# The Closed/Open Model for Lipase Activation. Addressing Intermediate Active Forms of Fungal Enzymes by Trapping of Conformers in Water-Restricted Environments†

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ABSTRACT: The behavior of prototypic fungal lipases in a water-restricted environment has been investigated by exploiting the reported experimental strategy that allows the trapping (freeze-drying) of the enzyme in the conformation present in aqueous solution and to subsequently assay it in nonaqueous media [Mingarro, I., Abad, C., and Braco, L. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92,* 3308−3312]. We now report, using simple esterification as well as acidolysis (triglycerides as substrates) as nonaqueous model reactions, that the presence of a detergent (*n*-octyl-*β*-glucopyranoside) in the freeze-drying buffer, at concentrations below the critical micellar concentration, generates different catalytically active (kinetically trapped) conformational states of the enzyme. These activated forms exquisitely discriminate between short- and long-chain fatty acids, suggesting that they can be correlated with intermediate conformations of the protein sufficiently open to permit the access of relatively small but not large substrates. Additional data obtained from aqueous solution activity measurements in the presence of detergent revealed that the fungal lipase retains an active conformation induced by high detergent concentration (30 mM) for a long period of time, a 'memory effect', which is stabilized in the absence of a well-defined interface by few detergent molecules. Together these results provide support to a model of lipase action involving several equilibrium states (closed, intermediate, and open), which can be modulated by the composition of the microenvironment, i.e., by the detergent concentration.

Lipases (triacylglyceride ester hydrolases, EC 3.1.1.3) constitute a diverse and ubiquitous family of enzymes that catalyze hydrolysis of triglycerides (*1*). One unique feature of these enzymes is the higher activity that they display when the substrate solubility is exceeded, giving rise to a lipid−water interface (*2*, *3*). This characteristic, called interfacial activation, is necessary for full lipase activity. This feature has also been postulated to be due to either an optimal orientation and/or hydration state of the lipid in the aggregate where the enzyme is structurally invariant (substrate models) (*4*), or an optimized active site geometry through conformational rearrangements in the lipase (enzyme models) (*5*). Studies of the molecular basis of interfacial activation have further resulted in the reconciliation of these two models by showing that both the nature of the substrate as well as conformational changes in the protein may contribute to the enzyme's unique behavior at the lipid−water interface (*6*).

Progress in the understanding of interfacial activation has been achieved from X-ray crystallographic studies of different lipases. Among others, the three-dimensional lipase structures from several organisms have been resolved: *Rhizomucor miehei* (*7*), human pancreas (*8*), *Geotrichum candidum* (*9*, *10*), *Candida rugosa* (*10*), *Rhizopus delemar* (*11*), *Pseudomonas glumae* (*12*), *Humicola lanuginosa* (*11*, *13*), *Penicillium camembertii* (*14*), *Candida antartica* (*15*), and *Chromobacterium viscosum* (*16*). All exhibit an α/*β* hydrolase fold, and it was found that the active sites containing the typical Ser-His-Asp(Glu) esterase catalytic triad are shielded from the solvent by a flexible protein structure element, often referred to as the 'lid' or the 'flap'. In the case of *Rhizomucor miehei* and human pancreatic lipases, it was possible to obtain the inactive (closed) forms of the protein (*7*, *8*) and also the active (open) ones when crystallized in the presence of a covalent inhibitor (*17*) or micelles (*18*). These structures have provided a detailed description of the structural rearrangements that are thought to mimic those occurring at the oil−water interface (*18*). Thus, it is supposed that in water the enzyme is in the 'closed' conformation where the lid covers the active site, hindering any substrate or solvent molecules from entering. When the lipid is bound, the lid then rotates around the hinge regions so that the active site becomes exposed to solvent, yielding the 'open' conformation (*19*, *20*). This conformational change should be involved in the interfacial activation of the enzyme (the two-state model for lipase activation) (*7*, *8*, *18*). Some functional consequences of these changes are the following: (i) access of the substrate to an otherwise buried active site, (ii) exposure of a large nonpolar surface at the entrance to the active site for interaction with the interface and substrate binding, and (iii), in some cases, formation of the oxyanion hole critical for the stabilization of the transition state during catalysis. It has been further proposed for some fungal lipases that an equilibrium exists

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between these two forms in aqueous solution, even in the absence of a true oil−water interface (*10*, *11*). In the presence of the interface, the opened form will be the predominant enzyme conformation in solution (*21*). In this regard, activated (open) forms of lipolytic enzymes have been kinetically trapped and retained in nonaqueous media by the so-called strategy 'trapping in the presence of interfaces' (TPI)[1] (*22*, *23*). The rationale was to trap the enzyme in a presumably activated form (i.e., when it is bound to an amphiphile−water interface) by freeze-drying and to further assay it (after washing out the amphiphile with an anhydrous solvent) in water-restricted environments (*22*). In these environments, the trapped conformation of the enzyme is expected to be preserved by virtue of the known enhanced conformational rigidity of proteins in anhydrous solvents (*24*). This rationale entails the complementary notion that the same lipase will be trapped in a nonactivated (closed) conformation in the absence of interfaces (i.e., that of the lipase immediately before freeze-drying). Micelles of the amphiphile *n*-octyl-*β*-D-glucopyranoside (n-OG) were used for activation of pancreatic lipase, which create an interface able to induce the conformational changes associated with interfacial activation in lipases from varied origins (mammalian, fungal, and bacteria) (*22*).

However, additional experimental data from lipases of various sources are not easily explained assuming a simple all-or-nothing (open/closed) model. In fact, in the *Candida rugosa* (*10*), *Humicola lanuginosa*, and *Rhizopus delemar* (*11*) lipase structures, the lid is not closed, even in the absence of a substrate. In the latter enzyme, two distinct positions of the lid, closed and intermediate, were found in the crystal, and it was postulated that might reflect a conformational heterogeneity of the lipase in solution. In *Candida rugosa*, the involvement of the carbohydrate residues in stabilizing the open conformation, as well as the limited number of hydrogen bonds on the hydrophilic side of amphipathic helices, suggests a number of highly populated open-state conformations (*21*). Intermediate conformations of lipases have been considered to account for the catalytic behavior of *Staphylococcal* enzymes using carbamate substrates (*25*). Also, the backbone dynamics of *Fusarium solani pisi* cutinase studied by a variety of nuclear magnetic resonance experiments revealed internal motions in the substrate binding site (*26*). These results are interpreted to suggest that the observed mobility likely represents the interconversion between open and more closed conformations. In relation to this, previous work in our laboratory using TPI strategy was also suggestive of a conformational heterogeneity for porcine pancreatic lipase (*23*). On the other hand, for a fungal enzyme from *Rhizomucor miehei*, theoretical normal mode analysis performed in both low and high dielectric media (*27*) and molecular dynamics simulation (*28*) have been able to characterize partial opening and closing of the lid.

In the present study, the use of our strategy in the presence of n-OG concentrations below the critical micellar concentration (cmc) has enabled, for the first time, detection of intermediate conformations for fungal lipases in aqueous solution which have been trapped in different activated states. The intrinsic activity of these intermediate forms versus both nonactivated and fully activated (in the presence of micelles) enzymes has been evaluated in a water-restricted environment using both simple esterification and acidolysis (triglycerides as substrates) as model reactions. It is evident that the nonaqueous approach seems ideally suited to provide insights into the requirements for the enzymatic activity or specificity of the enzyme toward monomolecularly dispersed natural substrates (*22*, *23*, *29*, *30*). Experiments which assay enzyme activity in aqueous solutions containing detergent also support a model including several lipase states in equilibrium.

## MATERIALS AND METHODS

*Materials.* Triglycerides, *n*-octyl-*β*-glucopyranoside (n-OG), fatty acid-free bovine serum albumin, olive oil, oleic acid, and *p*-nitrophenyl propionate ester (PNP-propionate) were purchased from Sigma. All other chemicals were from Aldrich or Merck (analytical grade or purer). Reactives for electrophoresis or chromatography like acrylamide, DEAE-Sepharose, or Sephadex G-100 were from Sigma or Pharmacia. All the organic solvents were of HPLC grade (Merck). The solvents used for nonaqueous assays were desiccated with 3 Å molecular sieves (Merck) to bring their water content below 0.01%, as determined by Karl Fisher titration.

*Enzyme Purification.* The four lipases used: *Candida rugosa* (CRL), *Geotrichum candidum* (GCL), *Humicola lanuginosa* (HLL), and *Rhizopus delemar* (RDL), were purified as previously described by Aires-Barros et al. (1994) (*31*). In brief, commercial powders supplied by Sigma (CRL) and Amano Co. (GCL, RDL, and HLL) were chromatographed on DEAE-Sepharose and Sephadex G-100. The purification process was followed in each case by electrophoresis. For lipase quantitation, Coomassie Blue-stained discontinuous SDS−PAGE gels were analyzed with an LKB 2202 Ultroscan laser densitometer using bovine serum albumin as a standard.

*Preparation of Activated and Nonactivated Lipase Samples by the Trapping Strategy.* The experimental procedure was basically the same as has been previously reported (*22*). Each lipase was incubated (before freeze-drying) for about 3−4 min at 4 °C, in 10 mM Tris-HCl buffer, pH 7.5, containing different n-OG concentrations from 0 (for the nonactivated, control enzyme) to 30 mM (fully activated enzyme). Liquid nitrogen was used for freezing. To remove the amphiphile after freeze-drying, the samples were washed at least 3 times with toluene/ethanol, 90:10 (v/v), and vacuum-dried for 2 h. The solvent was selected on the basis of amphiphile solubility and to minimize any possible deleterious effect on the enzyme. In fact, it was verified that unwashed control enzyme preparations yielded a similar activity in nonaqueous medium as washed controls. The completeness of n-OG removal was quantitatively assessed by analytical reversed-phase high-performance liquid chromatography using a Lichospher 100 RP-18 column (Merck) eluted with methanol/water, 90:10 (v/v). The critical micelle concentration (cmc) of n-OG in 10 mM Tris-HCl buffer, pH 7.5, was determined

[1] Abbreviations: cmc, critical micellar concentration; CRL, *Candida rugosa* lipase; GCL, *Geotrichum candidum* lipase; DEAE, diethylaminoethyl; HLL, *Humicola lanuginosa* lipase; HPLC, high-performance liquid chromatography; n-OG, *n*-octyl-*β*-D-glucopyranoside; PNP-propionate, *p*-nitrophenyl propionate; RDL, *Rhizopus delemar* lipase; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TPI, trapping in the presence of interfaces.

according to the procedure described by Miguel et al. (*32*). As expected, the cmc slightly increased with decreasing temperature from 24 mM (room temperature) to 27.5 mM (4 °C).

*Lipase Assays in Nonaqueous Medium.* Acidolysis of triglycerides by oleic acid and direct esterification of oleic acid and 1-hexanol in anhydrous isooctane were performed as previously reported (*22*, *23*). A given amount of washed lipase powder (freeze-dried from different n-OG concentrations) was suspended in a substrate(s)-containing organic solution, placed into a screw-capped vial, sonicated for 10 s, and shaken in an oven at 40 °C for acidolysis reaction and at 25 °C for direct esterification. Periodically, a 25 $\mu$L aliquot was withdrawn from the reaction mixture and centrifuged at 12000*g* for 3 min, and the supernatant was analyzed by HPLC. The liquid chromatograph (Waters) was equipped with M-510 solvent-delivery systems, an automated gradient controller, a U6K universal injector, and a Waters 410 differential refractometer. The reverse-phase Lichrospher 100 RP-18 column (Merck) was isocratically eluted with acetonitrile/dichloromethane/tetrahydrofuran/acetic acid, 70:22:20:8 (v/v), for long-chain triglycerides (*33*), or with methanol for short-chain ones at a flow rate of 1 mL/min. Simultaneous disappearance of the substrate(s) and appearance of the product(s) were monitored to obtain the reaction rates.

*Lipase Assays in Aqueous Medium.* Lipase activity in aqueous solution was determined by different methods depending on the substrate used: (i) Spectrophotometric assay. PNP-propionate hydrolysis (0.25 mM) in a 100 mM Tris-HCl buffer, pH 7.5, was followed at 25 °C with a Shimadzu UV 160 recording spectrophotometer, by the absorbance increase at 400 nm (*p*-nitrophenol formation) using a molar extinction coefficient of 16 000 $M^{-1}$ $cm^{-1}$. Substrate stock solution (25 mM) was prepared in acetonitrile. The final concentration of acetonitrile was always kept below 1% in the assay solution so that it would not affect the lipase activity. Spontaneous hydrolysis was automatically corrected using the same solution without enzyme as a control. (ii) Titrimetric assay. The activity toward triacetin was monitored potentiometrically with a Crison MicroTT 2050 recording pH-stat system. Typically, 5 mL of a solution containing 200 mM triacetin, 10 mM Tris-HCl, 10 mM NaCl, 5 mM $CaCl_2$, equilibrated at pH 7.5, was placed in a thermostated cuvette (27 °C) with a stirrer. The reaction was followed by titration of the released fatty acid with 30−50 mM sodium hydroxide. (iii) Turbidimetric assay. The artificial lipoprotein substrate solution was prepared as previously described (*34*). Briefly, 1 mL of 5% of bovine serum albumin in distilled water was incubated for 30 min at 30 °C with 40 $\mu$L of an olive oil emulsion. The olive oil emulsion was obtained by mixing 97 mL of olive oil with 1 mL of 2% arabic gum solution. Different amounts of lipase were added to the assay solution containing 0.2 mL of artificial lipoprotein solution and 0.8 mL of 200 mM Tris-HCl buffer, pH 7.6. The activity was monitored by the turbidity decrease at 660 nm of an initial absorbance between 0.9 and 1.2.

## RESULTS AND DISCUSSION

*Trapping of Lipase Conformers in Nonaqueous Medium from n-OG Aqueous Solutions.* Typically, enzymes to be used in nonaqueous media are freeze-dried from pH-adjusted buffer solutions in order to optimize enzyme conditions (*29*, *35*). In the TPI strategy previously developed by our group (*22*, *23*), a similar approach is used in which a conformational optimization is made based on the lipase interfacial activation (*22*). In brief, the enzyme in contact with the interface (micelles or vesicles) is induced toward the active (open) conformation in aqueous solution, and then is trapped by rapid freeze-drying of this solution. Consequently, an activated lipase form in the resulting lyophilized powder is obtained, which will be further retained in nonaqueous media by means of conformational rigidity (*24*). The question that arises is whether a detergent below its cmc (premicellar concentration) can induce activated forms of lipases which then can be trapped and retained in water-restricted media. Toward this end, experiments were carried out using n-OG concentrations between 0.25 and 20 mM in the freeze-drying buffer solution. The acidolysis of tributyrin (T4) by oleic acid (transesterification reaction) in anhydrous isooctane was chosen as a nonaqueous model reaction to evaluate the consequences of our strategy. Enzyme samples were used as a powder suspension in the organic medium, as in previously reported work (*23*). Although in our strategy the activity is assayed in nonaqueous media, it should be keep in mind that the activity in nonaqueous media reflects the conformational state that lipase has in the freeze-drying buffer. Figure 1 depicts, as an example, the time course of fatty acid transesterification mediated by CRL as a function of the amphiphile concentration in the freeze-drying buffer. Interestingly, a variable extent of enzyme activity was shown in anhydrous isooctane in such a way that increasing n-OG concentration gives rise to significant increased activity. As expected, maximum activity in organic solvent was obtained using detergent concentrations in the freeze-drying buffer above the cmc (30 mM). It was also verified that higher detergent concentration (40 mM) yields enzyme activity similar to those detected at 30 mM concentration. On the contrary, no appreciable transesterification was detected (even after 24 h of incubation) for the control sample prepared in the same conditions but in the absence of amphiphile (nonactivated lipase).

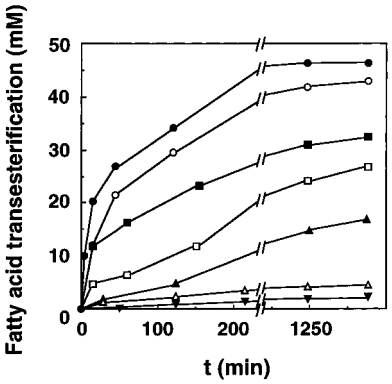


FIGURE 1: Time course of fatty acid transesterification for CRL in nonaqueous medium as a function of the aqueous n-OG concentration in the freeze-drying buffer. Detergent concentrations were: (▼) 0 mM, nonactivated (control) lipase; (△) 1 mM; (▲) 5 mM; (□) 7.5 mM; (■) 10 mM; (○) 20 mM; (●) 30 mM. The model reaction assayed was the acidolysis of tributyrin (10 mM) by oleic acid (50 mM) in anhydrous isooctane. The washed enzyme powder concentration in nonaqueous medium was 20 mg/mL.

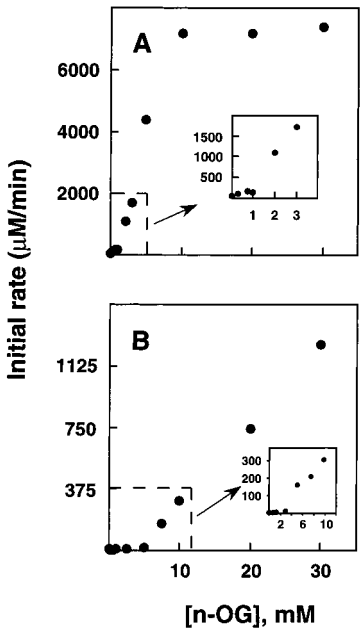


FIGURE 2: Enzyme activity for CRL in organic solvent using (A) direct esterification between 1-hexanol (100 mM) and oleic acid (100 mM) in anhydrous *n*-hexane and (B) acidolysis of tributyrin (10 mM) by oleic acid (50 mM) in anhydrous isooctane, as a function of n-OG concentration in the freeze-drying buffer. Inset: detail of initial rate dependence on detergent concentration. The washed enzyme powder concentration was 20 mg/mL.

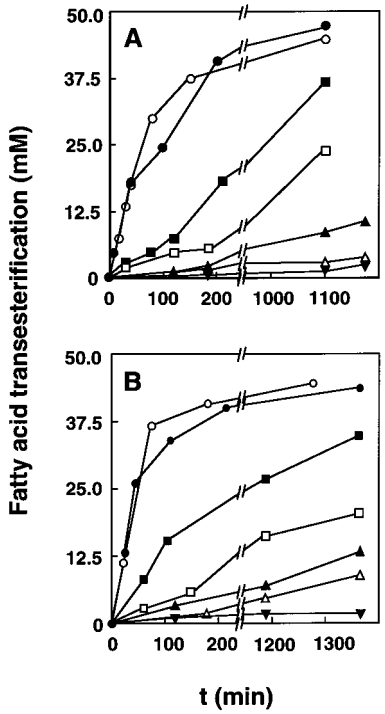


FIGURE 3: Time course of fatty acid transesterification for CRL (A) and RDL (B) in nonaqueous medium as a function of n-OG concentration in the freeze-drying buffer. Detergent concentrations were: (▼) 0 mM, nonactivated (control) lipase; (△) 0.75 mM; (▲) 2.5 mM; (□) 5 mM; (■) 7.5 mM; (○) 20 mM; (●) 30 mM. The model reaction assayed was the acidolysis of tricaprylin (10 mM) by oleic acid (50 mM) in anhydrous isooctane. The washed enzyme powder concentrations in nonaqueous medium were 20 and 15 mg/mL, respectively.

To compare different reactions in nonaqueous media, the esterification between 1-hexanol and oleic acid in anhydrous isooctane was also investigated (Figure 2A). A noticeable rate enhancement was obtained even at very low detergent concentrations (<2 mM) relative to tributyrin conversion (Figure 2B). For both reactions, it seems that a minimum detergent concentration was necessary to observe enzyme activation (1 mM for the esterification reaction and 3 mM for transesterification). In addition, both cases demonstrated a remarkable enzyme activation below the cmc value, suggesting that a small amount of detergent in the freeze-drying aqueous solution should be able to induce catalytically active conformations. It should be noted that an inherent advantage of our strategy in nonaqueous medium is that the substrate is totally soluble in the organic solvent, avoiding interference by interfaces or detergent in the enzymatic assay. On the other hand, the approach allows us to analyze the effect of detergent on enzyme conformation in the aqueous solution without interference by the substrate. The differences observed in the n-OG concentration needed for enzyme activation in a given reaction (Figure 2) would be ascribed to steric restrictions related to the substrate size.

*Selectivity of Trapped Lipase Conformers toward Triglyceride Chain Length.* Substrate steric restrictions in nonaqueous media have been previously reported for several lipases in the absence or presence of interfaces in the freeze-drying buffer. In particular, for porcine pancreatic lipase, it was found that enzyme activity was dependent on the triglyceride substrate size (*23*). Thus, we shifted our attention to a longer substrate such as tricaprylin (T8) and also to another fungal lipase, RDL. CRL is a representative enzyme of family 5 (yeast lipases), and RDL is a member of family 4 (filamentous fungi lipases) (*36*). In Figure 3, panels A and B depict for CRL and RDL, respectively, comparison of the time course of acidolysis of tricaprylin by oleic acid in anhydrous isooctane as a function of n-OG concentration in the freeze-drying buffer. Both lipases proved to be remarkably active, with significant substrate conversion for enzyme samples activated with detergent at 0.75, 2.5, 5, and 7.5 mM concentrations. Although the extent of activation was quite similar for both lipases (Figure 4), RDL was able to catalyze the acidolysis reaction even in enzyme samples prepared from very low detergent concentration (<5 mM, Figure 4B). For n-OG concentrations above 2.5 mM, the CRL activity for substrate T8 (Figure 4A) was always slightly lower than for T4 (Figure 2B). The presence of n-OG micelles (30 mM concentration) in the freeze-drying buffer results in the higher activity detected in nonaqueous medium for both enzymes (Figures 3 and 4). This functional form of lipase (maximum activity) can be related to a 'fully' open-lid active (kinetically trapped) conformation based on the mechanism of lipase activation at interfaces (*18*, *21*) as suggested previously for lipases from diverse sources (*23*, *30*). However, as shown for the small substrates (Figure 2), the most surprising result is that the activated forms induced by low concentrations of detergent were able to catalyze the acidolysis of the T8 substrate. If interfacial activation is the result of lid opening at detergent concentrations below the cmc, different enzyme forms (presumably less open) that can be correlated with catalytic activity will be trapped and retained in nonaqueous media. Substrate steric restrictions, as well as differences in the dynamic motion of the lid in aqueous solution due to the particular nature of the enzyme, can account for the changes observed in organic solvent.

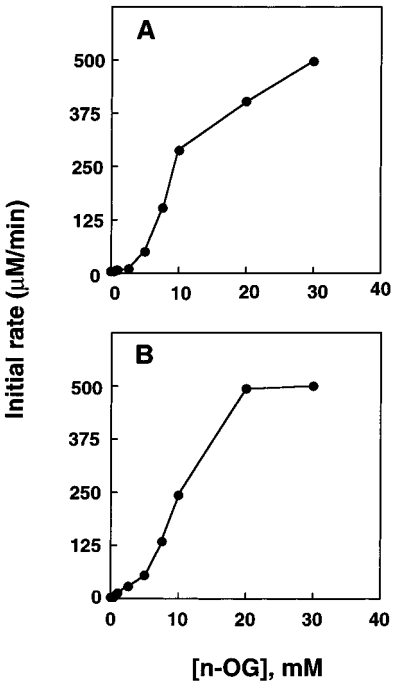


FIGURE 4: Comparison for CRL (A) and RDL (B) of the initial rate of fatty acid transesterification for n-OG-activated lipase as a function of the n-OG concentration in the freeze-drying buffer. The model reaction and other experimental conditions were as in Figure 3.

To reinforce our interpretation of the above results, several experiments were carried out using CRL as a model lipase and a series of saturated triglycerides as substrates. Figure 5 shows the CRL-mediated acidolysis of triglycerides (with acyl chain lengths between 4 and 18 carbon atoms) by oleic acid in anhydrous isooctane. Initially, three different n-OG concentrations (5, 7.5, and 10 mM) were used in the buffer solution previous to lyophilization. In these conditions, the lipase activity in organic solvent was dramatically dependent on the substrate size (Figure 5A). The figure shows how the lipase activity decreases as the acyl chain length increases with a critical size around 12 carbon atoms. This strong discrimination between triglycerides seen in the nonaqueous media was maintained in all ranges of detergent concentrations used, with a remarkable rate enhancement for a given triglyceride as detergent concentration increases. Also noteworthy is that the nonactivated lipase shows detectable activity toward both T4 and T8 triglycerides whereas no activity could be detected with long-chain substrates even after 20 h of incubation. These results agree with previous observations using nonactivated porcine pancreatic lipase (*23*) since these forms would be somehow sufficiently open to permit the access of relatively small (but not large) substrates. In fact, when this study was extended to analyze a broad range of n-OG concentrations (all below the cmc), it was revealed that for each triglyceride a different detergent concentration was required to observe a catalytically competent lipase form (Figure 5B). Thus, a clear dependence upon substrate size was shown in such a way that with an increase in substrate size more detergent was needed to detect enzyme activity. These results seem to indicate that, in the presence of detergent, different active forms of the lipase were trapped with conformations ranging from relatively closed to more open, permitting the binding of triglycerides with short, medium, or long chains. A progressive opening

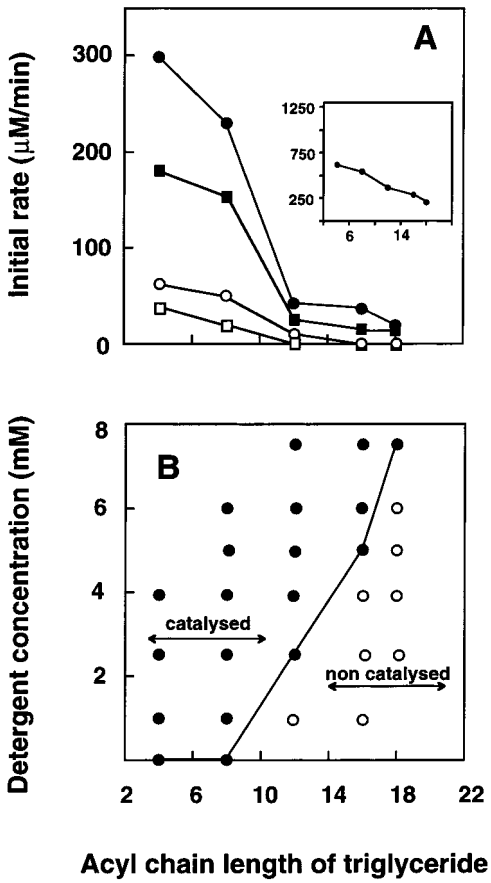


FIGURE 5: Triglyceride acyl chain length dependence in organic solvent using CRL. (A) Initial rate as a function of acyl chain length using different n-OG concentrations in the freeze-drying buffer: (□) 0 mM, nonactivated (control) lipase; (○) 5 mM; (■) 7.5 mM; (●) 10 mM. The inset shows the micelle-activated enzyme. (B) Diagram showing the detection of activity as a function of both the detergent concentration in the freeze-drying buffer and the triglyceride assayed. Filled circles and unfilled circles represent the detected and nondetected activity, respectively. The line shows the detergent concentration necessary to observe activity for each triglyceride. The assay conditions of acidolysis were as in Figure 1.

of the lid would explain the occurrence of these different lipase conformational states exhibiting different accessibilities to the catalytic hole. In this regard, detergent-induced conformational changes have been recently described for HLL using fluorescence spectroscopy (*37*). Premicellar assemblies contacting the hydrophobic surface of HLL have been proposed that should have long enough residence times to allow lid opening, exposure of the hydrophobic cleft, and concomitant stabilization of an open conformation.

Collectively, these findings suggest that activation of fungal lipases seems to be easier than previously thought, probably due to the motion of the lid. In fact, detergent molecules in aqueous solution (below cmc) may be able to induce intermediate enzyme activated states which can be kinetically trapped and further assayed in water-restricted media. Noteworthy is the fact that in our experiments the chain length selectivity was almost completely lost when the fungal lipase (CRL, RDL) was freeze-dried from buffer containing micellar n-OG concentration (see inset in Figure 5 as an example). In this case, a similar preference was observed for short- or long-chain fatty acids as previously described for porcine pancreatic lipase (*23*), probably due

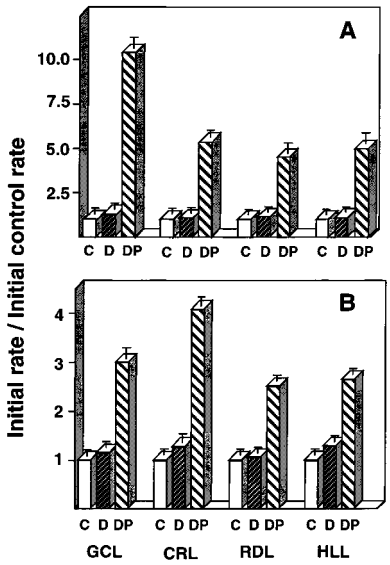


FIGURE 6: Effect of 30 mM n-OG preincubation in aqueous solution on lipase activity. Lipase activity of control (C), 'direct' 0.75 mM n-OG (D), and detergent preincubated (DP) samples for GCL, CRL, RDL, and HLL. Lipase activity for the hydrolysis of 0.25 mM PNP-propionate (A) and the hydrolysis of 200 mM triacetin (B). The lipase activity is expressed as the ratio between the initial rate observed for each enzyme and the control initial rate. Enzyme concentrations were as follows: 1.2 $\mu$g/mL (A) and 16 $\mu$g/mL (B) for GCL; 45 ng/mL (A) and 13 $\mu$g/mL (B) for CRL; 1 $\mu$g/mL (A) and 10 $\mu$g/mL (B) for RDL; 2.3 $\mu$g/mL (A) and 12 $\mu$g/mL (B) for HLL.

to a micelle-induced open conformation alleviating putative steric restrictions.

*Can the Conformational Heterogeneity of Lipase Be Detected Directly in Enzymatic Assays in Aqueous Medium?* Because different lipase conformers can be trapped in active conformation in nonaqueous media after freeze-drying lipase/detergent from buffer solution, the attention was shifted to ascertain if activation induced by n-OG can be detected directly in the aqueous solution. In a first attempt to search a similar behavior to that observed in nonaqueous media, an experiment in aqueous solution was performed in which lipase was preincubated for 10 min with micelles of n-OG (30 mM) before the enzymatic assay. Aliquots of 25 $\mu$L were withdrawn and diluted 1:40 (0.75 mM amphiphile concentration) in buffer solution. Then the enzyme activity of diluted lipase was assayed toward either PNP-propionate or triacetin substrates. Figure 6 shows esterolytic activity using PNP-propionate, 0.25 mM (panel A), and lipolytic activity using triacetin, 200 mM (panel B), for four fungal lipases (GCL, CRL, RDL, and HLL). GCL belongs to the same enzyme family as CRL (family 5), whereas HLL and RDL are members of family 4 (*36*). A noticeable increase in activity was observed for all cases in which the lipases were preincubated with detergent (DP) in comparison with detergent-free controls (C). To discern if the activation found was entirely due to the 0.75 mM n-OG remaining from the dilution, a nonpreincubated sample containing 0.75 mM n-OG ('direct' lipase, D) was also assayed. It should be mentioned that, the final enzyme concentration was the same in every sample for each given lipase. A modest rate of hydrolysis was observed for the 'direct' lipase relative to the control one for the four fungal enzymes (Figure 6). Since the most pronounced rate enhancement was only seen after

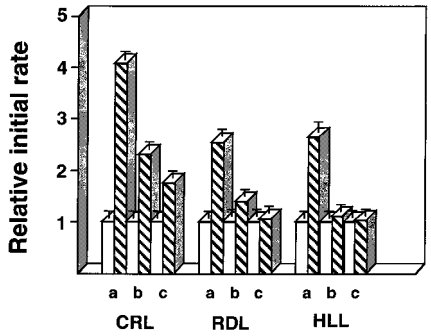


FIGURE 7: Dilution effect after n-OG preincubation on lipase activation. Relative initial rates of 0.75 mM 'direct' (open bars) and detergent preincubated (dashed bars) samples for lipases CRL, RDL, and HLL as a function of dilution used: (a) 1:40, (b) 1:100, and (c) 1:300. The relative initial rates were obtained as a ratio between each lipase initial rate and the control rate. The activity was assayed using 200 mM triacetin, and the enzyme concentrations were as in Figure 6.

the step of preincubation with detergent, it can be attributed to the previous treatment with the n-OG micelles and is not due to a direct effect of the free detergent molecules that remain after dilution. This treatment gives rise to a more active lipase not only toward PNP-propionate substrate (Figure 6A) but also toward triacetin isotropic solution (Figure 6B). We observed up to 10-fold activation, suggesting that the preincubation induces an active (open) conformation that is maintained during the assay despite the dilution of the sample.

To further deepen our understanding of this activation that we called a memory effect (the enzyme remembers the micelle-induced conformation), additional experiments were carried out in aqueous solution. To determine the importance of the remaining detergent, higher dilutions of the preincubated sample were explored for CRL, RDL, and HLL lipases, using triacetin as substrate (Figure 7). Significantly, in all cases, the activation progressively declines as dilution of the sample increases (from 1:40 to 1:300). The same changes were observed with other enzyme concentrations which ruled out any effect due to protein dilution. Such behavior observed for triacetin as well as for PNP-propionate (not shown) indicates that a minimum amount of detergent interacting with the enzyme must be required to maintain a conformational state exhibiting considerable enzyme activity. If the open form is stable for a period of time, at a moderate level of dilution there could be rapid rebinding of detergent (perhaps retained in the active site), and an open state could be stabilized in the absence of a well-defined interface. This conclusion is supported by the observation that a high level of detergent dilution diminishes the effect (Figure 7). Along these lines, an interaction between n-OG molecules and a pancreatic lipase, very close to the catalytic site and involving both polar and hydrophobic interactions, has been described (*38*). Also, for HLL it has been proposed that in conditions below the cmc a detergent (pentaoxyethylene octyl ether) can intercalate into the active site, even while the lid remains closed (*37*). So, now, the question is: Why does the 'direct' lipase not achieve an activated state similar to that obtained after dilution from detergent preincubated (DP) sample? It should be mentioned that the DP lipase has been previously induced to an open conformation by micelle-preincubation which will facilitate that detergent molecules can interact

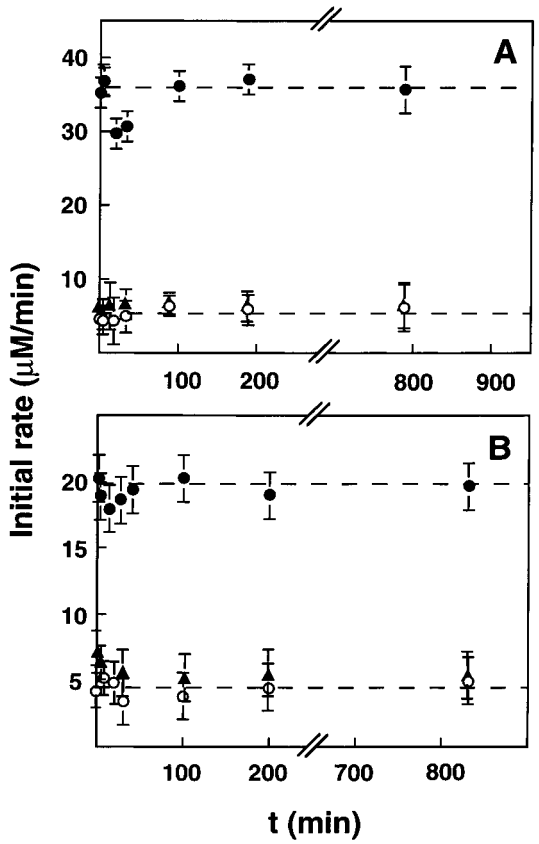


FIGURE 8: Progress of lipase esterolytic activity with time using GCL (A) and RDL (B). Lipase activity of control (open circles), 'direct' (filled triangles), and detergent preincubated (filled circles) samples at different times after dilution. The activity was followed by hydrolysis of PNP-propionate, 0.25 mM. Enzyme concentrations were 2 $\mu$g/mL and 10 ng/mL, respectively.

closely enough to stabilize the activated state observed after dilution (perhaps fully open or partially open conformations).

Other series of experiments are related to the stability of the enzyme-activated state. To elucidate how long the activated lipase could remain in this state, the lipase was diluted 1:40, and the activity was assayed by adding the substrate at different times. As an example, Figure 8 shows the esterolytic activity observed using GCL (panel A) and RDL (panel B), for the DP, C, and D samples when the activity was assayed at different times using PNP-propionate as substrate. A loss of the activation effect was expected to be observed after several minutes. However, surprisingly, it was found that lipase remained in the activated state for long incubation times. This effect (memory) was maintained even 13 days after dilution and was absent both in the control and in the 'direct' lipase for the same incubation period. Other fungal lipases (CRL, HLL) and lipase from *Pseudomonas fluorescens* also exhibit this memory effect (results not shown). It can be reasonably proposed that after a full lid opening induced by micelles, a hydrophobic patch will be exposed, and few detergent molecules will be 'sequestered' mainly by a hydrophobicity-driven mechanism. They will have long enough residence times to keep the enzyme activated as long as 13 days or more (see Figure 8). Support for our interpretation is provided from X-ray crystallographic studies of both filamentous fungi lipases (*11*, *13*, *17*, *39*) and yeast lipases (*9*, *10*, *40*): (i) In general terms, from X-ray analysis it is proposed that the interfacial activation is

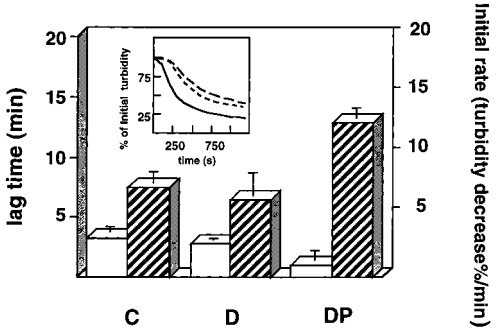


FIGURE 9: Kinetic study using artificial lipoprotein as substrate and RDL in aqueous solution. Lag time (open bars) and activity (dashed bars) of control (C), 'direct' 0.3 mM n-OG (D), and detergent preincubated (DP) lipases. In this case, the enzyme activity is shown as the percentage of turbidity decrease per minute taking as 100% the initial turbidity of the sample. Inset: time course of the reaction for the three lipase samples: control (···), 'direct' (- - -), and detergent preincubated (—). The enzyme concentration was 0.1 $\mu$g/mL.

achieved by the displacement of the lid which increases the hydrophobic surface in the vicinity of the catalytic site. (ii) The structure of the active site is very similar among the filamentous fungi enzymes (HLL, RDL, *Rhizomocur miehei*) as well as *G. candidum* (*39*), with a long hydrophobic scissile fatty acid binding site located inside the binding pocket (*36*). (iii) Observations for CRL structures (*10*) suggest a discrete opened state (an active state or an intermediate one, close to the active conformation), and once opened, the lid remains opened for some time (*21*). (iv) Significant lid mobility has been postulated for HLL and RDL in the absence of an oil−water interface (*11*, *39*), and the crystal structure of RDL indicates that specific molecular interactions with hydrophobic molecules (e.g., a detergent) present in the crystallization media affect/or stabilize the lid in an intermediate conformation (*11*). (v) The X-ray crystal structures also suggest how an open conformation for CRL can persist for months in aqueous solution at 4 °C (*41*); dissolving CRL in 50% 2-propanol followed by dialysis to remove 2-propanol increased the activity and the enantioselectivity toward carboxylic acids by converting the closed form of CRL to the open form.

It was also interesting to test the micelle-induced lipase activation in aqueous solution with a substrate that provides a different environment, e.g., an artificial lipoprotein. In this experiment, preincubation of lipase with 30 mM n-OG was performed, and aliquots of 10 $\mu$L of detergent preincubated enzyme were placed in the cuvette containing the artificial lipoprotein substrate (1 mL). The lag time and the enzyme activity for RDL in the C, D, and DP samples are shown in Figure 9. The inset this figure shows the time-course of the reaction for each of these three samples. Interestingly, the detergent preincubated enzyme gives rise to higher activities as well as shorter lag times. The differences observed in enzyme activation between lipoprotein (Figure 9) and PNP-propionate (Figure 6A) or triacetin (Figure 6B) substrates could be due to their physicochemical features. Although 200 mM triacetin is below its saturation point, it constitutes an isotropic solution in the form of monomers and micelles (*42*), and, consequently, a population of enzyme molecules may already be in the open form during the assay even in the control sample (free-detergent assay). Therefore, the catalytic activity of the detergent preincubated (DP) lipase

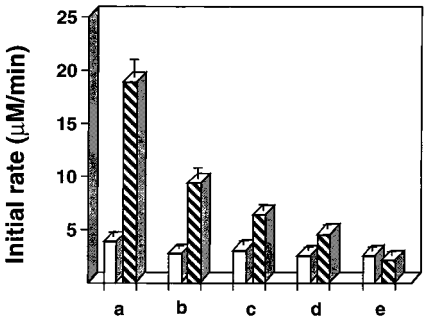


FIGURE 10: Effect on GCL lipase activity of n-OG preincubation below the cmc. Lipase activity of 'direct' (open bars) and detergent preincubated (dashed bars) samples for the hydrolysis of PNP-propionate, 0.25 mM. Detergent concentration was (a) 30 mM, (b) 20 mM, (c) 10 mM, (d) 7.5 mM, or (e) 3 mM. Enzyme concentration was 1 $\mu$g/mL.

relative to the control (C) one is lower for triacetin than for PNP-propionate as shown for the four fungal enzymes (Figure 6). The same argument could be used to explain the small differences between detergent preincubated and free-detergent lipases when using artificial lipoprotein as substrate. In the presence of artificial lipoprotein that seems like true emulsion, the interface can induce lid opening, and the expected change in activity after preincubation with micelles is smaller. On the other hand, the observation that preincubation with detergent shortens the lag time with a water-insoluble substrate strengthens the notion that detergents can promote an active conformation with a more accessible active site that easily interacts with the substrate.

Experiments of preincubation of lipase with n-OG concentrations between 3 and 20 mM were also performed in order to detect lipase-activated forms induced by premicellar detergent concentrations in direct assays in aqueous solution. Lipase was preincubated with the detergent for 10 min, and aliquots (25 $\mu$L) were withdrawn and diluted 1:40 (0.075−0.5 mM amphiphile concentration). Different substrates and fungal lipases were used, and the enzymatic activity of the diluted sample was compared with the corresponding 'direct' ones. As an example, Figure 10 shows for GCL the hydrolytic activity using as substrate PNP-propionate, 0.25 mM, for samples preincubated with 3, 7.5, 10, and 20 mM n-OG. The enzymatic activity observed after preincubation with 30 mM n-OG is also included for comparison. Interestingly, enzyme samples preincubated with n-OG at concentrations below the cmc show catalytic activity slightly higher than that observed for the 'direct' lipase. Note that maximum activity is shown for micelle preincubated enzyme (open lid conformation). Thus, different lipase forms (presumably with less opened lid) that can be correlated with the enzyme catalytic activity could be stabilized by the detergent in the absence of substrate (preincubation step), consistent with the results from trapping in nonaqueous media. However, for other fungal enzymes (e.g., CRL, RDL), using PNP-propionate or triacetin as substrates, activation effects below the detergent cmc in direct assays in aqueous solution were difficult to detect (not shown). A higher flexibility of the protein structure and lid lability of these lipases would generate intermediate states too unstable and short-lived to be observed directly in the aqueous assay. In addition, the presence of aggregated substrate (triacetin) and/or putative effects due to the remaining 1% of acetonitrile (PNP-propionate) could determine an overall higher catalytic

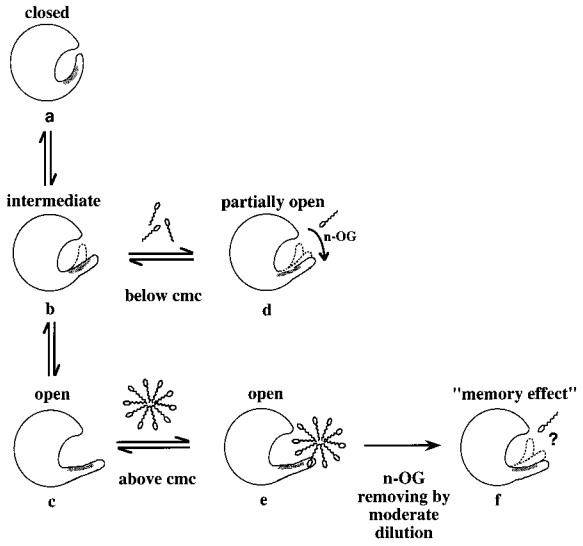


FIGURE 11: Proposed model for the conformational equilibrium of fungal lipases in solution. The simplified scheme illustrates the activation induced by n-OG detergent molecules below and above the cmc, and the memory effect.

activity (more open forms) in the control ('direct') enzymes and, consequently, lower differences in activity with respect to detergent preincubated samples. Therefore, the nonaqueous medium can be regarded as a unique way to analyze the inherent activity of the different intermediate lipase conformations because they are kinetically trapped in stable states.

*Equilibrium Model for Lipase Activation Including Closed, Intermediate, and Open Forms*. Taken together, our results are consistent with the model depicted in Figure 11. The hypothetical model is based on an equilibrium in aqueous solution where lipase intermediate conformations are postulated between the closed and the open states of the enzyme. In a preexisting equilibrium between closed (a), intermediate (b), and open (c) conformations, the closed form will be predominant in the absence of n-OG detergent. At n-OG concentrations below the cmc, a stabilization of partially opened lipase conformers might exist (d), with a more open lid as detergent concentration increases. During incubation with n-OG, 30 mM (above cmc), the detergent micelles mainly stabilize completely opened forms of the lipase (e). These different lipase states (a−e) will be trapped after freeze-drying of the buffer solution, and their conformations will be further retained in anhydrous solvents. Thus, our kinetic studies carried out in water-restricted medium provide support for the proposed model. In fact, we have shown that in the absence of detergent, although nonactive (closed, a) forms of the enzyme are predominantly trapped, some active (slightly opened, b) conformers also exist which preferably react with short-chain triglyceride substrates (Figure 5A, bottom curve). This demonstrates that in the absence of an oil−water interface there is a subtle equilibrium between the (a) and (b) enzyme conformations. The conformers (d) and (e) trapped in the presence of detergent correspond to different stabilized activated forms of the lipase. This would imply a more or less continuous range of conformations where intermediate positions of the lid might be postulated in premicellar environments (d), that will be completely open in the micellar milieu (e). When lipase activity measurements were conducted in aqueous solution after preincubation of the enzyme with n-OG micelles followed by moderate

dilution, our results show that the lipase retained the active conformation induced by high detergent concentration (memory effect) for a long period of time (f). Higher dilutions resulted in the loss of this activation state and led us to think that a minimal amount of detergent interacting with the enzyme is needed to stabilize an opened conformational state with significant catalytic activity (f).

In light of the above results, it can be concluded that detergent molecules (in the absence of interface) may play an important role in both inducing and maintaining activated states of lipases. Detergents or bile salts may do more for lipase activity than simply act as inhibitors by preventing binding to the substrate surface. Such a conclusion is conceivable in light of recent evidence obtained from human pancreatic lipase (*43*). It has been revealed that the presence of either bile salts or a small proportion of water-miscible organic solvents (called activators compounds) considerably enhances the enzymatic activity of pancreatic lipase in the absence of aggregated substrate, likely favoring lid opening (*43*). On the other hand, as suggested for various fungal lipases (*11*, *21*, *26*, *44*) the flap helix can move as a whole by rotating around hinge regions, thereby opening and closing the binding site. In this respect, experiments have been carried out in our laboratory using biophysical techniques (CD, FT-IR) and protease sensitivity assays, but no significant differences were observed between diluted DP, 'direct', and control samples that reflect minor changes in the overall secondary protein structure of these lipases.

Finally, it is worth mentioning some observations from crystallographic studies of fungal lipases (HLL and RDL) where detergents were proved to be critical for the growth of X-ray-quality crystals (*11*). It was postulated that the small amounts of detergents used in the crystallization medium may have occasioned the displacement of the lid from their closed position (*21*), and that a significant population of molecules exhibit intermediate conformations of the lid caused by the bound detergent (*11*). In this regard, our results in a water-restricted environment provide the first direct demonstration of protein intermediate (kinetically trapped) activated states of lipases, which strongly supports previous suggestions about lipase conformational heterogeneity in solution. As the detergent can alter the conformational equilibrium of the lipase (Figure 11), it can be reasonably expected that the confluence of both lipase−detergent interactions and crystal-packing factors may determine lid stabilization of a given intermediate conformation as shown by X-ray crystallography (*11*). However, not only amphiphilic molecules but also other additives of the crystallization media, e.g., organic compounds, seem to be crucial in the adoption of a 3-D structure (reviewed in ref *43*) in a variety of lipases. This indicates that the 'history' of sample preparation has an effect on enzyme conformation. Thus, the question remains open as to whether these enzymes, even in the absence of aggregated substrate, may open the lid due to changes in the physicochemical parameters of the medium (*10*, *43*). How different conformational states can be dictated by the 'history' of sample preparation has been widely demonstrated in our laboratory for a membrane protein prototype from studies in organic solvent and lipid milieu (*45*−*48*). In addition, the importance of microenvironment parameters to achieve a thermodynamically stable ion-conducting functional conformation (*49*), or to control the interfacial activation of lipases (*50*, *51*), has been addressed. In light of the present results, it cannot be disregarded that an environment-dependent modulation of the activity of lipases involving the stabilization of intermediate, catalytically competent, conformations could be an additional exquisite mechanism for enzyme regulation with relevant biological significance, even in the absence of a well-defined interface. The trapping approach offers a useful tool for future exploration in biomimetic surroundings which may unveil novel facets in the properties of this peculiar type of enzyme, and enrich our understanding of the mechanisms that trigger the acquisition of catalytically active lipase conformations.

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