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Interfacial activation-based molecular bioimprinting of lipolytic enzymes

(lipases/phospholipase A_2/nonaqueous media)

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ABSTRACT **Interfacial activation-based molecular (bio)-imprinting (IAMI) has been developed to rationally improve the performance of lipolytic enzymes in nonaqueous environments. The strategy combinedly exploits (*i*) the known dramatic enhancement of the protein conformational rigidity in a water-restricted milieu and (*ii*) the reported conformational changes associated with the activation of these enzymes at lipid–water interfaces, which basically involves an increased substrate accessibility to the active site and/or an induction of a more competent catalytic machinery. Six model enzymes have been assayed in several model reactions in nonaqueous media. The results, rationalized in light of the present biochemical and structural knowledge, show that the IAMI approach represents a straightforward, versatile method to generate manageable, activated (kinetically trapped) forms of lipolytic enzymes, providing under optimal conditions nonaqueous rate enhancements of up to two orders of magnitude. It is also shown that imprintability of lipolytic enzymes depends not only on the nature of the enzyme but also on the "quality" of the interface used as the template.**

Nonaqueous enzymology has emerged in the last decade as a promising research field (for reviews, see, for example, refs. 1–3), not only because of a large number of widely recognized advantages (2) but especially as a result of the realization that enzymes placed in nearly anhydrous environments can exhibit exciting features, such as an increased conformational rigidity, which confers on the protein greatly enhanced (thermo)stability (4), or a profoundly altered substrate specificity (5). In particular, one interesting facet of the exploitation of the drastically lowered flexibility of a protein in very low-water media is the so-called ligand-induced enzyme "memory" (or simply ligand memory) (6), presumably based on inducing, upon ligand binding, an enzyme conformational change that (after freeze-drying of the solution) will be preserved in the lyophilized sample when exposed to anhydrous solvents. This strategy, also referred to as molecular (bio)imprinting (7, 8), has been extended to nonenzymic proteins and other macromolecules (9, 10) and shares a conceptual analogy with the previously known molecular imprinting of polymers (for a review, see ref. 11). In the case of enzyme (bio)imprinting, only scarce efforts have been reported so far, which have focused on a very few related proteases and made use of competitive inhibitors (amino acid derivatives) as print molecules (6–8). Although these preliminary results seem indeed encouraging in terms of an activity enhancement or even an apparent (stereo)selectivity alteration in the organic milieu, the nature of the presumptive conformational changes induced has not been characterized, and the molecular reasons for the experimental observations are still open to debate (12).

Paradoxically, in this context, no effort aimed at assessing the possibility of imprinting lipolytic enzymes has been reported yet, which contrasts with the extraordinary profusion during the last few years of nonaqueous studies of lipases. Catalysis by lipolytic enzymes is characterized by the so-called interfacial activation (13), manifested as a pronounced activity increase upon substrate aggregation [i.e., over the substrate critical micelle concentration (cmc)]. It has long been proposed that this activation should involve some discrete conformational changes of the soluble enzyme in fixing itself at the substrate surface (14). Recent evidence derived mainly from x-ray crystallographic studies in the case of triglyceride lipases (for review, see, for example, refs. 15 and 16), and from solution Fourier transform infrared spectroscopy and especially NMR experiments in the case of phospholipase A_2 (PLA2) (17, 18), has shed light on this point by showing that for different lipolytic enzymes characterized (from mammalian as well as microbial origin), relevant conformational changes or structural rearrangements can be neatly appreciated when comparing the structure of the free enzyme with that of an enzyme–micelle (or enzyme–inhibitor) complex. As an illustrative example, human pancreatic lipase has been crystallized in two conformational states (19, 20): a "closed" (inactive) one, in which the catalytic triad in the active site is covered by a helical "lid" (flap), and an "open" (active) one, obtained by crystallization in the presence of micelles, in which the lid has been displaced, adopting a totally different conformation and exposing the catalytic residues. Very interestingly, this lid displacement and other entailed conformational changes ultimately result in both providing space in the active site for lipid docking and shaping the catalytic machinery ("induced fit") (15, 20, 21).

On this basis, we considered the exciting possibility of rationally generating "activated" lipolytic enzyme powders [in analogy to the reported activated lipase crystals (22, 23)] by molecular imprinting, which hopefully would exhibit an improved catalytic activity in nonaqueous media.† The rationale was to "trap" (freeze-dry) the enzyme in a presumably activated form [i.e., when it is bound to (or even acting on) an amphiphile–water interface] and to further assay it (after "washing out" the print amphiphile with an anhydrous solvent) in water-restricted environments where its imprinted conformation is expected to be preserved. We next present the results of implementing this strategy, which we coined interfacial activation-based molecular (bio)imprinting (IAMI) of lipolytic enzymes. To explore and optimize the imprintability of lipo-

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Abbreviations: AF, activation factor; IAMI, interfacial activation-based molecular (bio)imprinting; EPC, egg yolk phosphatidylcholine; LPC, egg yolk lysophosphatidylcholine; n-OG, *n*-octyl β-D-glucopyranoside; CrL, *Candida rugosa* lipase; FsC, *Fusarium solani* cutinase; GcL, *Geotrichum candidum* lipase; ppL, porcine pancreatic lipase; RmL, *Rhizomucor miehei* lipase; ppPLA2, porcine pancreatic phospholipase A_2; SUV, small unilamellar vesicle; TX-100, Triton X-100; PC, phosphatidylcholine; cmc, critical micelle concentration.

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lytic enzymes, six enzymes (five of them certainly prototypic in that their three-dimensional structure has been solved) were selected: porcine pancreatic PLA2 (ppPLA2); porcine pancreatic lipase (ppL); three fungal lipases, from *Rhizomucor miehei* (RmL), *Candida rugosa* (CrL), and *Geotrichum candidum* (GcL); and *Fusarium solani* cutinase (FsC). For ppPLA2, recent solution NMR experiments have demonstrated that relevant conformational rearrangements occur upon binding of the enzyme to substrate-like inhibitor-containing micelles; one such rearrangement results in the induction of a tighter, more competent catalytic network (18). In the case of RmL (22, 23) and CrL (24, 25), two conformational states (closed and open) of each enzyme have also been crystallized, and it has been clearly established that lid displacement, which uncovers the catalytic site, is directly involved in the mechanism of their interfacial activation. A similar mechanism seems to be inferred for GcL from recent data obtained from crystals of an apparently closed (occluded) form of the enzyme (26). Finally, we selected FsC as a negative control because it does not exhibit interfacial activation, apparently as a result of the absence of a lid and the presence in the "free" enzyme of a *preformed* oxyanion hole (27).

EXPERIMENTAL PROCEDURES

Materials. ppPLA2 was a kind gift from Novo Nordisk or was purchased from Sigma. RmL and GcL were generously provided by Novo Nordisk and Amano, respectively; ppL and CrL, as well as α-chymotrypsin and subtilisin, were obtained from Sigma. Purified, recombinant FsC was a gift from Unilever. All phospholipids, amino acid derivatives, and PG2000-200 controlled pore glass beads (mesh 120–200) were purchased from Sigma. Synthetic surfactants as well as all other chemicals used were obtained from commercial suppliers (Aldrich, Merck, Sigma, or Serva) and were of analytical grade or purer. All organic solvents employed (Merck) were of analytical or HPLC grade. "Anhydrous" solvent refers here to a solvent dessicated with 3-Å molecular sieves (Merck) to bring its water content below 0.01%, as determined by Fisher titration.

Enzyme Imprinting. For sample preparation, each enzyme was incubated (before freeze-drying) for about 1–2 min at 4°C in either an amphiphile-containing (for imprinted enzyme) or amphiphile-free (for control enzyme) buffer. The buffers used were 10 mM Tris·HCl (containing 0.2 mM Ca^{2+}) at pH 8.0 in the case of ppPLA2 and 10 mM Tris·HCl at pH 7.5 in the case of triacylglycerol lipases. In experiments where liposomes were used as print interfaces, the phospholipid vesicles were prepared by standard protocols, and their lipid content and integrity were determined as reported (28). After freeze-drying, activated and nonactivated samples were treated in an identical manner. Thus, imprinted and control enzyme powders were washed (stirring of the suspension followed by centrifugation steps) at least three times with an anhydrous solvent (or solvent mixture): typically, in the case of ppPLA2, anhydrous benzene or benzene/ethanol, 95:5 (vol/vol), for *n*-octyl β-D-glucopyranoside (n-OG) and all phospholipids and anhydrous ethyl acetate for Triton X-100 (TX-100), and in the case of lipases, anhydrous benzene/ethanol, 90:10 (vol/vol), or ethyl acetate. The solvent was carefully selected in each case 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. After washing, the samples were vacuum dried for at least 3 h.

Nonaqueous Assays. The nonaqueous assays were carried out using the following model reactions: in the case of ppPLA2, the hydrolysis of egg yolk phosphatidylcholine (EPC) in water-saturated chloroform (29), and in the case of lipases, different esterifications in anhydrous solvents. To start the reactions, a given amount of washed (imprinted or control) enzyme powder was suspended in a given volume of the substrate-containing reaction medium in a stoppered screw-capped vial, sonicated for 10 s, and shaken at 250 rpm at 25°C. The reaction progress was accurately followed by periodically withdrawing 10-μl aliquots of the reaction mixture, which, in the case of ppPLA2, were treated and analyzed by normal-phase HPLC as described (29), and in the case of lipases were analyzed by HPLC on a reverse-phase Lichrospher 100 RP-18 column (Merck) isocratically eluted with methanol or by GC using standard derivatization procedures for the fatty acids. In all cases, simultaneous disappearance of substrate(s) and accumulation of product(s) was followed. In the case of ppL, colipase was not added to the lyophilization buffer since bile salts were not used in any experiment. Other details are given in the corresponding legends to the figures. The results shown are representative or correspond to the mean of four independent experiments involving two batches of enzyme.

RESULTS AND DISCUSSION

In a preliminary screening, we found that different nonsubstrate, synthetic as well as natural, amphiphiles (used as print molecules) were able to enhance the ppPLA2 hydrolytic activity in organic solvent (water-saturated chloroform). Using selected amphiphiles, we next investigated the dependence of the activation response of ppPLA2 in nonaqueous medium on the aqueous surfactant concentration. According to our hypothesis, a marked increase in the activation factor (AF; defined here as the ratio of the nonaqueous initial reaction rate for the imprinted relative to the nonimprinted enzyme) should be expected upon appearance of interfaces. We were gratified to find that this seemed to be the case for n-OG (cmc in the millimolar range), as illustrated in Fig. 1*A*. This effect was not appreciable for egg yolk lysophosphatidylcholine (LPC) (Fig. 1*B*) (cmc in the micromolar range), though activation was also saturable at concentrations well above the cmc.

Interestingly, ppPLA2 could be imprinted more efficiently when using phospholipid substrates as templates, organized as either micelles or liposomes. Fig. 1*C* shows the dependence of the AF on the aqueous concentration of either the micelle-forming diheptanoyl phosphatidylcholine (PC) (notice the activation enhancement occurring over the cmc) or EPC small unilamellar vesicles (SUVs). In this case, it was verified that at the moment of freezing the enzyme solution, some substrate hydrolysis had taken place, although it was not completed. Thus, since ppPLA2 had apparently been frozen while *macroscopically* acting on the substrate, the imprinted phospholipase conformation could be virtually envisaged, as an average, as that of the enzyme "caught at work." On the other hand, imprinting with vesicles of natural PCs (EPC) or mixtures of pure PCs (L-dimyristoyl-PC/L-dioleoyl-PC, 1:1 molar ratio) was more efficient than that with single, pure species (see Fig. 1*D*). This behavior could be accounted for on the basis of the present knowledge that activation of ppPLA2 is tightly related to the topological microheterogeneity of the membrane lipid matrix (30), so that enzyme penetration into the interface (and hence activation) seems facilitated by bilayer surface defects (e.g., mismatches) or phase separation. Furthermore, when ppPLA2 was imprinted either with L-dipalmitoyl-PC (substrate) or D-dipalmitoyl-PC (not a substrate) SUVs, a marked difference in the AF value was observed; the latter was approximately 4–5 (Fig. 1*D*). Interestingly, the above results suggest a certain correlation between the "quality" of a given phospholipid vesicle as a substrate for the enzyme and its imprinting ability. It appears evident that the mere presence of any lipid–water interface is not a sufficient condition to obtain a reasonable imprinting, but rather the interfacial quality of the substrate must be taken into account. In fact, doping of TX-100 micelles (acting as

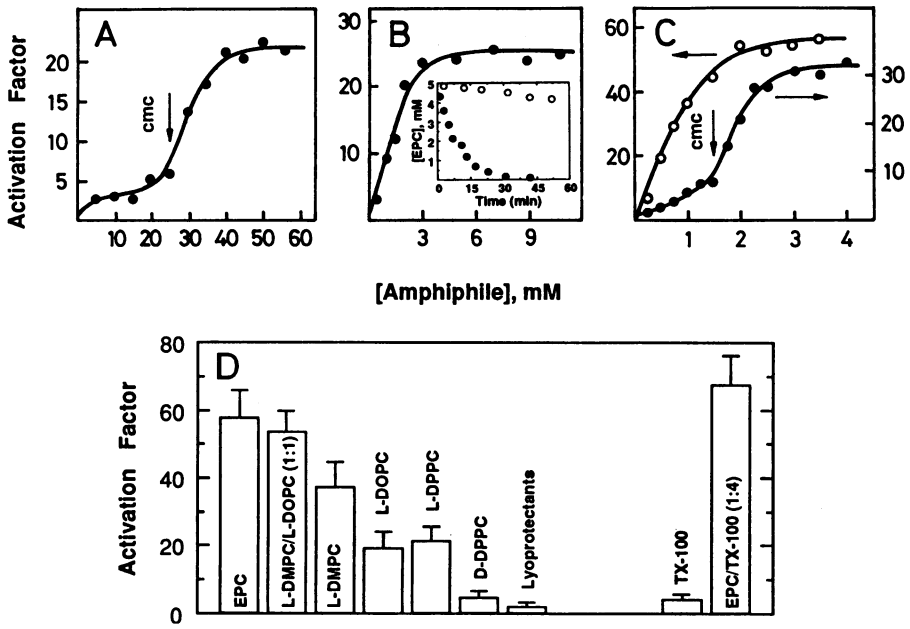

FIG. 1. Dependence of the imprinting-derived activation response of ppPLA2 in nonaqueous medium on the aqueous concentration (prior to freeze-drying) and nature of the template amphiphile. The model reaction assayed was hydrolysis of EPC (5 mM) in water-saturated chloroform. The washed enzyme powder (either imprinted or control) concentration in nonaqueous medium was 1 mg/ml. A smooth curve was drawn for each amphiphile for clarity. The maximum AF values for n-OG (*A*), LPC (*B*), the micelle-forming diheptanoyl-PC (*C*; ●), or EPC SUVs (*C*; ○) were 21 ± 5, 27 ± 6, 32 ± 5, and 57 ± 8, respectively. (*B Inset*) An example of the time course of EPC hydrolysis catalyzed by either control ppPLA2 (○) or ppPLA2 imprinted with 3 mM LPC (●). (*D*) Dependence of AF for ppPLA2 on the phospholipid composition of the vesicles (the total phospholipid concentration was in all cases 2 mM). The effect of lyoprotectants was tested by incorporating either lactose (10 mM) or sorbitol (100 mM) to the lyophilization buffer. Also included is the activation enhancement obtained upon doping of TX-100 micelles (8 mM, aqueous concentration) with the substrate EPC (2 mM). L-DPMC, L-dimyristoyl-PC; L-DOPC, L-dioleoyl-PC; DPPC, dipalmitoyl-PC.

"neutral diluent") with EPC (20 mol %) resulted in a considerable increase in the AF value, even higher than that obtained by imprinting with EPC SUVs at the same molar phospholipid concentration (Fig. 1*D*). In this regard, it is indeed tempting to qualitatively associate the modest imprinting obtained with TX-100 alone and the notable activation upon doping of the micelles with EPC (Fig. 1*D*) with the two steps proposed by Peters *et al.* (18) for activation of ppPLA2 in solution [i.e., an initial conformational change occurring upon binding of the enzyme to a (nonsubstrate) micellar interface and the acquisition of a catalytically active conformation upon binding a substrate or competitive inhibitor at a lipid–water interface].

Next, to investigate the generality of IAMI for lipolytic enzymes, we tested the imprintability of several triglyceride lipases, in a similar way as for ppPLA2. Fig. 2 depicts, as an example, the dramatic rate enhancements in two ester syntheses obtained with n-OG-imprinted ppL relative to its nonimprinted counterpart. Other amphiphiles or mixtures, routinely at concentrations moderately above the cmc, were able to generate to a varied extent an activation response of the lipase (Fig. 3*A*), though a rationalization for the selectivity observed, in terms of amphiphile charge or nature, is not immediately obvious. Interestingly, RmL, CrL, and GcL could be also activated by different amphiphiles but exhibited a distinct preference with respect to the template (Fig. 3*B*). In particular, it might be suggested that the poorer AF values obtained for GcL relative to the other lipases seem consistent with the known difficulty to open its lid and crystallize the enzyme in an activated form; in fact, the so far available three-dimensional structures appear to correspond in all cases to a closed form of the enzyme (26). In this sense, our imprinted GcL could presumably represent the first reported noncrystallized, manageable activated form of this enzyme.

Since it has recently been reported that the use of lyoprotectants during freeze-drying can result in enzymatic rate enhancements in nonaqueous medium (see, for example, refs. 12 and 32), several experiments were performed to confirm that the *main* contribution to the observed activation of lipolytic enzymes must involve the actual generation of a permanently activated conformation of the enzyme rather than a simple (general or even specific) lyoprotection effect.

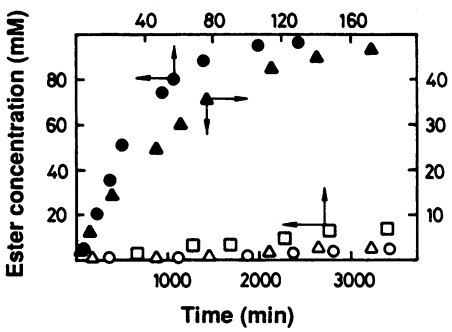

FIG. 2. Comparison of the time course of nonimprinted (open symbols) and imprinted (filled symbols) ppL-mediated esterification between oleic acid (100 mM) and either benzyl alcohol (100 mM) (○, ●) or cyclohexanol (100 mM) (△, ▲) in anhydrous *n*-hexane. n-OG (40 mM) was used as a template. Washed enzyme powder (either imprinted or control) concentration in nonaqueous medium was 25 mg/ml. For comparison, the time course of esterification of oleic acid (100 mM) and benzyl alcohol (100 mM) in anhydrous *n*-hexane is shown when ppL immobilized on controlled pore glass beads was used (at the same nonaqueous concentration as for the control powdered enzyme) (□). Immobilization was carried out as reported (31), except that after enzyme adsorption from a surfactant-free solution the beads were immediately freeze-dried and further washed several times (with benzene/ethanol, 90:10), identically as the control powdered ppL. Both horizontal axes refer to incubation time, in min, and both vertical axes to product concentration, in mM. The arrows indicate the axis scales used in each model reaction: the left and upper axis scales correspond to those assays with benzyl alcohol (●, ○, and □), whereas the right and lower axis scales correspond to those assays with cyclohexanol (▲ and △).

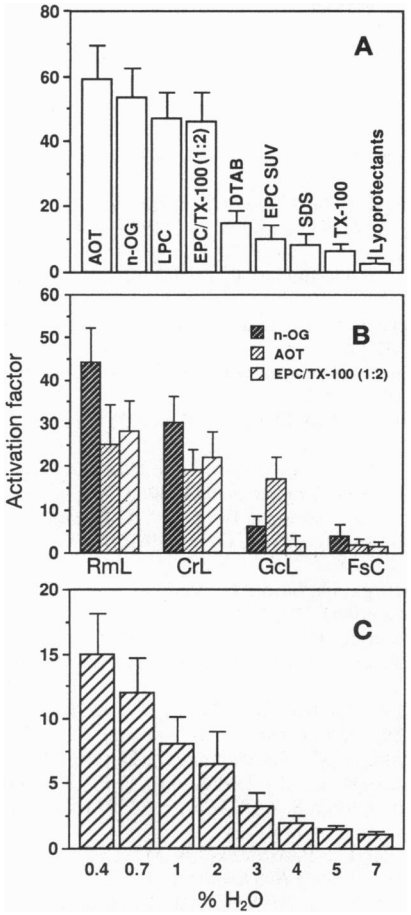

FIG. 3. Imprintability of several lipases by different amphiphiles. (*A*) Imprinting-derived activation response of ppL in nonaqueous medium for different amphiphile templates present in the lyophilization buffer. Aqueous amphiphile concentrations were 5, 40, 3, 2, 20, 2, 13, and 6 mM for sodium bis(2-ethylhexyl)sulfosuccinate (AOT), n-OG, LPC, EPC in mixed micelles, dodecyltrimethylammonium bromide (DTAB), EPC liposomes, SDS, and TX-100, respectively. The model reaction used for the nonaqueous assay (10 mg/ml of washed enzyme powder) was the esterification of oleic acid (100 mM) and 1-hexanol (100 mM) in anhydrous *n*-hexane. Also included is the result of incorporating in the lyophilization buffer different lyoprotectants—e.g., sucrose (2%) or sorbitol (2%). It was verified that conversion by control ppL after 80 h of incubation was >90%. (*B*) Comparison of the activation generated by different amphiphiles on the lipases RmL, CrL, and GcL as well as FsC. The aqueous amphiphile concentrations, model reaction, and conditions for the nonaqueous assay were the same as in *A*, except in the case of GcL where 2,2,4-trimethylpentane was used as reaction medium instead of *n*-hexane. Enzyme powder concentrations were 60, 10, 20, and 2 mg/ml for RmL, CrL, GcL and FsC, respectively. (*C*) Erasability of the n-OG-imprinted ppL memory as a function of the water percentage in the reaction medium. The model reaction was the ppL (20 mg/ml of enzyme powder)-mediated hydrolysis of triolein (10 mM) in 2-propanol.

Thus, when the enzymes were freeze-dried in the presence of lyoprotectants such as lactose, sucrose, or sorbitol, no significant rate enhancement for ppPLA2 and at most a 2- to 3-fold increase for ppL [in agreement with the range of values reported for other lipases (12, 32)] were obtained (see Figs. 1*D* and 3*A*). On the other hand, the lipase memory was progressively lost as the water content of a water-miscible solvent (and hence the protein conformational flexibility) was increased, as shown for ppL in Fig. 3*C* using triolein hydrolysis as a model reaction (the decrease in AF was found to be in part the result of a diminution in imprinted ppL activity). Finally, when cutinase was tested, no significant activation could be observed for this enzyme when treated with different print amphiphiles

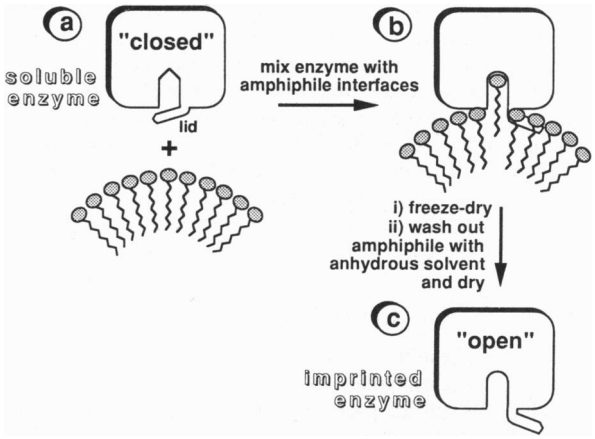

FIG. 4. Simplified scheme illustrating the strategy of the interfacial activation-based molecular imprinting of a lipase. (*a* and *b*) The enzyme is in aqueous solution but in different conformation. (*c*) The open conformation of the imprinted lipase represents an activated enzyme manageable in water-restricted environments. Notice the two types of postulated conformational changes induced in the imprinted protein, which involve the lid and the catalytic site.

(Fig. 3*B*), which seems to rule out a *specific* lyoprotection (presumably exerted by an amphiphile molecule bound to the enzyme's active site during the freeze-drying process) as the main cause of the activation observed for the other lipases. In addition, results such as the exquisite modulation of the ppPLA2 activation response by the quality of the phospholipid interface in SUVs (see Fig. 1*D*) are also more comfortably explained as a result of an actual enzyme imprinting than of amphiphile protective effects. In any case, a partial contribution of specific lyoprotection cannot be discarded in general in the activation of lipolytic enzymes obtained by imprinting with interfaces.

Some additional results in the characterization of imprinting of lipolytic enzymes are worth mentioning. Thus, activated ppL powder yielded a significantly higher reaction rate when assayed for the esterification of benzyl alcohol with oleic acid in *n*-hexane than its nonimprinted counterpart immobilized (and thus monodispersed) on a porous support (see Fig. 2). As for enantioselectivity, it was verified, using a previously reported CrL-mediated esterification between L- and D-menthol and lauric acid in isooctane (33) as the model reaction, that imprinting resulted in a considerable increase of activity towards the L-isomer (preferred isomer) without appreciable loss of enzyme enantiodiscrimination. Finally, when interface-unrelated, nonlipolytic enzymes such α-chymotrypsin and subtilisin were assayed for an *N*-acetyl amino acid ester transesterification model reaction, it was verified that they could not be activated under conditions optimal for imprinting of lipases.

Taken together, our results are consistent with the proposed imprinting hypothesis and strongly support that the IAMI consists of the induction and permanent retention in anhydrous environments of an activated (kinetically trapped) conformation of a lipolytic enzyme, closely associated to its interfacial activation. In light of the present structural information, the following considerations can be reasonably made. In the case of lipases, two distinct types of conformational rearrangements may be involved in the imprinting phenomenon, whose contribution obviously must be dependent on the nature of the particular enzyme and template used: (*i*) a lid displacement (opening), which alleviates the accessibility restrictions that the substrate(s) can find in the nonimprinted enzyme, and (*ii*) an adjustment of the catalytic machinery which remains locked in the activated enzyme in a more competent topology relative to the nonactivated counterpart. Interestingly, this adjustment need not necessarily be induced

by the binding of the ligand (amphiphile) to the enzyme's active site, since at least for human pancreatic lipase it has been established that formation of the oxyanion hole is a direct consequence of lid reorganization (20). In the case of ppPLA2, imprinting might involve the acquisition of a catalytically active conformation and, in particular, the ligand-induced formation of a catalytically competent, extensive network of hydrogen bonds (18). The above concepts are schematized in Fig. 4, which illustrates the possible conformational rearrangements postulated in the case of imprinting of a lipase.

CONCLUSIONS

This work represents a promising approach to rationally engineer lipolytic enzymes for nonnatural environments. This approach is versatile, provides under optimal conditions a dramatically enhanced performance, and seems extensible to other enzymes (we have succeeded in activating several lipases of yet unresolved three-dimensional structure). Although this methodology deserves further exploration to assess its full potential, our results permit us to envisage a number of potentially important, far-reaching implications: (*i*) It represents a straightforward way to obtain manageable, noncrystallized activated forms of lipolytic enzymes that are fully operative in water-restricted media. (*ii*) It might be also helpful in assisting the screening of candidate amphiphiles for an eventual successful crystallization of other activated lipases. (*iii*) We expect that this strategy will stimulate and guide the exploration for imprintability of other membrane-associated proteins (not necessarily lipases), an exciting possibility given the repertoire of enzymes known to act at lipid–water interfaces. (*iv*) Finally, since our results clearly suggest that lipases may have been markedly underexploited in many nonaqueous biotechnological applications (i.e., a closed, rather poorly active form of the enzyme may have been employed), a considerable practical benefit might be derived from the IAMI implementation in numerous nonaqueous bioconversions, including those in supercritical fluids or in the gas phase.

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