, 214–223.
[24] Taipa, M.A., Liebeton, K., Costa, J.V., Cabral, J.M.S. and Jaeger, K-E. (1995) Biochim. Biophys. Acta 1256, 396–402.
[25] Rúa, M.L., Schmidt-Dannert, C., Wahl, S., Sprauer, A. and Schmid, R.D. (1997) J. Biotechnol. 56, 89–102.
[26] Alberghina, L. and Lotti, M. (1997) in: Methods in Enzymology (Rubin, B. and Dennis, E., Eds.), Vol. 284, pp. 246–250, Academic Press, San Diego, CA.
[27] Brunger, A.T. (1993) in: X-PLOR: A system for X-ray Crystallography and NMR, Yale University Press, New Haven, CT.
[28] Jones, T.A., Zou, J.Y., Cowan, S.W. and Kjeldgaard, M. (1991) Acta Cryst. Sect. A 47, 110–119.
[29] Turner, N.A., Needs, E.C., Khan, J.A. and Vulfson, E.N. (2001) Biotechnol. Bioeng. 72, 108–118.
[30] Martinelle, M. and Hult, K. (1994) in: Lipases: their structure, biochemistry and application (Woolley, P. and Petersen, S.B., Eds.), pp. 159–180, Cambridge University Press, Cambridge.
[31] Lotti, M., Tramontano, A., Longhi, S., Fusetti, F., Brocca, S., Pizzi, E. and Alberghina, L. (1994) Protein Eng. 7, 531–535.
[32] Kraulis, P.J. (1991) J. Appl. Crystallogr. 24, 946–950.