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Regulation of the interfacial activation within the *Candida rugosa* lipase family

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The fungus *Candida rugosa* produces several lipase isoenzymes and the 3D structure of three was solved (Lip1, Lip2 and Lip3). In an aqueous solution, the hydrophobic catalytic cavity of these lipases is buried under a flap that blocks the entrance of the substrate. In the hydrolysis of triacetin, the limiting step of the catalytic process was the activation of the enzyme and only the existence of the highly hydrophobic interface provided by hexane was able to shift the equilibrium towards the open conformation. In the case of Lip1, the hexane interface was crucial and once the open conformation was stabilised, Lip1 was as efficient as Lip3 for the hydrolysis of triacetin. Lip2 isoenzyme behaves more similarly to mLip3 reinforcing the higher structural and functional similarity between these isoenzymes. Inhibition experiments carried out under non-kinetic conditions allowed to correlate the higher flexibility of the closed flap and the higher hydrophobicity of the catalytic pocket of mLip3 with the greater facility of this isoenzyme to become activated by interfaces of different chemical nature. Both factors might allow a more intense penetration of mLip3 into the interfaces. In these systems, we observed a unique behaviour of Lip2, enzyme that although in a monomeric state and provide with an analogous flap structure to that of Lip1 or Lip3 underwent a very fast activation even in the absence of supramicellar concentrations of surfactants. We determined that the inhibitor itself forms micelles and hypothesised that they might provide an adequate interface for Lip2 activation. Copyright © 2009 John Wiley & Sons, Ltd.

Supporting information may be found in the online version of this paper.

Keywords: lipase; *Candida rugosa*; interfacial activation; E600; diethyl pnitrophenyl phosphate

INTRODUCTION

Lipases (triacylglycerol lipase EC 3.1.1.3) catalyse the hydrolysis of triacylglycerides at the interface between the insoluble substrate and water. Although the naturally occurring triacylglycerides are the preferred substrates, lipases also catalyse the enantio- and regioselective hydrolysis of a wide range of natural and synthetic esters.[1]

A unique feature of lipases is their increase in catalytic activity at the water–lipid interface, a phenomenon known as interfacial activation.[2] The activation has been associated to a conformational change in which a lid or flap, consisting of at least one α-helix, opens by rotating around its hinge regions. In the inactive closed conformation, the flap covers the active site avoiding its exposure to the aqueous solvent. However, in the presence of the interface the flap opens (active conformation) making the active site accessible to the substrate. At the same time, a large hydrophobic surface is exposed that supposedly interacts with the lipid interface.[3–7]

The structural basis for the lipase activation can be more complex since not all lipases with a flap exhibit this phenomenon and conversely, lipases without a flap or with a virtually absent one show interfacial activation. On the other hand, the activation of the *Staphylococcus hyicus* lipase is dependent on the substrate.[8]

Candida rugosa lipases (CRLs) constitute the most complex family of microbial lipases described so far. It is constituted by at least seven lipase genes (lip1–lip7) from which five (lip1–lip5) have been fully characterised.[9–11] The comparison of the predicted amino acid sequences indicated a high similarity within family ranging from 77 to 88% for pairs of proteins.

The crystal structures of Lip1 both in an open[12] and in a closed conformation[7] and that of the Lip3 complexed with cholesteryl linoleate[13] have been solved. Lip2 structure has been solved in our group in the closed conformation.[14] The comparative analysis of their structures revealed that the main differences were localised in the flaps and substrate binding pockets. For these reasons, this lipase family constitutes a good candidate to deepen in the study of the interfacial activation process.

In previous papers, we have purified and characterised three CRL isoenzymes Lip1, Lip2 and Lip3, the last in two aggregation states, monomer (mLip3) and dimer (dLip3) resulting from the association of two identical Lip3 monomers in the open conformation.[15–17] We identified several interactions that modulate the conformational flexibility of the flap that covers the catalytic domain in the isoenzymes and influence their interaction with lipolytic substrates.[14,17]

This work was aimed to compare the activation process of the three CRL isoenzymes in the presence of non-catalytic interfaces provided by surfactants and organic solvents.

Several authors have reported that the addition of water-miscible organic solvents increased the enzymatic activity of a

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Abbreviations used: *CRL, Candida rugosa lipase; TA, triacetin; E600, diethyl p-nitrophenyl phosphate; Mr, molecular weight.*

number of lipases acting on isotropic solutions of triacylglycerides. These results correlated well with a conformational transition between the open and closed form of the lipase promoted by the solvent.[18,19]

On the other hand, interfaces can be generated using surfactants above their critical micelle concentration (CMC) and their activating effect determined in parallel with the inhibition kinetics of the isoenzymes with the irreversible inhibitor E600 (E600 inhibition requires an exposed active site and therefore is indicative of the flap opening).

Although lipase–surfactant interactions are mainly hydrophobic, a considerable divergence in the lipase activation might be observed using ionic or non-ionic detergents.[20] In this work, we used bile salts derivatives as ionic and zwitterionic surfactants, as they are structurally similar to the cholesterol moiety of cholesteryl esters which are good substrates for the CRLs, particularly Lip2 and mLip3.[21] Triton X-100 was chosen as non-ionic surfactant.

MATERIALS AND METHODS

Materials

Lipase type VII from *C. rugosa*, diethyl *p*-nitrophenyl phosphate (E600), sodium cholate, sodium deoxycholate and Triton X-100, were purchased from Sigma Chemicals Co. (St. Louis, USA). A lyophilised crude extract (UAB extract) from a pilot plant scale *C. rugosa* fed-batch fermentation[22] was kindly suministrated by the Departament d'Enginyeria Química from the Universitat Autònoma de Barcelona (Spain). Tributyrin and triacetin were from Fluka (Deisenhofen, Germany). CHAPS was from Amresco (Solon, Ohio). All other chemicals were of analytical grade.

Lipase purification

Lip1 and Lip3 were purified from Sigma type VII powders (Sigma) according to Rúa and Ballesteros (1994)[23] and Rúa *et al*. (1993),[15] respectively. Lip2 was purified from lyophilised UAB powders according to Pernas *et al*. (2000).[16]

Determination of CMC

The CMC of detergents (sodium cholate, sodium deoxycholate, Triton X-100 and CHAPS) and E600 were determined by measuring the interfacial tension (γ) of the solutions in a tesiometer K9 (Krüss, USA). Stock solutions of each detergent and E600 were prepared in Tris/HCl 25 mM (pH 7.5) containing NaCl 150 mM and 4% (v/v) of acetonitrile. Afterwards, dilutions were prepared directly in a thermostatted vessel at 30 °C provided with a magnetic stirrer. The interfacial tension was measured and plotted *versus* the surfactant concentration. The CMC coincided with the intersection point of the two lines that divide the curve of surface tension as a function of the surfactant concentration.[24]

Enzyme assays

Lipase activity was measured in a pH-stat (Methrom, Switzerland) at 30 °C and pH 7.0 using a tributyrin emulsions (114 mM) stabilised with gum arabic as described in Pernas *et al*. (2000).[16]

Kinetic measurements

They were carried out following the initial hydrolysis rate of triacetin in a pH-stat. The assays were performed in 5 mM Tris/HCl buffer (pH 7.0) containing 0.1 M $CaCl_2$ at 30 °C and variable amounts of triacetin (from 35 mM to 1.06 M). All assays were done keeping the same stirring speed, while care was taken to avoid the formation of air bubbles in the reaction vessel. The reaction was started with the addition of the enzyme and at least triplicates of each assay were made. One activity unit was defined as the amount of enzyme that released 1 μmol of fatty acids per min. The solubility of triacetin in the reaction conditions was estimated measuring the turbidity as described in Ferrato *et al*. (1997).[8] The lipase/esterase activity ratio (TA_{lip}/TA_{est}) for each lipase was determined by dividing the specific activity above (1.06 M) and below (0.18 M) the solubility limit of triacetin.

E600 Inhibition

The inactivation of CRL lipases by E600 was performed in 25 mM Tris/HCl (pH 7.5) containing 150 mM NaCl and 4% (v/v) acetonitrile. E600 was directly added from stock solutions prepared in acetonitrile. The mixture was incubated at 30 °C and aliquots were withdrawn at different times. Remaining activity was titrimetrically determined at pH 7.0 using the tributyrin emulsion as substrate. In the experiments, the lipase concentration was kept constant (5×10^{-6} M) and the molar ratio of inhibitor to lipase, defined as R, was varied increasing the E600 concentration. When required, the inhibition was performed in the presence of different surfactants at concentrations above their respective CMCs. The surfactants were dissolved in 25 mM Tris/HCl (pH 7.5) containing 150 mM NaCl. Control experiments in which the inhibitor was omitted were also performed in order to check the stability of the incubated lipases under the different assayed conditions.

Protein concentration

It was determined by the Lowry method using BSA as standard.

RESULTS AND DISCUSSION

Activation under kinetic conditions

In previous works, we determined the hydrolysis kinetics of triacetin catalysed by three CRL isoenzymes.[17,21] We found that Lip3 was the most active on this substrate along the whole substrate concentration range and Lip1 the less active. Nonetheless, the three isoenzymes had a low activity when the substrate concentration was maintained below its solubility limit what was correlated with the prevalence of the closed conformation of the enzymes in the aqueous medium. The TA_{lip}/TA_{est}, considered as a good criterion to define the esterasic/lipasic character within the CRL family,[17] follows the order Lip1 ≫ Lip2 ≈ Lip3 (43.7, 7.3 and 7.6, respectively). The higher 'esterase-like character' of Lip2 and Lip3 was associated with the superior flexibility of their flaps.

Several authors have reported that the addition of water-miscible organic solvents increased the enzymatic activity of several lipase acting on isotropic solutions of triacylglycerides. These results correlated well with a conformational transition between the open and closed form of the lipase promoted by the solvent.[18,19]

In a first attempt, we determined that several water-miscible organic solvents (acetonitrile, isopropanol and tert-butanol) were not effective with the CRL isoenzymes (not shown). In contrast, a non-polar solvent as hexane, which could provide a highly hydrophobic interface, was a good activator for CRLs acting on soluble triacetin. Figure 1 shows the effect of 25% (v/v) hexane on the kinetics of Lip1, Lip2 and Lip3 isoenzymes. For comparative purposes, the kinetics without hexane are also provided.[17,21] In all cases, increases in activity were obtained along the whole triacetin concentration range although they were particularly high below the solubility limit of the triacetin. The introduction of the hydrophobic interface provoked a transition between lipolytic or sigmoidal kinetics to Michaelis–Menten kinetics.

The strongest activation effect was achieved for Lip1, enzyme that reached activity levels similar to those of Lip3 both below and above the solubility limit of triacetin. Thus, although in the presence of the organic solvent the maximum activity (TA_{lip}) was around 100 U/mg protein for both isoenzymes, in its absence they were 26.7 and 81.7 U/mg for Lip1 and Lip3, respectively.

On the other hand, Lip2 activity on insoluble triacetin was closer to that of Lip1 (TA_{lip} was 26.7 and 28.2 U/mg for Lip1 and Lip2, respectively). Nonetheless, increases in activity upon addition of hexane were more similar to those of mLip3 (TA_{lip} increased 3.3 –Lip1-, 1.5 –Lip2- and 1.3-fold –mLip3- and TA_{est} 128 –Lip1-, 6.8 –Lip2- and 8.5-fold –mLip3-), although the absolute values of specific activity were higher for Lip3 than for Lip2. These results showed once again the closer kinetic similarity of Lip2 and mLip3 isoenzymes as it has been observed with a longer fatty acid chain triacylglyceride (tributyrin) and cholesteryl oleate.[21]

The effect of the so-called quality of the interface has been regarded as an important factor of the catalytic process of lipases.[25] In this sense, it has been pointed out that the high mutual solubility of triacetin and water probably results in the formation of a diffuse and poorly hydrophobic interface.[26] This hypothesis has been useful to explain the lack of interfacial activation of the *R. miehei* lipase with triacetin, enzyme that however does show activation with other substrates.[27] These results emphasised the importance of the enzyme binding step in the overall lipolysis rate and suggested that the low activity of Lip1 on triacetin in the absence of hexane, compared to that Lip3 (Fig. 1), was mainly determined by the defective adsorption of the former enzyme to the interface. The higher conformational rigidity of the closed Lip1 flap as compared to that of Lip2 or Lip3 together with the lower hydrophobicity of its catalytic cavity could be the key factors that restrict Lip1 adsorption to the triacetin interface.

As we were interested on studying the effect of different interfaces on the interfacial activation within the CRL family, next we carried out a series of inhibition experiments using E600 in micellar systems. The micelles, provided by inert surfactants

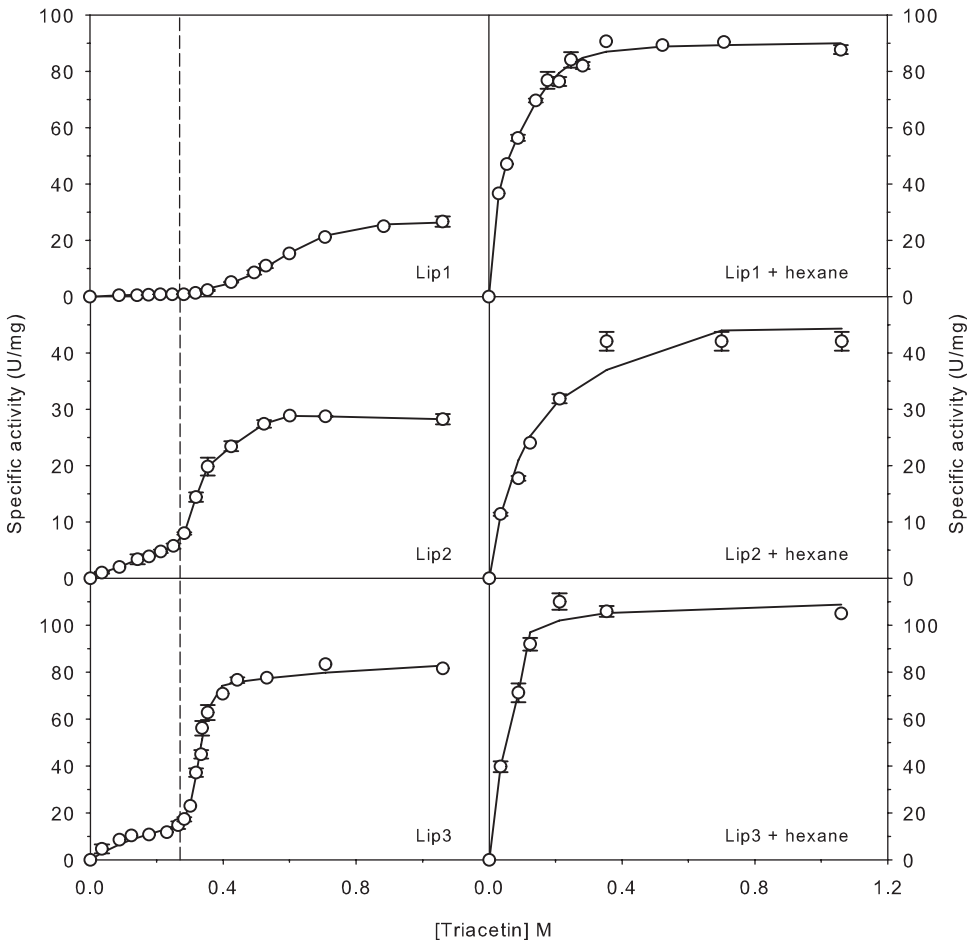

Figure 1. Dependence of specific activity on triacetin concentration in the absence and presence of 25% hexane. Lipase concentration: 80 μM. The vertical dashed lines indicate the solubility limit of triacetin

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above their CMCs, played two main roles: to provide an interface for the partitioning of the inhibitor from the aqueous phase and a surface for the lipase binding in the active form.

Effect of the inhibitor/enzyme ratio on the inactivation rate of *C. rugosa* lipases

Inhibition rates of Lip1 and mLip3 were determined in a previous study and found to be very slow in aqueous media although faster for Lip3 in accordance with the postulated higher flexibility of its flap.[17] Our first objective was to extend this study to the isoenzyme Lip2, which has been recently crystallised in our group, under the same experimental conditions previously used for Lip1 and mLip3 (ratio inhibitor/enzyme = 1000, 30 °C). Table 1 shows the results obtained for the three monomeric isoenzymes and, for comparative purposes, for the dimeric form of Lip3 (dLip3).

Surprisingly, Lip2 inhibition at different inhibitor/enzyme ratios (R) was more similar to that obtained with dLip3 and clearly faster than that of the other monomeric isoenzymes. Thus, 1 h incubation at $R = 100$ was enough to completely abolish the activity of Lip2 or dLip3 (Fig. 2A) but it was necessary to increase R up to 1000 and to include an interface (sodium deoxycholate above its CMC) to be able to inactivate Lip1 or mLip3 (Fig. 2B).

The inhibition of Lip2 was not dependent on the substrate used to follow the kinetics. Thus, the same inhibition pattern obtained with emulsified tributyrin was obtained with a soluble substrate (triacetin below its solubility limit) (not shown). We also checked that Lip2 inhibition was irreversible as judged by the activity assay (emulsified tributyrin) with the inhibited enzyme after exhaustive dialysis.

Due to the fast inhibition of Lip2, the effect of the interfaces on the inactivation rates was carried out only with Lip1 and mLip3. A more detailed study of Lip2 inhibition is described below.

Effect of the chemical nature of the interfaces. Activation under non-kinetic conditions

Previously, we determined the CMCs of each assayed surfactant under the experimental conditions used in the inhibition experiments (refer Methods). In all the cases, the CMC values determined by us were lower than those reported in the bibliography, probably due to the different media composition (temperature, salt and/or organic solvents concentrations, etc.) (Table 2).

Table 1. Half-life for inactivation ($t_{1/2}$) of the CRL isoenzymes

CRL isoenzyme	$t_{1/2}$ (min)	r^2
Lip1	169.1	0.971
Lip2	3[a]	—
mLip3	83.2	0.986
dLip3	5[a]	—

Conditions: [enzyme] = 5×10^{-6} M; $R = 1000$, 30 °C, pH 7.5, without detergents.
[a] For Lip2 and dLip3 isoenzymes, $t_{1/2}$ (the time needed to reach 50% lipase inhibition) was determined directly from the grafic due to neither of them adjust to a pseudo-first-order process.

Lip1 and mLip3 were incubated at 30 °C with E600 ($R = 1000$) in the presence of anionic (sodium cholate and deoxycholate), zwitterionic (CHAPS) and neutral (Triton X-100) surfactants in both below and above their respective CMC. To ensure the presence of micelles surfactants concentration was almost five times superior at their CMC.

Results obtained are shown in Table 3 in terms of the half-life for inactivation ($t_{1/2}$) for either Lip1 or mLip3. In all cases, the inactivation followed pseudo-first order kinetics. In addition, none of the assayed detergents had an effect on the $t_{1/2}$ value of Lip1 or mLip3 when present at concentrations below their CMC (not shown). However, above the CMC all of them provoked a noticeable decrease on $t_{1/2}$ compared to the control in the absence of surfactants. In these conditions, the values determined for mLip3 were always lower than those of Lip1, indicating that irrespective the charge of the surfactant a higher rate of inhibition by E600 (faster interfacial activation) was observed for mLip3. This result strongly support our hypothesis that the low conformational flexibility of the flap that covers the catalytic domain of Lip1 isoenzyme is the main constrain for its activation[17] and, apparently, more important than the chemical nature of the interface.

Nonetheless, a closer look to Table 3 highlights fine differences in the efficiency of the assayed surfactant as activating interfaces.

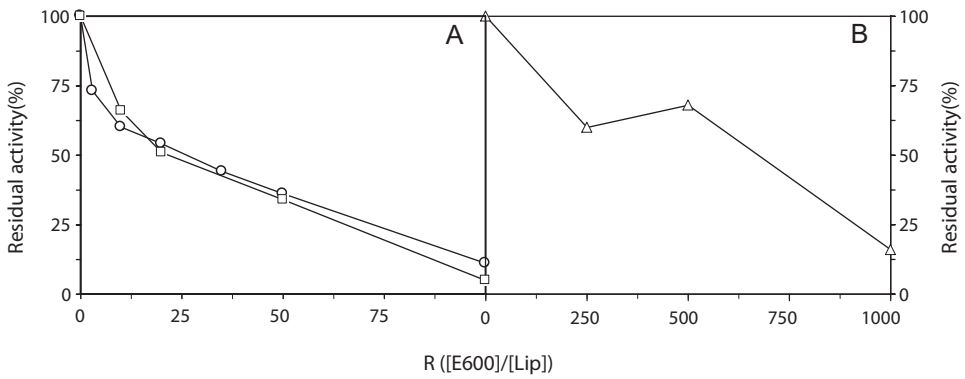
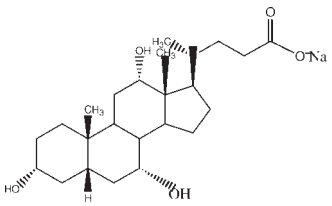
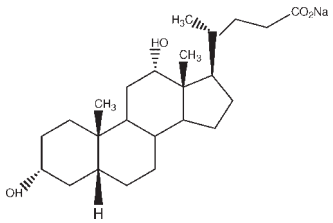
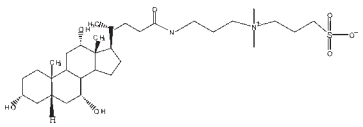
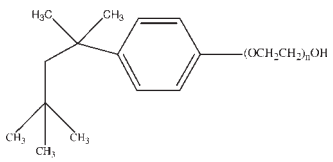


Figure 2. (A) Inhibition of Lip2 (circles) and dLip3 (squares) by E600 in aqueous medium. (B) Inhibition of Lip1 (triangle) in the presence of 3 mM sodium deoxycholate. The concentration of each enzyme was 5×10^{-6} M and R was varied by changing the E600 concentration. Results expressed as residual activity after 60 min of incubation. No inhibition was observed in the absence of E600 (not shown)

Table 2. Structure and CMC of the surfactants (experimental and reported)

Surfactant	Structure	CMC (mM)	CMC (mM) References
Sodium cholate		2.33 ± 0.010	11–13[31] 7–16.2[35]
Sodium deoxycholate		0.69 ± 0.005	3–10[31] 2–4[35,36]
CHAPS		1.24 ± 0.008	6.5[35]
Triton X-100		0.14 ± 0.001	0.25[35] 0.3–0.6[37]

Thus in the case of Lip1, the best (lower $t_{1/2}$ values) were those formed by the neutral (Triton X-100) and zwitterionic (CHAPS) surfactants. The absence of a net charge in the interface might favour the approaching of the isoenzyme and subsequent binding to the interface. This hypothesis would be in accordance with the observed defective binding of this isoenzyme to the highly polar interface formed by the substrate triacetin.

In the case of mLip3, the differences among the surfactants were higher than those observed for Lip1, although the interface formed by the neutral surfactant was also the best ($t_{1/2} = 3.9$ min). Concerning the bile salts derivatives, $t_{1/2}$ was significantly lower when the interface was provided by sodium deoxycholate ($t_{1/2} = 4.5$ min). Thus, with the second anionic bile salt or the zwitterionic one $t_{1/2}$ increased up to 18 and 27.2 min, respectively.

Bile salts are rigid molecules, shaped like a flattened ellipsoid possessing dissimilar sides: in the common bile salts the β-face is hydrophobic whereas the α-face, which possesses the hydroxyl groups, is hydrophilic. In the micelles formed by bile salts, the hydrophobic faces are considered to be in contact with each other while the hydrophilic faces are directed to the aqueous environment.[28–30] In this sense, an important difference between the sodium deoxycholate and the other two bile salts

Table 3. Effect of the chemical nature of the interface on the half-life for inactivation ($t_{1/2}$) of Lip1 and mLip3 isoenzymes

		Lip1				mLip3			
Conditions	[Surfactant] mM	$t_{1/2}$ (min)	k' (min^{-1})	r^2	hlr (×)	$t_{1/2}$ (min)	k' (min^{-1})	r^2	hlr (×)
None	—	161.1	$4.8\ 10^{-3}$	0.984	—	83.2	$8.3\ 10^{-3}$	0.986	—
Sodium cholate	12.0	42.8	$1.6\ 10^{-2}$	0.996	3.8	27.2	$2.5\ 10^{-2}$	0.980	3.1
Sodium deoxycholate	3.0	32.95	$2.1\ 10^{-2}$	0.993	4.9	4.5	$15.5\ 10^{-2}$	0.988	18.5
CHAPS	12.0	26.4	$2.6\ 10^{-2}$	0.984	6.1	18.0	$3.8\ 10^{-2}$	0.997	4.6
Triton X-100	1.2	20.2	$3.4\ 10^{-2}$	0.998	8.0	3.9	$17.6\ 10^{-2}$	0.989	21.3

Conditions: [enzyme] = 5×10^{-6} M; $R = 1000$, 30 °C, pH 7.5.
hlr (×): half-lives ratio in absence and presence of detergent.

used in this work is the removal of one hydroxyl group in the α-face (Table 2) that causes an increase in the overall hydrophobicity of the sodium deoxycholate micelles.[31] We suggest that this situation might facilitate and/or stabilise the opening of the flap of the isoenzyme with the highest flap flexibility and hydrophobicity of the catalytic pocket (mLip3).

This hypothesis is in agreement with previous fluorescence studies that postulate a distinct localisation of Lip1 and mLip3 in reverse micelles of AOT (sodium bis-(2-ethylhexyl) sulphosuccinate).[32] The authors also found the more hydrophobic Lip3 isoenzyme localised in a more apolar environment thus avoiding the effect of the anionic interface formed by the AOT.

Interfacial activation of Lip2 isoenzyme

Figure 3 shows the inhibition kinetic of Lip2 in an aqueous medium at $R = 1000$. As previously mentioned, Lip2 behave more similarly to dLip3 (its kinetic is also included in the figure as comparison) than to the other monomeric CRLs and the inhibition was very fast in the absence of the interface provided by the surfactants. The inhibition curve for Lip2 (and dLip3) did not follow first order kinetics. Instead, a biphasic kinetic was obtained and after prolonged incubation times we could still observe a residual activity. The time needed to reach 50% lipase inhibition ($t_{50\%}$) was very close for Lip2 and dLip3 (3 and 5 min, respectively).

In regard to dLip3 the reason for the fast inhibition in aqueous media was the free access of the inhibitor to the active site.[17] As it is know from the reported crystal structure of dLip3, the dimerisation generated four openings at the interface whose size was big enough to allow the entrance of inhibitor molecules to the active sites.[13,17,33]

In this sense the results obtained for Lip2 suggested that, in aqueous solution, the enzyme mainly existed as an open conformer. Nevertheless, no evident factor came out for the stabilisation of the open conformation of Lip2 in the aqueous media as this enzyme was eluted in gel filtration chromatography as a monomer[16] and the crystal structure of Lip2 does not provide evidences of dimer formation[21] as in the case of dLip3. In addition and opposite to dLip3, Lip2 showed interfacial activation with partially soluble substrates (triacetin and tributyrin), as does the other monomeric isoenzymes –Lip1 and mLip3–.[17,21]

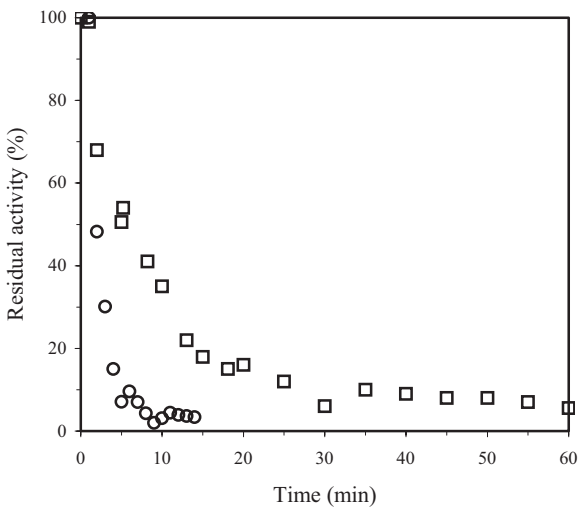


Figure 3. Time course of inactivation of Lip2 (circles) and dLip3 (squares) with E600. Lipases (5×10^{-6} M) were incubated with 5×10^{-3} M E600 ($R = 1000$). Results expressed as residual activity as a function of time. No inhibition was observed in the absence of E600 (not shown)

Our results might suggest that for Lip2 the inhibitor itself was shifting the equilibrium from closed to open conformation. If so, the question should be if E600 was merely modifying (inhibiting) the open conformation of Lip2 and/or was forming a kind of micelle-like structures able to trigger the lipase activation. It has been reported that molecules of similar size as E600 are able to form micelles, normally with a low aggregation number.[34]

To investigate weather or not the E600 could form micelles in the aqueous media, we determined the variation of the superficial tension as a function of the inhibitor concentration. The inhibitor was prepared in the same buffer used to perform the lipases inhibition (25 mM Tris/HCl (pH 7.5) containing 150 mM NaCl and 4% (v/v) acetonitrile) and its concentration varied from 5×10^{-3} M to 0.4×10^{-3} M (refer Methods). The two linear segments of the plots corresponding to the monomeric and micellar forms of the compound intersected at the critical concentration (not shown), which resulted to be 3.4×10^{-3} M.

This result indicated that, in the experimental conditions ($R = 1000$; E600 $= 5 \times 10^{-3}$ M), we were working in the presence of E600 micelles that could promote the activation of Lip2. In order to check this hypothesis, we carried out the inhibition at lower R values ($R = 500$ and 50) that allowed concentrations of the inhibitor closer (2.5×10^{-3} M) and well below to its CMC (0.25×10^{-3} M), respectively. As expected, in both experiments we observed significant decreases in the inhibition rate ($t_{50\%}$) as R decreased (Table 4). Nonetheless, as the E600 was also the inhibitor, these decreases could not be exclusively attributed to the elimination of the activating interface at the lowest R values. To solve this problem, next we introduced a new interface in the experiments that had probed to be efficient for the other monomeric CRLs. Thus, we carried out new series of inhibitions at the three values of R (50, 500 and 1000) in the presence of 3 mM of sodium deoxycholate (above its CMC). Results are shown in Fig. 4. It is clear that the surfactant interface allowed a faster inhibition of Lip2 but the strongest effect was obtained at the lowest value of R. Concentrations below the sodium deoxycholate CMC did not affect the inhibition kinetic (not shown).

These results suggested that the high $t_{50\%}$ value determined at $R = 50$, in the absence of deoxycholate micelles, was not due to a low inhibitor concentration in the system but more to the absence of an activating interface and reinforce our hypothesis that the inhibitor itself could form interfaces able to trigger Lip2 inhibition.

Table 4. Half-life for Lip2 inactivation ($t_{50\%}$) at different R values

R	$t_{50\%}$ (min)
50	51.0
500	9.0
1000	4.1

Conditions: [enzyme] $= 5 \times 10^{-6}$ M; 30 °C, pH 7.5.

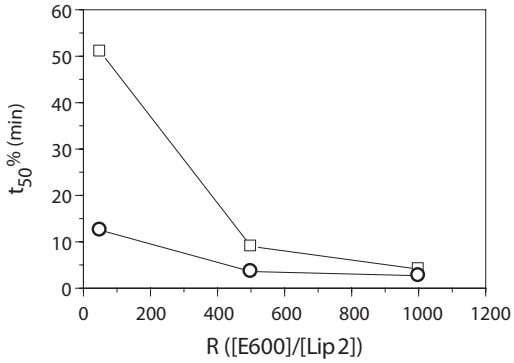


Figure 4. Dependence of the time for 50% inhibition ($t_{50\%}$) at different values of *R* in the presence (circles) and absence of micelles of sodium deoxycholate (squares)

At the present it is difficult to explain why Lip2 isoenzyme can be activated by micelles formed probably by only a small number of molecules, particularly when neither Lip1 nor mLip3 seemed to be affected by them. It should be pointed out that, for example, at $R = 1000$, $t_{1/2}$ was 169.1 and 83.2 min for Lip1 and mLip3, respectively, in the absence of surfactants. Under these conditions, $t_{50\%}$ was only 4.1 min for Lip2. Further work is being done in our laboratory exploring new inhibitors and interfaces to find additional evidences to explain the different behaviour of Lip2 isoenzyme in micellar systems.

In conclusion, we have correlated structural differences in the flap stabilisation and hydrophobicity of the binding pocket of three CRL isoenzymes with their facility to be activated by interfaces provided by a substrate or by non-catalytic interfaces. We found that, at least for Lip1, the limiting step of the catalytic process was the activation of the enzyme and only the existence of a highly hydrophobic interface was able to shift the equilibrium towards the open conformation of the lipase. In contrast, the superior flexibility of the closed flap of mLip3 and the higher hydrophobicity of its catalytic cavity allow a more intense penetration into the interfaces and faster activation of mLip3. Lip2 showed a quite unique behaviour and, apparently, was less dependent on the quality of the interface (hydrophobicity, charge) to stabilise its open conformation.

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