Trapping of Different Lipase Conformers in Water-Restricted Environments†

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ABSTRACT: Based on a recently reported strategy to rationally activate lipolytic enzymes for use in nonaqueous media [Mingarro, I., et al. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 3308−3312], we compared the behavior in water-restricted environments of activated *vs* nonactivated forms of different lipases toward their natural substrates, triacylglycerols. To this end, nine lipases from varied origins (mammalian, fungal, and bacterial) were assayed using simple acidolyses as nonaqueous model reactions. The experimental results for several (though not all) lipases, discussed in the light of current structural and functional information, were collectively consistent with a model where, depending on the "history" of sample preparation, basically two different conformers (open and closed) of the lipase can be trapped (and assayed) in the nonaqueous medium. In particular, for a few prototypic lipases investigated in more detail, the following were shown: (i) the activation strategy permitted them to rationally overcome their reported reluctance to convert saturated, long-chain triglycerides, providing quantifiable nonaqueous rate accelerations of up to 3 orders of magnitude; (ii) the activated conformer exhibited a markedly higher ability than its nonactivated counterpart to bind a ligand (nonhydrolyzable phospholipid) in the nonaqueous medium; and (iii) a clearly distinct selectivity profile toward the substrate chain length was obtained for either conformer.

Traditionally, lipases (triacylglycerol hydrolases, EC. 3.1.1.3) have constituted a paradigmatic target for nonaqueous studies for many reasons, including their function at oil−water interfaces, their typically water-insoluble substrates, or their impressively broad utility in nonaqueous bioconversions [for general, recent reviews on nonaqueous enzymology, see e.g. Dordick (1991), Gupta (1992), Wescott and Klibanov (1994), Bell et al. (1995), Braco (1995), and Carrea et al. (1995)]. However, both a reconsideration and a reassessment of the fundamental behavior of lipases in nonaqueous media seem necessary in light of the emerging wealth of structural and mechanistic information on lipolytic enzymes. Only recently, the molecular mechanism of *interfacial activation* of lipases (i.e., the remarkable increase in enzyme activity when their substrates are presented as micelles or lipid droplets) has begun to be deciphered, mainly from crystallographic studies [for recent reviews, see Lawson et al. (1992), Cambillau and van Tilbeurgh (1993), Derewenda and Sharp (1993), Carrière et al. (1994), Derewenda (1994), Kazlauskas (1994), Rubin (1994), Winkler and Gubernator (1994), and Egloff et al. (1995a,b)].

For a number of selected, prototypic lipases from diverse origin, it has been possible to elucidate and structurally compare inactive (or "closed") and active (or "open") forms of the enzyme, crystallized in the absence or presence, respectively, of covalent inhibitors (or micelles) (Brzozowski et al., 1991; van Tilbeurgh et al., 1993; Grochulski et al., 1994; Egloff et al., 1995a,b). This comparison has led to the proposal that interfacial activation is associated in general (though not for all lipases) with the displacement (opening) of a lid covering the active site, which in turn results in (i) an exposure to the solvent of the otherwise buried catalytic machinery (increasing substrate accessibility), (ii) the creation of a nonpolar surface around the lipase active site entrance, and (iii) even in some cases the formation of the "oxyanion hole" critical for the stabilization of the transition state intermediate during catalysis. In light of all this knowledge, it is evident that many questions arise regarding lipase behavior in nonaqueous media, for instance: Is the enzyme conformation in a lid-closed or lid-opened state? Is it possible to place the enzyme in the organic milieu in either of the two conformations? and in this case would activity or any other property be significantly different?

To address these and other issues, we have recently developed a rational approach which permits us to obtain (trap) activated forms of lipolytic enzymes, manageable in nonaqueous environments (Mingarro et al., 1995; Braco & Mingarro, 1996). Originally inspired from a methodological viewpoint in the so-called molecular (bio)imprinting of enzymes applied to proteases (Russell & Klibanov, 1988; Ståhl et al., 1990, 1991) and dehydrogenases (Johansson et al., 1995), our strategy of activation of lipases can be structurally rationalized in simple terms according to a model based on their mechanism of activation at interfaces. In brief, if relevant, activating conformational changes (associated to interfacial activation) are induced in a lipase in solution *by addition of interfaces* (e.g., micelles or vesicles), rapid freeze-drying of this solution will trap an activated (presumably open) form of the enzyme in the resulting lyophilized

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powder, which will be further retained ("remembered") in nonaqueous media by virtue of the known dramatically enhanced conformational rigidity of proteins in anhydrous solvents (Zaks & Klibanov, 1984). Evidently, this rationale entails the complementary notion that the same lipase lyophilized from the same solution *in the absence of interfaces* will be trapped in a nonactive (closed) conformation (i.e., that of the lipase immediately before freeze-drying).

Our preliminary results (Mingarro et al., 1995; Braco & Mingarro, 1996), collectively consistent with this model, were obtained with a few selected lipases using simple esterification reactions for the nonaqueous assays. The aim of the present investigation was to explore and characterize in more detail the phenomenon of activation of lipases by *trapping in the presence of interfaces* (TPI),[1] extending the repertoire of lipases tested and especially centering our attention on triacylglycerols as substrates. This, for the first time, will provide us with an opportunity to evaluate and compare the intrinsic activity of activated *vs* nonactivated lipase conformations toward monomerically dispersed, but long-chain, natural substrates of the enzyme. In addition, it will permit us to rationally address the reasons underlying the reported (but so far unexplained) reluctance of some lipases when suspended in organic solvents to catalyze the conversion of their natural substrates (Basheer et al., 1995; Goto et al., 1995).

EXPERIMENTAL PROCEDURES

Materials. Porcine pancreatic lipase (ppL), *Candida rugosa* lipase (CrL), all the triglycerides, oleic acid, *n*-octyl β-D-glucopyranoside (n-OG), Triton X-100, (TX-100), lactose, sorbitol, PG2000−200 controlled pore glass (CPG) beads (mesh 120−200), and fatty acid-free bovine serum albumin (BSA) were purchased from Sigma. *Rhizopus arrhizus* lipase (RaL), *Aspergillus niger* lipase (AnL), and *Pseudomonas fluorescens* lipase (PfL) were obtained from Fluka, and *Rhizopus delemar* lipase (RdL), *Rhizopus javanicus* lipase (RjL), *Rhizopus niveus* lipase (RnL), and *Pseudomonas sp.* lipase (PsL) were generously provided by Amano. 1,2-*O*-dihexadecyl-*sn*-glycero-3-phosphorylcholine (DHPC) was obtained from Bachem. All other chemicals were from Merck or Aldrich and of analytical grade or purer. All the organic solvents were of analytical or HPLC grade (Merck). The term "anhydrous" solvent refers to a solvent desiccated with 3-Å molecular sieves (Merck) to bring its water content below 0.01%, as determined by Karl-Fisher titration.

Preparation of Activated and Nonactivated Enzyme Samples. The protocol followed was basically the same as in a preliminary study where a screening of experimental conditions was carried out for optimization of the method (Mingarro et al., 1995). In brief, each lipase was incubated (before freeze-drying) for about 1−2 min at 4 °C, either in 10 mM Tris-HCl buffer, pH 7.5, *in the presence* of micellar n-OG (40 mM) (for the activated enzyme), or in the same buffer *in the absence* of amphiphile (for the nonactivated, control enzyme). During and after freeze-drying (typically overnight), both activated and nonactivated samples were rigorously treated in an identical manner. After a washing step (to remove the amphiphile) with benzene/ethanol, 90:10 (v/v), repeated three times, both samples were vacuum dried for 2 h.

Nonaqueous Assays. Unless otherwise stated and for a strict comparison with the data (using powdered, nonactivated lipases) from Goto et al. (1995), the acidolysis of tripalmitin (10 mM) by oleic acid (50 mM) in anhydrous isooctane at 40 °C was selected as a model reaction. As previously reported (Mingarro et al., 1995), a given amount of washed lipase powder (either activated or nonactivated) was suspended in a given volume of substrate(s)-containing organic solution in a stoppered vial, sonicated for 10 s, and shaken (or stirred with a magnetic bar) at 250 rpm in an oven at 40 °C; periodically, a 25-μL aliquot was withdrawn from the reaction mixture and centrifuged at 12000*g* for 3 min, and the supernatant analyzed chromatographically by HPLC in combination with gas chromatography. The liquid chromatograph (Waters) was equipped as described before (Mingarro et al., 1994; 1995). The reverse-phase Lichrospher 100 RP-18 column (Merck) was isocratically eluted with acetonitrile/dichloromethane/tetrahydrofuran/acetic acid, 70:22:20:0.8 (v/v), for long-chain triglycerides (Cho & Rhee, 1993), or with methanol for short-chain ones. Gas chromatographic determinations were carried out as in Goto et al. (1995), using a J&W Scientific DB-1 capillary column. Routinely, simultaneous disappearance of the substrate(s) and appearance of the product(s) were followed. For every lipase assayed, the nonaqueous enzyme concentration was optimized to maximize the rate enhancement obtained by the activation treatment.The activation factor (AF), as defined elsewhere (Mingarro et al., 1995), refers to the ratio of the nonaqueous initial reaction rate for the activated relative to the nonactivated lipase.

Aqueous Assays. When determined for comparative purposes, the activity of ppL in aqueous medium on different triglycerides was monitored potentiometrically with a Crison MicroTT 2050 recording pH-stat system, using TX-100 as an emulsifier (detergent:substrate ratio of 5). Typically, 5 mL of a solution containing the emulsified substrate (15 mM), 2.5 mM Tris-HCl, 10 mM NaCl, and 5 mM $CaCl_2$, equilibrated at pH 8.0, was placed in a thermostated cuvette (25 °C), and the reaction was followed in a similar manner as in Mingarro et al. (1994).

Stability Measurements. To determine the stability of either sample conformation (activated or nonactivated) in the nonaqueous milieu, enzyme samples (of either type) were preincubated in pure isooctane for different periods of time (from 0 to 22 h), and their residual activity was then assayed as mentioned above after addition (zero time) of a solution of the substrates (tripalmitin and oleic acid) in isooctane to get the required final concentration. Alternatively, the loss of the acquired activation was assayed as a function of the water content of the medium as in Mingarro et al. (1995) using triolein as a substrate in a water-miscible solvent.

[1] Abbreviations: AF: activation factor; AnL: *Aspergillus niger* lipase; CPG: controlled pore glass; CrL: *Candida rugosa* lipase; DHPC: dihexadecylphosphatidylcholine; hpL: human pancreatic lipase; HPLC: high performance liquid chromatography; n-OG: *n*-octyl β-D-glucopyranoside; PfL: *Pseudomonas fluorescens* lipase; ppL: porcine pancreatic lipase; PsL: *Pseudomonas sp.* lipase; RaL: *Rhizopus arrhizus* lipase; RdL: *Rhizopus delemar* lipase; RjL: *Rhizopus javanicus* lipase; RnL: *Rhizopus niveus* lipase; SDS−PAGE: sodium dodecyl sulfate−polyacrylamide gel electrophoresis; T4: tributyrin; T6: tricaproin; T8: tricaprylin; T10: tricaprin; T12: trilaurin; T14: trimyristin; T16: tripalmitin; T18: tristearin; TPI: trapping in the presence of interfaces; TX-100: Triton X-100.

Enzyme Immobilization. ppL was adsorbed onto CPG beads as previously reported for other enzymes (Braco et al., 1992), except that after adsorption of the lipase from the amphiphile-free buffered solution (10 mM Tris-HCl, pH 7.5) the beads were immediately freeze-dried and washed with anhydrous solvent in an identical manner as the control powdered sample.

Binding Assays. The general strategy to assay the binding of DHPC to either activated or nonactivated ppL in the nonaqueous medium was based on that described by Braco et al. (1990) for nonenzymic proteins. In the present case, a given amount of washed lipase (either activated or control), typically 65 μM, was suspended in a solution of the ligand at the desired concentration in toluene/chloroform, 8:2 (v/v), sonicated for 10 s, and then vigorously stirred at 25 °C. After 2 h of incubation (a time determined to be sufficient for binding equilibrium to be reached for both activated and nonactivated enzymes), a 20-μL aliquot was withdrawn and centrifuged in an Eppendorf tube at 12000*g* for 3 min, and 10 μL of the supernatant was analyzed by HPLC using previously described chromatographic conditions for phosphatidylcholine quantitation (Mingarro et al., 1994). For lipase quantitation, Coomassie Blue-stained discontinuous SDS−PAGE gels were analyzed with an LKB 2202 Ultroscan laser densitometer using BSA as a standard.

As reported elsewhere (Mingarro et al., 1995), in the case of ppL colipase was not added since bile salts were not used in any experiment. Typically, the results shown are representative or correspond to the mean of three independent experiments. Other details are given in the corresponding legends to figures.

RESULTS

Micellar n-OG was selected for activation in the present study because it was found to be among the optimal amphiphiles for several lipases in a preliminary screening (Braco et al., 1995; Braco & Mingarro, 1996). On the other hand, the acidolysis of tripalmitin by oleic acid in isooctane was chosen as a nonaqueous model reaction to evaluate the consequences of our activation method since some lipases have been reported to be unable to catalyze this particular conversion when suspended in the reaction medium (Basheer et al., 1995; Goto et al., 1995). Unless otherwise stated, enzyme samples (activated or nonactivated) were used as a powder suspension in the organic medium, as in previously reported work with lipases (Mingarro et al., 1995; Braco & Mingarro, 1996), or related studies of ligand-induced activation of proteases (Russell & Klibanov, 1988; Ståhl et al., 1990, 1991) or dehydrogenases (Johansson et al., 1995).

Consequences of Activation by TPI on Lipase Ability to Convert Triglycerides. In Figure 1, Panels A and B depict, for RaL and RdL, respectively, the comparison of the time course of conversion of tripalmitin mediated by either the nonactivated (open symbols) or the activated (filled symbols) lipase. As an example, Figure 2 details in the case of RaL the extent of acidolysis *vs* hydrolysis (this latter practically negligible in the anhydrous solvent) (Figure 2A) and the percentage of the different products formed (Figure 2B) as a function of time. Remarkably, as opposed to a disappointingly slow reaction rate for the control enzymes, conversion was almost completed (>90%) after only 2 h when using

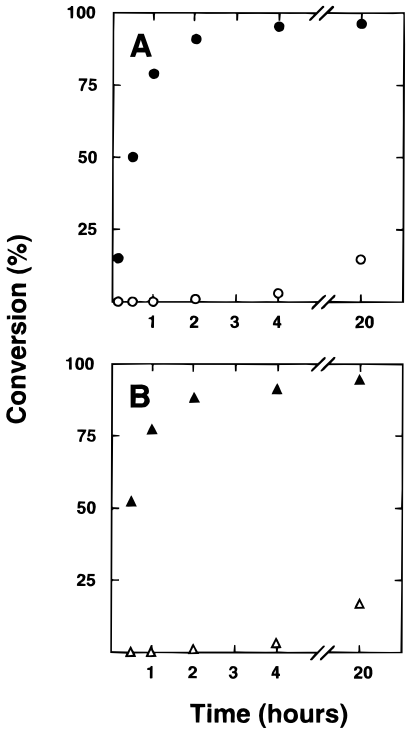

FIGURE 1: Comparison, for RaL (A) and RdL (B), of the time course of tripalmitin conversion by the nonactivated (open symbols) and the n-OG-activated (filled symbols) lipase. The model reaction assayed (see Experimental Procedures) was the acidolysis of tripalmitin (10 mM) by oleic acid (50 mM) in anhydrous isooctane at 40 °C. Washed enzyme powder concentrations were 10 and 5 mg/mL for RaL and RdL, respectively.

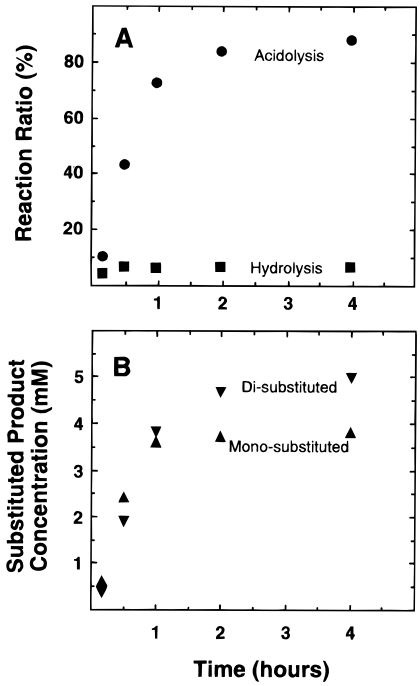

FIGURE 2: Detail of product appearance during the reaction progress in Figure 1A. (A) Time-dependent extent of acidolysis *vs* hydrolysis. (B) Concentration of substituted products formed as a function of time. Other details as in Figure 1A.

the activated lipases. Thus, activation of lipases by TPI results in dramatic nonaqueous rate enhancements, not only in simple esterifications (Mingarro et al., 1995; Braco & Mingarro, 1996) but also when triglycerides are used as substrates for acidolyses.

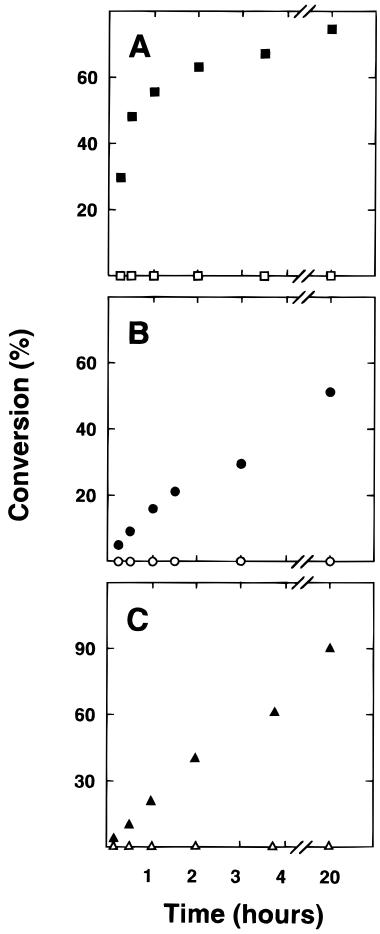

FIGURE 3: Comparison, for ppL (A), CrL (B), and RnL (C), of the time course of tripalmitin conversion by the nonactivated (open symbols) and the n-OG-activated (filled symbols) lipase. Washed enzyme powder concentrations were 25, 25, and 10 mg/mL, respectively. The model reaction and other experimental conditions as in Figure 1.

Gratified by these results, we shifted our attention to two other lipases, ppL and CrL, reported to be surprisingly refractory to catalyze the same acidolysis reaction (Basheer et al., 1995; Goto et al., 1995). Indeed, in our hands both nonactivated lipases also showed reluctant, as no appreciable conversion was detected after 20 h of incubation in either case (Figure 3, panels A and B, open symbols). In fact, no product formation could be detected for ppL even after 72 h (not shown). On the contrary, when subjected to the TPI procedure, both lipases proved to be remarkably active, with significant conversions even after 2 h (Figure 3, panels A and B, filled symbols). A similar result was obtained for RnL (Figure 3C), i.e., the TPI treatment converted an apparently inactive lipase into a very active one. The previous observation that ppL and CrL control samples prepared under exactly the same conditions as in the present study were able to catalyze (although slowly) esterification reactions using small substrates (Mingarro et al., 1995; Braco & Mingarro, 1996) suggests that the inability observed toward tripalmitin is substrate-related and might arise at least in part from steric restrictions. Interestingly, since it has been reported for both the human homologue of ppL (hpL) and CrL that their interfacial activation is the result (among other causes) of lid opening (van Tilbeurgh, 1993; Grochulski et al., 1994), it can be reasonably proposed that the reluctance (experimentally observed by others and ourselves) of nonactivated ppL or CrL to convert tripalmitin in nonpolar solvent is a consequence of the inaccessibility of the occluded active site in the closed conformations of these enzymes to such a relatively bulky substrate. In the activated enzymes, a trapped conformation with a more open lid would alleviate these restrictions, resulting in a significant activity (Figure 3).

Table 1: TPI-Derived Activation Factor Values for Lipases from Different Origin in the Acidolysis between Tripalmitin (10 mM) and Oleic Acid (50 mM) in Anhydrous Isooctane at 40 °C

lipase	activation factor
porcine pancreatic (ppL)	ND[a]
Candida rugosa (CrL)	ND
Rhizopus niveus (RnL)	ND
Rhizopus arrhizus (RaL)	75 ± 8
Rhizopus delemar (RdL)	60 ± 5
Rhizopus javanicus (RjL)	15 ± 3
Aspergillus niger (AnL)	5 ± 2
Pseudomonas fluorescens (PfL)	13 ± 4
Pseudomonas sp. (PsL)	0.9 ± 0.3

[a] ND, not determined, as no substrate conversion could be detected for the nonactivated lipase (see Figure 3).

To investigate the generality of the TPI activation phenomenon, other lipases from diverse sources were also tested under the same conditions. Table 1 summarizes the values obtained for the AF. Although notable rate accelerations were obtained for lipases from different origin, it is evident that the extent of activation depends on the particular nature of the enzyme. For those lipases yielding a very high AF value, but not yet crystallized in closed and open conformation, an explanation for the observed activation based on our model may also plausibly be suggested. On the other hand, it may not be surprising that for PsL there was apparently no activation by TPI (though the enzyme was fairly active in converting tripalmitin), as some lipases from bacterial origin have been reported not to exhibit interfacial activation (Jaeger et al., 1994; Schmid et al., 1995). In any case, n-OG might not be optimum for activation in all cases, though a search for amphiphile optimization is beyond the purpose of this work.

Comparison of Lipase Activation Obtained by TPI or by General "Protection" Methods. Diverse experimental strategies have been documented in recent years which permit an enhancement of enzyme activity in nonaqueous media [summarized in Braco & and Mingarro (1996)]. In particular, two simple approaches have been reported based on a *protection* of the enzyme against denaturation: (i) freeze-drying of the enzyme solution in the presence of lyoprotectants (Lamare et al., 1992; Adlercreutz, 1993; Arakawa et al., 1993; Dabulis & Klibanov, 1993), and (ii) lyophilization in the presence of a moderately high concentration of inorganic salts (KCl) (Khmelnitsky et al., 1994). Both methods allow nonaqueous rate enhancements, though by possibly different mechanisms: lyoprotectants apparently protect against reversible denaturation during freeze-drying whereas inorganic salts (intimately mixed with the protein in the lyophilized solid matrix) are believed to afford protection against organic solvent aggression.

In order to reinforce our interpretation of the above results on the basis of our model, and to rule out a mere protection effect during the trapping protocol as the main cause of the activation observed, several additional experiments were carried out using ppL and RdL as model lipases. Figure 4

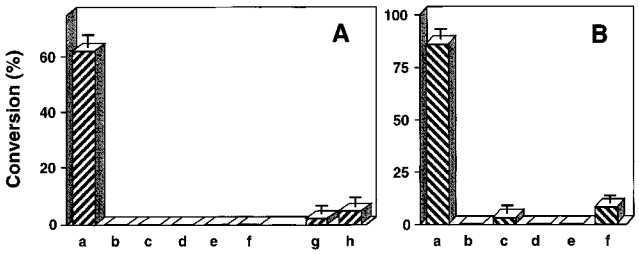

FIGURE 4: Comparison of the activating effect of different experimental strategies (see text) on the ability of ppL (A) or RdL (B) to catalyze the acidolysis of tripalmitin by oleic acid. The strategies were as follows: addition to the lyophilization buffer of (a) 40 mM n-OG (good activator); (b) nothing (control lipase); (c) 2% lactose; (d) 2% sorbitol; (e) 5 mg/mL KCl; (f) 25 mg/mL KCl; (g) 10 mM TX-100 (poor activator); (h) the enzyme was immobilized by adsorption onto CPG beads. The nonaqueous lipase concentration was the same in all cases. The conversion values correspond to 2 h of reaction. The rest of experimental conditions as in Figures 1 and 2.

compares the tripalmitin conversion after 2 h of incubation, obtained with ppL (Figure 4A) or RdL (Figure 4B) either activated by TPI or "protected" (and hence implicitely "kept more active") by the use of either different lyoprotectants, or KCl at different concentrations in the range previously recommended for maximal effect using proteases (Khmelnitsky et al., 1994). Meaningfully, the TPI treatment was unique in conferring a marked activity to both enzymes, relative to the use of sugars or salt additives. Notice the marginal conversion (about 4−8%) detected at this short time only in the case of RdL, when treated with protectants (Figure 4B).

Differential Ligand Binding by Activated and Nonactivated ppL in Nonaqueous Medium. Ligand binding studies in nonaqueous media have been previously reported for several proteins (enzymic or not), where the binding extent and selectivity were compared for the protein lyophilized in the absence or presence of the assayed ligand or a related one (Russell & Klibanov, 1988; Braco et al., 1990; Dabulis & Klibanov, 1992). On the other hand, "soaking" experiments with enzyme crystals suspended in organic solutions of different ligands have been recently used to study binding selectivity in anhydrous solvents (Yennawar et al., 1995). We next investigated whether trapping a lipase in the presence of interfaces could result in an alteration of its binding ability in nonaqueous medium. For this purpose, we selected ppL and the (nonhydrolyzable) phospholipid DHPC as model lipase and ligand, respectively, for two reasons: (i) because the ligand is relatively bulky (but not a substrate) and might be used *a priori* as an accessibility probe for the active site region, and especially (ii) because a phospholipid molecule was found bound in the active site of the open form of the homologous hpL crystallized from a phospholipid/detergent mixed micellar solution (van Tilbeurgh et al., 1993). According to the TPI activation model and the above-mentioned active site accessibility considerations, it could be reasonably expected that activated (trapped in open conformation) ppL exhibited an increased ability to bind soluble DHPC in the nonaqueous milieu, relative to its nonactivated counterpart (trapped in closed conformation).

Figure 5A shows that, under optimized experimental conditions, binding of DHPC by nonactivated ppL was indeed negligible relative to that by the activated lipase. When a titration experiment was performed, binding by activated ppL proved to be saturable at moderately high ligand concentration whereas binding by the control lipase remained negligible in the whole range of DHPC concentrations assayed (Figure 5B). When the data from the binding isotherm for activated ppL were subjected to Scatchard plot (assuming a single class of binding sites), a straight line was obtained (inset in Figure 5B) with a correlation coefficient of >0.98, a K_d for the interaction of 1.9 ± 0.3 mM ($n = 3$), and an estimated 8 ± 2 DHPC molecules bound per molecule of lipase at saturation ($n = 3$). In the case of nonactivated ppL, no reliable fitting is shown since binding values were negligible and fell within experimental error. Although apparently high at first sight, the estimated number of bound ligand molecules is not surprising taking into account the known propensity of phospholipids to aggregate (through head contacts) in nonpolar solvents (Datta et al., 1992) and our recent observation, in a study of phospholipase A_2 in nonaqueous media, that phosphatidylcholine molecules tended to cluster at the entrance of the active site hydrophobic cavity of the enzyme causing a marked substrate inhibition (Mingarro et al., 1994).

It might be still argued that the observed differences in binding by activated and nonactivated ppL may be due to interfacial activation-unrelated, unspecific effects such as: (i) unprotection of the control lipase during freeze-drying, somehow altering the active site conformation; or (ii) variation in the extent of protein−protein contacts in the washed, activated lipase powder (relative to the nonactivated), due to the mere presence of "space"-providing molecules (e.g., of surfactant) during lyophilization. These

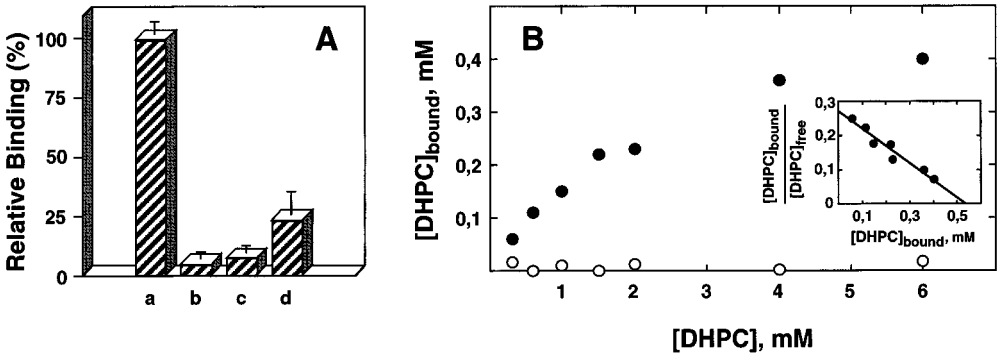

FIGURE 5: (A) Comparison of the ability to bind DHPC (2 mM) in nonaqueous medium [toluene/chloroform, 8:2 (v/v)] of n-OG-activated ppL (a), with respect to ppL samples treated in other ways: control lipase (b), freeze-dried in the presence of 2% lactose (c) or 10 mM TX-100 (d). (B) Binding isotherms for activated (filled circles) and nonactivated (open symbols) ppL. The nonaqueous lipase concentration was 65 μM. Inset: Scatchard analysis from the data for activated ppL in panel B.

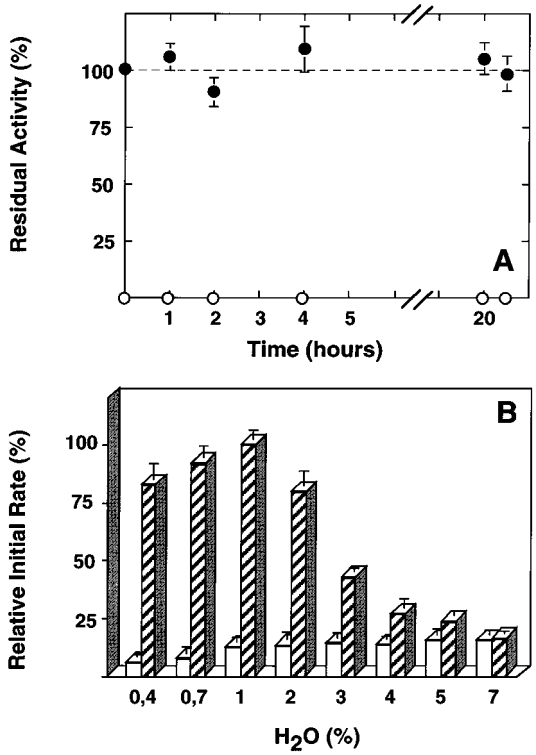

FIGURE 6: (A) Stability of the TPI-derived activation of ppL in anhydrous isooctane as a function of incubation time (filled circles). For comparison, it is also shown that incubation in the organic solvent of the nonactivated ppL counterpart does not confer on it the ability to convert tripalmitin (open circles). The assay for residual activity was carried out under the conditions of Figure 3A. (B) Loss of ppL acquired activation (dashed bars) by gradual increase of the water content of the solvent (2-propanol). For comparison, the effect of water addition to a nonactivated ppL sample is also shown (open bars). The model reaction was the hydrolysis of triolein (10 mM), and the lipase concentration was 20 mg/mL.

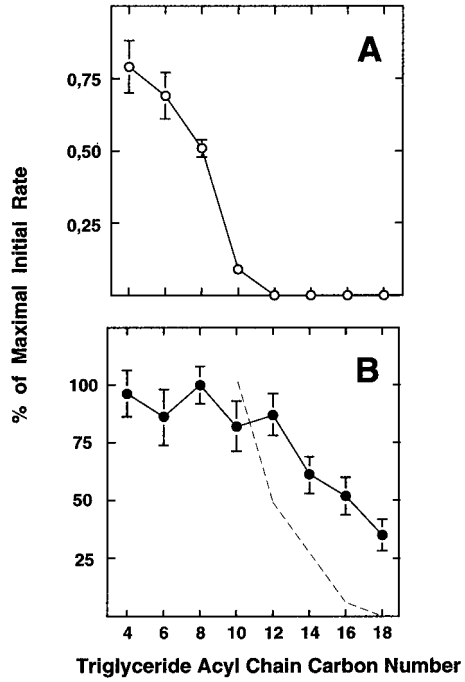

FIGURE 7: Dependence on the substrate chain-length of the ability of either nonactivated (A) or n-OG-activated (B) ppL to catalyze the acidolysis of different triglycerides (10 mM each) by oleic acid (50 mM) in anhydrous isooctane. The rest of experimental conditions as in Figure 3A. The maximal initial rate (100%) in the figure [that for activated ppL (panel B) on tricaprylin (T8)] corresponds to a value of 15.2 μM/(min·mg of washed enzyme powder). For comparison, the relative rates of hydrolysis in aqueous medium of different emulsified triglycerides (from T10 to T18) by ppL (Lin et al., 1986) have been included (dashed line) and are essentially the same as our results (not shown to not overcrowd the figure) using Triton X-100 as emulsifier (see Experimental Procedures).

possibilities, however, seem to be ruled out by the following experiments. First, DHPC binding by a ppL sample freeze-dried from a lactose (lyoprotectant)-containing solution was similar to that of the control enzyme (Figure 5A). Second, when a poor "activator" such as TX-100 micelles (Mingarro et al., 1995) was added (instead of n-OG) to the lyophilization buffer, there was a reasonable correlation between the poor activation obtained (Figure 4A) and the reduced DHPC binding relative to the n-OG-activated lipase (Figure 5A).

Stability of the Acquired Activation in Organic Media. It is well documented that a protein suspended in an anhydrous solvent is conformationally very rigid [see, e.g., Affleck et al. (1992), Burke et al. (1993), Hartsough and Merz (1993), and Desai and Klibanov (1995)]. To investigate the stability in the organic milieu of the trapped activated conformation of the lipase, we decided to measure the loss of enzymatic activity rather than to try to directly address unfolding/denaturation events by using conventional techniques (e.g., circular dichroism or differential scanning calorimetry), since this latter approach (in general more appropriate for proteins in solution) is not possible or at least not easily tractable for protein suspensions in nonaqueous media. For this purpose, experiments were carried out using ppL as a model to know whether the lipase lost its TPI activation by merely being incubated in the organic milieu (prior to the activity assay) or as a result of gradually increasing the water content of the medium. Figure 6A shows that, for activated as well as nonactivated ppL, preincubation of the enzyme samples in anhydrous isooctane (even for 1 day) did not result in any significant variation of their respective activities, i.e., no loss for the activated enzyme but no apparent gain for the control enzyme. On the other hand, in a water-miscible solvent the triglyceride hydrolysis rate of activated ppL was progressively lost as the water content of the medium increased (Figure 6B). Interestingly, whereas the activity of the control lipase monotonically increased in the range studied with increasing water proportion, that of the activated enzyme dropped to equal that of its counterpart (Figure 6B). This result would suggest that the trapped activated conformation of the lipase is retained at low water content (where conformational rigidity is elevated) but may be "relaxed" at higher water proportion by the flexibilizing effect of water.

Comparison of Activated and Nonactivated Lipase Selectivity toward Triglyceride Chain-Length. Since several nonactivated lipases seem unable to catalyze the conversion of tripalmitin in isooctane (Figure 3), the question arises whether this reluctance will be exhibited by the enzyme regardless of the substrate triglyceride assayed. Using ppL as a model and a series of saturated triglycerides as subtrates, we quite surprisingly found that the nonactivated enzyme's activity was dramatically dependent on the substrate size (Figure 7A): while from T18 to T12 no product formation could be detected even after 20 h of incubation (in triplicate independent measurements), from T8 to T4 a clear (though small) activity was observed; the activity toward T10 was only marginal, this substrate being somewhat critical between

Table 2: Dependence on the Triglyceride Acyl Chain Length of the TPI-Derived Activation Factor for ppL-Mediated Acidolysis between the Substrate (10 mM) and Oleic Acid (50 mM) in Anhydrous Isooctane at 40 °C

triglyceride	activation factor	triglyceride	activation factor
tributyrin (T4)	122 ± 10	trilaurin (T12)	ND[a]
tricaproin (T6)	126 ± 13	trimyristin (T14)	ND
tricaprylin (T8)	197 ± 21	tripalmitin (T16)	ND
tricaprin (T10)	920 ± 67	tristearin (T18)	ND

[a] ND, not determined, the same meaning as in Table 1 (see Figure 7).

the "small" and "large" ones. Interestingly, when a parallel study was carried out with activated ppL (Figure 7B), the enzyme was able to readily convert *all* the substrates, with remarkable rate enhancements in all cases (see Table 2). Thus, the observed reluctance of the lipase is indeed not inherent in the enzyme but rather a feature of its nonactivated form. It is also worth noticing that for a "compromised" substrate such as T10 the nonaqueous reaction rate was enhanced by TPI treatment as much as by 3 orders of magnitude (Table 2), a 10-fold improvement relative to the previously reported enhancements using more simple (esterification) model reactions (Mingarro et al., 1995; Braco & Mingarro, 1996).

Finally, some additional results are worth being briefly mentioned. Thus, the remarkable difference in nonaqueous rate between activated and nonactivated ppL was practically canceled when both enzyme samples were redissolved in (amphiphile-free) buffer, relyophilized, and reassayed in the nonpolar solvent. On the other hand, the activation obtained by immobilization (adsorption) of ppL on glass beads (the enzyme is monodispersed and therefore more accessible than in the powder) was very small compared to that achieved by TPI treatment of the same amount of lipase (Figure 4A). Moreover, when n-OG was added *a posteriori* to the nonaqueous reaction medium containing nonactivated ppL, no tripalmitin conversion was detected after 2 h, thus ruling out an activation effect due to the presence of residual molecules of amphiphile even after multiple washing of the lipase powder.

DISCUSSION

The Trapping Strategy. Since the first reports (about five years ago) clearly establishing that two discrete conformational states (closed and open) might be crucial for the understanding of the mechanism of interfacial activation of lipases, nonaqueous studies of these enzymes (even the more fundamental ones) seem to have overlooked this fascinating, compelling information. The present study, as a natural extension of a previously developed strategy to trap activated lipolytic enzymes (Mingarro et al., 1995; Braco & Mingarro, 1996), is an effort to reexamine the behavior of lipases in nonaqueous media toward their natural substrates, in the light of current structural knowledge. Our results basically conform to a model where, depending on the "history" of sample preparation (essentially whether interfaces were present or not in the lyophilization buffer), two conformers of the enzyme, activated (open) and nonactivated (closed), can be trapped and further used in water-restricted media.

In practical terms, the activated form of the lipase will exhibit a nonaqueous rate remarkably higher than that of its nonactivated (control) counterpart, presumably as a result (depending on the lipase) of a more opened lid and/or a more competent catalytic machinery. Of course, there is no reason to expect that in the activated lipase the lid is as much opened as in the corresponding crystal open form, but in general terms TPI shoud result in an increased substrate accessibility to the active site; in fact, the observed trend that the bulkier the triglyceride the higher the AF obtained (Table 2) seems to support this assumption. On the other hand, it may be worth emphasizing the notion that in the case of lipases a full optimization of the enzyme powder must contemplate not only a "pH-memory" (Zaks & Klibanov, 1988) but especially an "interface-memory" effect. Of course, a notable activation of a lipase by TPI should be expected in principle *only* if the enzyme exhibits interfacial activation in aqueous medium (which is not the case for all lipases).

It has been proposed before in the case of phospholipases (Mingarro et al., 1994) as well as lipases (Holmquist, 1995) that nonaqueous studies represent an interesting way to investigate the *inherent* activity of a lipolytic enzyme toward different monomerically dispersed substrates (in particular, when dealing with long-chain natural substrates, phospholipids, or triglycerides, which are water-insoluble but readily soluble in organic solvents), since the observed activity on the "soluble" monomer can be uncoupled from other factors such as the contribution of the physical properties ("quality") of the interface or the adsorption of the enzyme to it. In this sense, our trapping approach has provided in the present work a simple means to compare the activity of *both* inactive and active lipase conformers toward a broad series of triglycerides (Figure 7), a rather untractable goal in aqueous medium. Some immediate conclusions can be drawn from this part of the study. When we compare (Figure 7B) the relative activity of ppL toward T10 to T18 triglycerides either in organic solvent (*for the activated lipase*) or in water using gum arabic (Lin et al., 1986) or a simple surfactant (our data) as emulsifier, a similar decreasing trend is observed: the longer the substrate chain the lower the lipase activity. This proves that activated (rather than nonactivated) lipases ought to be used in nonaqueous studies, especially when the data are to be compared with results from all-water medium. On the other hand, the reasonable activity exhibited by activated ppL on monomerically dispersed T18 (Figure 7B), as opposed to the null activity found in aqueous medium for the same enzyme on emulsified T18 (Lin et al., 1986; see also Figure 7B), is indicative that ppL has an intrinsic ability to use this substrate but might fail in water probably as a result of adsorption/inactivation problems at the interface in this emulsion system. In this regard, activation by TPI might be useful as an approach to test lipase activity on "solubilized" substrates, complementary to more conventional methods where the substrate is presented at an oil (or air)−water interface.

On the other hand, at first sight the strategy of activation of lipases by TPI may appear *on methodological grounds* a special case of the more general phenomenon of molecular bioimprinting, i.e., induction by a ligand (template) in water of a conformational change in the protein, which is further retained in the lyophilized powder in anhydrous media. However, bioimprinting (implemented in the case of proteases or dehydrogenases) and trapping of activated forms of lipases may represent issues of a somewhat different nature. Bioimprinting of proteases and dehydrogenases

(Russell & Klibanov, 1988; Ståhl et al., 1990, 1991; Johansson et al., 1995) has apparently resulted in general in dramatic alterations of substrate or cofactor selectivities, although the molecular interpretation of these experimental observations is open to debate (Dabulis & Klibanov, 1993; Klibanov, 1995; Yennawar et al., 1995); in any case, the rationale seems to be forcing ("tuning") the enzyme conformation in water to accept "unnatural" substrates or cofactors in organic solvent. In the case of lipases, rationalization is inherently simpler as the enzyme is trapped in either of two conformations implicated in its "natural" catalytic mechanism; the ligand (amphiphile interfaces) in aqueous solution is not expected to force the lipase into a "novel" conformation but only to shift the ratio of (closed and open) conformers.

From a broader perspective, our trapping strategy may add to the varied repertoire of efforts reported in recent years aimed at improving our understanding of the structure−activity relationships of biomolecules (from simple peptides to large multimeric enzymes) by trapping different conformers in frozen phases or in water-restricted environments. To name a few instances: the dimer−monomer conformational transition of linear gramicidins in model bilayers and reverse micelles has been assessed in our lab by HPLC using a strategy to trap both peptide conformers (in a nonpolar mobile phase) during chromatographic elution (Bañó et al., 1991; Salom et al., 1992, 1995); the trapping of both competent and incompetent conformers of triosephosphate isomerase in low-water content reverse micelles has been used in the investigation of folding and dimerization of this enzyme (Fernández-Velasco et al., 1995); or the structure of the open-channel form of the acetylcholine receptor has been recently determined by means of an elegant strategy involving the trapping (by rapid freeze-drying) of the acetylcholine-induced structural response of the receptor and the further electron microscopy analysis of the frozen membranes [Unwin, 1995; for a recent review on the topic see Moffat and Henderson (1995)].

Activated vs Nonactivated Lipase Conformers in Nonaqueous Media. It has been sporadically claimed or implicitely assumed (Martinelle & Hult, 1994; Holmquist et al., 1995) that just placing a free lipase in a (pure, typically nonpolar) organic solvent must open the lid, presumably as a result of hydrophobic stabilizing contacts between the inside of the lid and the solvent. This assumption, which might appear correct at first sight only on thermodynamic grounds, seems not warranted for several reasons. First, it is widely documented that proteins (lyophilized as well as crystalline) are much less flexible when suspended in organic solvents than in aqueous solution (Affleck et al., 1992; Hartsough et al., 1993; Desai & Klibanov, 1995); in particular, the conformational rigidity of enzymes in anhydrous nonpolar solvents seems to be dramatically enhanced relative to that in water (Burke et al., 1993). It is therefore very unlikely that in an anhydrous nonpolar solvent a lipase freeze-dried in a closed conformation (as it was in the lyophilization buffer) undergoes a rearrangement such as lid opening. One should rather expect that, on kinetic grounds, the lipase remains trapped in the closed form in the anhydrous milieu. Interestingly, in this regard, the results from a recent, thorough study by Louwrier et al. (1996) strongly support our view: it was basically shown that *Rhizomucor miehei* lipase (*freeze-dried from an amphiphile-free buffer*) was unable to exhibit interfacial activation *in* (predominantly anhydrous) organic solvents, a behavior explicitly interpreted as the result of the lipase having a closed lid in the suspended powder, the same conformation assumed to be adopted in solution in the lyophilization buffer from which the sample was prepared. Second, our stability data show that no significant changes in nonaqueous activity occur for either activated or nonactivated ppL by preincubation of the enzyme samples in isooctane even for 1 day (Figure 6A). This result is consistent with the assumption that closed and open forms of the lipase are indeed kinetically captured in the anhydrous solvent. On the contrary, the dramatic difference in activity between activated and control ppL is difficult to explain (see below) if one assumed that the lid is already opened in the control enzyme. Furthermore, the gradual decrease in reaction rate for activated ppL (toward the values of its nonactivated counterpart), as the content of water (and hence, its lubricating, flexibilizing effect) increases (Figure 6B), is also consistent with our model. This model, though, does not necessarily contradict the assumption in a recent nonaqueous study of *Humicola lanuginosa* lipase (Holmquist et al., 1995) that the enzyme (which was not subjected to any TPI procedure) might be in an open conformation in the organic medium; in fact, in that particular case the lipase was used as previously immobilized on a macroporous resin, and it would not be surprising that adsorption to this support caused some lid opening. In this respect, we verified (unpublished results) that TPI treatment of free *R. miehei* lipase yielded a preparation with a similar esterification activity to that of the enzyme immobilized on a macroporous resin (Lipozyme-IM).

Trapping of Activated Conformers vs Mere (Lyo)protection. It might be suggested, as an alternative mechanism for the nonaqueous rate enhancement observed, that a mere protection of the enzyme (by amphiphiles) occurs during freeze-drying or even in the organic solvent. Several pieces of evidence, however, are difficult to explain on the basis of a simple protection effect. (i) One is the fact that *Fusarium solani* cutinase [a lipase without lid and with a preformed oxyanion hole (Martinez et al., 1994)] was not significantly activated by any of several amphiphiles tested (Mingarro et al., 1995), or that, e.g., PsL was unaffected by the TPI treatment (Table 1); why would these enzymes not be protected? (ii) The acidolysis rate enhancements obtained for lipases from different origin (ppL and RdL), by using reported protection strategies or by enzyme immobilization (Figure 4), are negligible or marginal as compared to those afforded by TPI treatment. (iii) The dramatic difference in the binding of a phospholipid in nonaqueous medium by activated *vs* nonactivated ppL is strongly suggestive of a conformational change of the enzyme, exposing site(s) or residues previously unavailable. Still, it has been recently proved by means of solid-state NMR studies of the structural integrity of α-chymotrypsin and bovine pancreatic trypsin inhibitor, using a transition state analog probe (Burke et al., 1992) or in hydrogen isotope exchange experiments (Desai & Klibanov, 1995), that freeze-drying *in the absence of lyoprotectants* indeed caused a denaturation of about 40% of the active centers, although apparently there was essentially no appreciable further damage by incubation of the protein in nonpolar solvents (such as isooctane used in the present work). Therefore, assuming that these results can be roughly extrapolated to the case of lipases, it is not easy

to explain the remarkable AF values obtained by TPI (e.g., almost 1000-fold for T10), even comparing with additive-protected samples, on the mere basis of a "depressed" activity of nonactivated lipases due to inactivation (denaturation). In any case, a partial contribution to the activation observed of a specific protection by amphiphile molecule(s) located in the active site during lyophilization cannot be discarded.

Two or More Conformers? Finally, it is worth discussing some additional biochemically relevant implications of the present study. It has been reasonably proposed by different authors [see, e.g., Derewenda et al. (1994), Jennens and Lowe (1994), and Rubin (1994)], in part to account for the observed low (but not null) activity of lipases on monomolecularly dispersed substrates in aqueous medium, that an equilibrium might exist in solution between the two states (closed and open) of the enzyme, largely favorable to the closed conformer. Assuming this model, the low activity observed for several nonactivated lipases (relative to their activated counterparts) in the present and earlier work (Mingarro et al., 1995; Braco & Mingarro, 1996) would be due to the small fraction of open forms (present in solution before freeze-drying) trapped in the control enzyme powder. Addition of interfaces (micelles or vesicles) to the aqueous solution would displace the equilibrium toward the open form of the lipase, thus stabilizing this conformer (and activating the enzyme) which would be predominantly trapped upon freeze-drying.

Nevertheless, the scenario may be even more complicated. As has been recently suggested (Derewenda et al., 1994) on the basis of experimental evidence, the two-state ("all-or-nothing") model for lipase activation may be rather an oversimplification. Indeed, an unexpected conformational lability was observed in the lids of *H. lanuginosa* and RdL lipases, crystallized in the absence of substrates or inhibitors (in the latter enzyme, two distinct positions of the lid, closed and intermediate, were found in the crystal) (Derewenda et al., 1994); it was postulated that these results might reflect a conformational heterogeneity of the lipase in solution, implying a more or less continuous range of conformations where intermediate conformations of the lid might be populated. In this regard, the results in Figure 7A seem both suggestive and supportive of this more sophisticated model: the clear discrimination by nonactivated ppL against large substrates (T12 to T18) as opposed to relatively small triglycerides (T4 to T8) is consistent with the assumption that in the nonactivated sample intermediately open forms of the enzyme (reasonably populated in solution immediately before freeze-drying) may be trapped; these forms would be somehow sufficiently open to permit the access of relatively small (but not large) substrates. These results are not easily explained assuming a simple all-or-nothing model. Notice also that the reluctance of nonactivated ppL toward large triglycerides is not the result of enzyme inactivation [as it *can* convert small substrates (Figure 7A)] or of an intrinsic inability [as the activated counterpart readily uses them (Figure 7B)]. Interestingly, such a model including several states in equilibrium (closed, intermediate, and open) is conceivable in the light of recent circumstancial evidence obtained from a study of lactate dehydrogenase (Xue & Yeung, 1995), which by combining capillary electrophoresis and fluorescence microscopy has shown that the activity of *individual* electrophoretically pure enzyme molecules can significantly vary, plausibly owing to the presence in solution of several stable forms of the enzyme.

In conclusion, the present study provides the basis of both a conceptual and an experimental framework to address, probably more rigorously than has been done so far, further fundamental investigation of lipase behavior in water-restricted environments. In addition and from a more practical standpoint, our trapping strategy has also permitted to rationally overcome the reluctance of several lipases to catalyze conversions of undeniable biotechnological interest, with remarkable rate enhancements relative to the conventional (nonactivated) preparations. These results make the approach promising as a method to expand the range of substrates utilizable by lipases in nonaqueous media and should hopefully stimulate further research aimed at activating enzymes in water-restricted media. Work is in progress in this direction in our lab.

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