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Effects of a fungal lipase on membrane organization evaluated by fluorescence polarization

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Abstract

Triglyceride lipase from *Thermomyces lanuginosa* (TlL) binds to the non-substrate small (40 nm) unilamelar vesicles of 1,2-dimiristoylglycero-*sn*-3-phosphoglycerol (DMPG-SUVs) in a catalytically active structure, whereas it adopts a catalytically incompetent form in binding to zwitterionic 1,2-dimiristoylglycero-*sn*-3-phosphocholine (DMPC-SUVs) or to large (100 nm) unilamelar DMPG (DMPG-LUVs) vesicles. Steady-state anisotropy measurements with probes that localize at different positions in the membrane give information on the effects of TlL (and its mutants) on the mobility of the phospholipids. All TlL mutants insert into the DMPG-SUVs and increase lipid order at the headgroup region and at the hydrophobic core of the lipid bilayer as well. The increase of the rigidity of the membrane that occurs in the gel and liquid crystal states, results in an increase of the phase transition temperature (T_m). Kinetic experiments with monolayers of 1,2-dicaprin demonstrate the thermal stability of the enzyme in the range of temperatures of the phase transition. Mutations in the tryptophan (Trp) residues of TlL reduce activity of this enzyme and affect its interaction with the membrane. The membrane insertions of TlL mutants with other than Trp substitutions are much more shallower and produce only small increases of T_m, whereas mutation of lid-located Trp89 or mutation of any other Trp residue (117, 221, 260) result in a deeper penetration and significant increases of the T_m. Lipid dynamics of DMPC-SUVs or DMPG-LUVs are not affected by any of the TlL mutants, despite their strong binding to the lipids revealed by resonance energy transfer (RET). These results are consistent with the lipase–lipid penetration model in which the "lid" region of TlL inserts into the highly curved anionic interface, thus stabilizing the "open" or active enzyme conformation, whereas TlL binds to the surface of zwitterionic and large (small curvature) anionic vesicles in a "closed" (or inactive) conformation, without insertion of the lid.

Keywords: Thermomyces lanuginosa lipase; Fluorescence anisotropy; Interfacial activation; Lipid vesicles; Monolayers

Abbreviations: DPH, 1,6-diphenylhexa-1,3,5-triene; DMPC, 1,2-dimiristoylglycero-*sn*-3-phosphocholine; DMPG, 1,2-dimiristoylglycero-*sn*-3-phosphoglycerol; LUV, large unilamelar vesicles (100 nm); NBD-PE, *N*-(7-nitro-2-1,3-benzoxadiazol-4-yl) dioleoylphosphatidylethanolamine; PNPB, *p*-nitrophenyl-butyrate; POPC, 1-palmitoyl-2-oleoylglycero-*sn*-3-phosphocholine; POPG, 1-palmitoyl-2-oleoylglycero-*sn*-3-phosphoglycerol; RET, resonance energy transfer; TB, tributyrin; T_m, gel-to-liquid crystal transition temperature; TlL, *Thermomyces lanuginosa* lipase; SUV, small unilamelar vesicles (40 nm); TMA-DPH, 1-[4-(trimethylammonium) phenyl]-6-phenyl-1,3,5-hexatriene

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1. Introduction

Lipases (EC 3.1.1.3) are esterases that hydrolyze triglycerides to di- and monoglycerides. They differ from classical esterases in that their activity increases dramatically upon binding to the lipid surface formed by their water-insoluble substrates. This "interfacial activation" phenomenon was first described by Sarda and Desnuelle [1], and it is generally attributed to a conformational change in the enzyme leading to an increase in activity [2], as supported by X-ray crystallography studies of pure [3–8] and complexed enzymes [9–12]. These studies suggest two enzyme conformations: (a) with the active site covered by a "lid" that renders it inaccessible to substrate, (b) with the lid displaced in a fashion which not only opens access to the active site, but also exposes a large hydrophobic domain around the catalytic triad, necessary for substrate binding [13,14]. The first form, referred to as "closed conformation", corresponds to the lipase in aqueous solution, in the absence of an interface or organic solvent, and it has a very low lipolytic activity. The second form of the lipase is so-called "open conformation", adopted by the enzyme at the lipid interface formed by aggregated substrates, and it corresponds to the catalytically active form of the enzyme. Fungal lipase from *Thermomyces lanuginosa* (TlL) is a well-characterized 30 kDa, 269 amino acid interfacial enzyme, and several structures of this lipase have been reported [15–17]. They revealed an α-helical lid constituted by amino acids 82–96, which is blocking the access to the catalytic triad: Ser146-Asp201-His258, in the uncomplexed enzyme (closed form). The lid is displaced in the presence of covalently bound inhibitors or when co-crystallized with micelles. Although there are no direct structural data to show that the same conformational rearrangement is responsible for the activation of the enzyme at the lipid/water interface, biophysical studies indicate that TlL lipase adsorbs to the interface by the hydrophobic domain around the active site, and that the amphiphilic lid interacts with both the lipid interface and the surface of the enzyme molecule [18–21]. We have recently shown that TlL binds to small unilamelar vesicles (SUV) of anionic phospholipids, such as 1-palmitoyl-2-oleoylglycero-*sn*-3-phosphoglycerol (POPG), adopting a catalytically active conformation for the hydrolysis of partitioned substrate (e.g. tributyrin or *p*-nitrophenylbutyrate), with a more than 100-fold increase of the catalytic rate for the same concentration of substrate [20,22]. In contrast, TlL binds with similar affinity to zwitterionic vesicles (e.g. 1-palmitoyl-2-oleoylglycero-*sn*-3-phosphocholine, POPC) but in a catalytically inactive form [22]. As TlL binds also to the small-curvature large vesicles (LUVs) in the inactive form, independently of the lipid composition, it seems that the vesicle curvature, and not only its composition has an important effect on the enzyme activation. Spectroscopic experiments with the use of several TlL mutants (including inactive and tryptophan (Trp)-mutants), suggest that in anionic SUVs, the lid penetrates the membrane and is stabilized in the open conformation by a combination of electrostatic and hydrophobic interactions. It leaves the entrance of the active site free for the binding and hydrolysis of the substrate, which is partitioned at the lipid interface. Since the physical properties of the interface control the activity of the enzyme, we focused here on the effect of TlL on the fluidity of the lipid membrane. This was investigated by fluorescence polarization with the employment of different probes that are known to accommodate at different positions within the lipid bi- and mono-layer. Different mutants of TlL were studied, with the inactive mutant (iTlL) as the wild type analogue that minimizes the interferences resulting from the formation of the product. Mutants of all four Trp residues of TlL (in positions 89, 117, 221, and 260) were used to investigate their role in the protein–lipid interaction. Residues Trp117, Trp221, and Trp260, are known to be located in the regions not directly involved in the interfacial activation process, whereas Trp89—common to other pancreatic and fungal lipases—is located in the central part of the lid. In the crystal structure of the open form of TlL, Trp89 is in close contact with the acyl moiety of a transition state analogue bound to the active site [16], and is important for enzyme activity [23].

Results in this paper show that the open form of TlL, in small anionic vesicles, dramatically modifies lipid dynamics in the gel and the liquid crystal states of the membrane, increasing lipid order and the transition temperature. On the contrary, the closed form of TlL, in small zwitterionic vesicles and in large anionic vesicles, binds superficially to the interface, with no modification of the lipid order and phase behavior.

2. Material and methods

2.1. Chemicals

DMPC, DMPG, and NBD-PE were from Avanti Polar Lipids (Alabaster, AL); DPH and TMA-DPH were purchased from Molecular Probes (Eugene, OR). PNPB and 1,2-dicaprin were from Sigma. All chemicals were of the highest available purity.

2.2. Enzyme samples

T. lanuginosa lipase wild type; mutants: TlLS146A (inactive, with all four Trps: iTlL); TlLW117F-W221H-W260H (active with only one Trp89 (in the lid): TlLW89); TlLW117F-W221H-W260H-S146A (inactive with only one Trp89: iTlLW89); TlLW89L (active with structural Trp(s) 117, 221, 260, without Trp89 in the lid). All TlL samples were kindly provided by Dr. Allan Svendsen (Novo Nordisk A/S, Denmark). TlL was cloned, sequenced, and expressed as described elsewhere [24]. Site-directed mutagenesis in the lipase expression plasmid, and purification of the produced wild type and variants were conducted as previously reported [25]. Protein concentration was determined spectrophotometrically at 280 nm; for TlL and iTlL a molar extinction coefficient of 43,000 $M^{-1}\,cm^{-1}$ with MW of 32 kDa was used [26]; the extinction coefficient for iTlLW89 was 20,800 $M^{-1}\,cm^{-1}$ and for TlLW89L was 32,640 $M^{-1}\,cm^{-1}$ (data from Novo Nordisk).

2.3. Lipid vesicles

Small unilamelar vesicles of DMPG or DMPC, alone, with the substrate PNPB or with the fluorescent probes NBD-PE, DPH, or TMA-DPH, were prepared by evaporation of a mixture of the lipids and probes in $CHCl_3/CH_3OH$ (2:1 (v/v)); PNPB was added from stock solutions in tetrahydrofuran. The dried film was hydrated to a final lipid concentration of 20 mM, and then sonicated in a bath type sonicator (Lab Supplies, Hickesville, NY, Model G112SPIT) above the gel–fluid transition temperature until a clear dispersion was obtained (typically 2–4 min). To obtain large unilamelar vesicles of the same composition, the hydrated lipid film was submitted to five freeze-thaw cycles to ensure homogeneous mixture of the multilamelar vesicle suspensions (MLVs). The MLVs were extruded eight times through a series of three polycarbonate filters (Nucleopore), one of 200 nm and two of 100 nm pore size, in a high-pressure extruder (Lipex Biomembranes, Vancouver, BC). Vesicles were annealed for 30 min above their transition temperature before use. Vesicle size was measured by dynamic light scattering with a Malvern II-C autosizer. SUV vesicles had a mean diameter of 40 nm, and a narrow size distribution (polydispersity < 0.1), whereas LUVs mean diameter was 105 nm with approximately the same polydispersity.

2.4. Compression isotherms

The compression isotherms (area/pressure curves) were performed on a Langmuir film balance KSV5000 equipped with a Wilhelmy platinum plate. The thermostatted PTFE (Teflon) trough (surface area: 750 cm^2, volume: 1000 cm^3) and the plate were thoroughly washed before each run with hot water to avoid carry over of the lipid. Monolayers of 1,2-dicaprin were formed by microsyringe (Hamilton Co., Reno, NV) application of small drops of the spreading solution in $CHCl_3$ on the subphase of 10 mM Tris–buffer (pH = 8.0). After 10 min allowed for solvent evaporation, the monolayers were continuously compressed (symmetrical compression)—with an area reduction rate of 30 cm^2/min—up to the collapse pressure. Each run was repeated three times and the reproducibility was ± 1 $Å^2$ per molecule. The subphase was maintained at the desired temperature with a circulating water bath connected to the trough.

2.5. Kinetic protocols

The kinetics of hydrolysis of PNPB were monitored as the change in the optical density (OD) at 400 nm, corresponding to the absorption maximum of the *p*-nitrophenolate anion with molar extinction coefficient of 14,000, as described elsewhere [20]. Data acquisition and manipulation were carried out in a Shimadzu spectrophotometer (model UV-2401PC) with 1 s acquisition time. Measurements were done in 0.7 ml of 10 mM Tris, pH 8.0 at 25 °C in a quartz cuvette. The addition of the vesicles of DMPG (or DMPC) to the cuvette was followed by the

introduction of PNPB, and the reaction was initiated by addition of the lipase and gentle mixing of the whole solution. The experiments carried out without vesicles were done with special care during the stirring of the samples, as the vigorous mixing creates air bubbles, which provide an unwanted air–water interface for the partition of the substrate and the enzyme [20]. The effect of temperature on the reactions catalysed by TlL was investigated in monolayers of 1,2-dicaprin spread at the air/water interface. The lipolysis reactions were recorded on a fully automated monolayer system KSV5000 (KSV, Helsinki, Finland), using a zero-order thermostatted PTFE trough (reaction compartment area: 38 cm^2, volume: 40 ml; reservoir compartment length: 21.5 cm, width: 7.5 cm.). Substrate was spread from a stock solution in chloroform to achieve a surface pressure of 17 mN/m. The enzyme was added with a microsyringe to the reaction compartment which was constantly stirred. To maintain the surface pressure constant during hydrolysis, the mobile PTFE barrier was moving to compensate for the substrate molecules removed from the film by the enzyme. Surface pressure was measured using a Wilhelmy plate connected to an electrobalance. To avoid carryover of lipid and protein, the PTFE trough and the Wilhelmy plate were thoroughly cleaned before each run with hot water at >70 °C. The aqueous subphase contained 10 mM Tris at pH 8.0, adjusted to the desired temperature with a circulating water bath. Hydrolysis rates are represented as the slopes of the progress curves at the steady-state (mm/min).

2.6. Binding of TlL mutants to DMPC and DMPG vesicles

Binding of TlL enzymes to DMPG and DMPC vesicles containing 2% of NBD-PE was determined by the resonance energy transfer (RET) from Trp residues in the enzyme to the labeled interface phospholipid at 340 nm (excitation 280 nm). Experiments were conducted in an AB-2 spectrofluorimeter (SLM-Aminco). The enzyme was added from its water stock solution to a cuvette with 10 mM Tris at pH 8.0, to a final concentration of 2.47 μM, and fluorescence from tryptophan was monitored continuously with 2 s acquisition time. The labeled DMPG or DMPC vesicles were added (0.4 mM lipid) when a stable reading was achieved. After consequent stabilization of the baseline, a second aliquot of excess unlabeled vesicles (0.8 mM) was added to determine the possibility of enzyme migration between vesicles. Samples were stirred continuously during the measurements. The relative change in fluorescence, δF, is defined as $(F - F_0)/F_0$, where F_0 and F are the intensities without and with vesicles, respectively.

2.7. Fluorescence anisotropy of membrane lipids

Steady-state anisotropy measurements were carried out with the same instrument as the RET experiments, using L-format fluorescence polarizers. The dynamics of lipids in liposome membranes in the presence of TlL mutants was determined by measuring the degree of depolarization of the fluorescence emitted from the probes DPH, TMA-DPH, and NBD-PE [27]. The excitation and emission wavelengths were 365/425 nm for DPH and TMA-DPH (slit-widths: 4 nm), and 460/534 nm for NBD-PE (slit-widths: 4 nm). Labeled vesicles with 1% of the desired probe (133 μM lipid) were mixed with the enzyme (2.47 μM) in 10 mM Tris at pH 8.0, and anisotropy was measured automatically. For each sample, two cycles were done: a heating cycle from ~15 up to 42 °C, followed by a cooling cycle to the initial temperature, at 1 °C intervals, allowing for thermal equilibration. In some cases, vesicles were titrated with lipase at a constant temperature, below or above the T_m of the lipids. All solutions were stirred continuously during the measurements. The temperature was controlled with a peltier system piloted by a computer program (Microbeam S.A., Barcelona, Spain), and the temperature was registered with a thermocouple inserted into the cuvette. Fluorescence anisotropy (r) was calculated automatically by the software provided with the instrument, according to:

$$r = \frac{I_{Vv} - GI_{Vh}}{I_{Vv} + 2GI_{Vh}}$$

where I_{Vv} and I_{Vh} are the intensities of the emitted polarized light with the emission polarizer parallel or perpendicular, respectively, to the excitation polarizer. Anisotropy values were corrected for dependencies in the detection system (G-factor correction, $G = I_{Hv}/I_{Hh}$).

3. Results and discussion

3.1. Effect of DMPG and DMPC vesicles on the catalytic activity of TlL

We have previously shown that the catalytic activities of TlL, TlLW89, and TlLW89L are dramatically enhanced by POPG-SUVs, but not by POPG-LUVs or by POPC vesicles, either SUVs or LUVs [20–22,28]. Anionic POPG-SUVs act as a matrix surface to which the lipase binds in an active or "open" form for the catalytic turnover of partitioned substrate. With this system, the interfacial kinetic constants and equilibrium parameters for TlL can be meaningfully calculated, because the non-substrate matrix does not bind the active site of the enzyme, acting as a real neutral diluent. Moreover, binding to this lipid interface induces the opening of the lid that covers the catalytic triad of TlL in the absence of substrate (closed form) [20]. In this paper, we used vesicles of DMPG and DMPC, because the gel-to-liquid crystal phase transition temperature (T_m) for these lipids, at around 23 °C, facilitates the study of enzyme interactions both below and above the phase transition (the T_m for POPG or POPC is at −2 °C). In Fig. 1, TlL activity is shown as OD change, using PNPB as substrate. The rate of hydrolysis of PNPB below its solubility limit is very low (Fig. 1, curve b), but it is dramatically enhanced in the presence of DMPG-SUVs (curve a). This interfacial activation of TlL depends on the quality of the diluent interface, since large anionic vesicles, around 100 nm, of the same composition, do not support the activation. Also, zwitterionic DMPC vesicles did not have any effect on the rate of hydrolysis of PNPB (same as curve b). Similar results were obtained for TlLW89 and TlLW89L mutants (results not shown), although the rates were lower as expected for the Trp-mutants [22,23].

3.2. Effect of temperature on the activity of TlL

To determine the effect of TlL mutants on lipid dynamics of DMPG and DMPC vesicles below and above the T_m, samples were submitted to temperatures ranging from 15 to 42 °C. The effect of temperature on the catalytic activity of TlL was determined using monolayers of 1,2-dicaprin spread at the air/water interface at a constant surface pressure (π) of 17 mN/m. The use of monolayers was preferred over the previously described assay with PNPB substrate incorporated on DMPG-SUVs, in order to investigate the effect of the temperature on enzyme activity without the influence of lipid order and gel-to-liquid crystal phase transition. The substrate diglyceride 1,2-dicaprin has

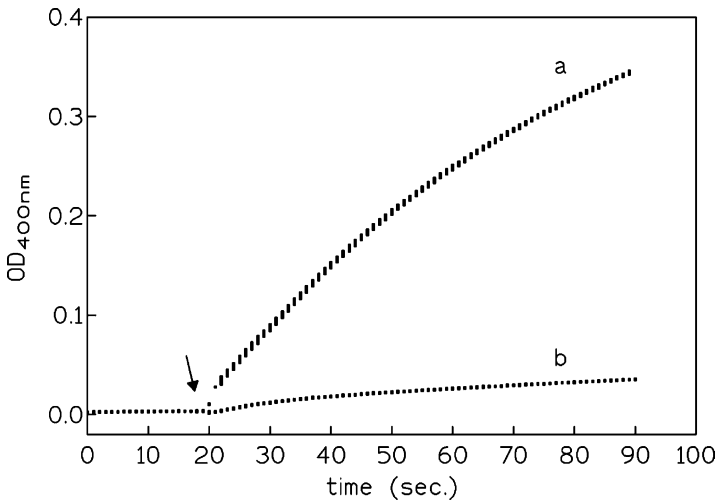

Fig. 1. Reaction progress curves monitored as change in optical density at 400 nm, for the hydrolysis of 18.7 μM PNPB in 10 mM Tris, pH 8.0 by 4 pmol of TlL, in the presence of 54 μM DMPG-SUVs (a), or in the presence of 54 μM DMPC-SUVs or DMPG-LUVs (b). Curves in the absence of vesicles were the same as (b). Vesicles were added first, followed by substrate and reaction was then initiated by addition of enzyme (indicated by an arrow).

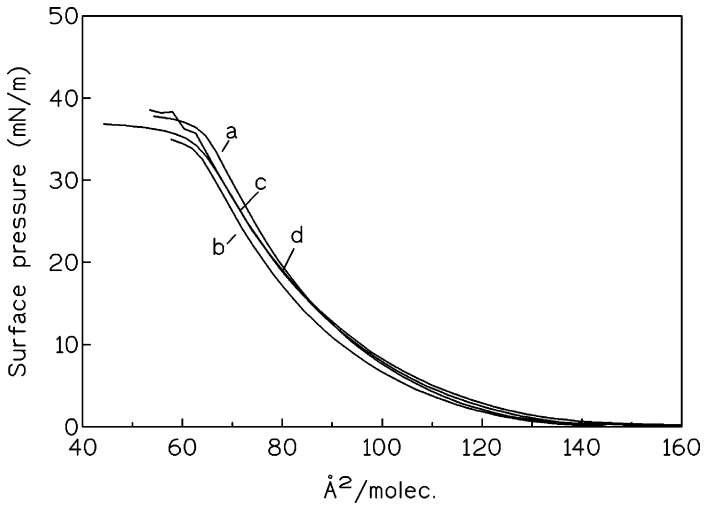

Fig. 2. Isotherms of surface pressure vs. mean molecular area for monolayers of 1,2-dicaprin spread at the air/water interface at different temperatures: (a) 20 °C, (b) 28 °C, (c) 36 °C, and (d) 40 °C. Subphase of 10 mM Tris at pH 8.0.

low melting temperature and it is in the liquid state at all the temperatures used in this work. To determine if the temperature had any effect on lipid organization, compression isotherms of 1,2-dicaprin were obtained at different temperatures. Representative pressure/area curves obtained at different temperatures are shown in Fig. 2, and are not significantly different. From the isotherms, the compressibility modulus (C_s^{-1}) was calculated as

$$C_s^{-1} = -A\left(\frac{\partial \pi}{\partial A}\right)_T$$

At 17 mN/m, C_s^{-1} was between 50 and 60 mN/m independently of the temperature, which corresponds to a liquid phase according to Davies and Rideal [29]. As shown in Fig. 3, the catalytic activities of TlLW89

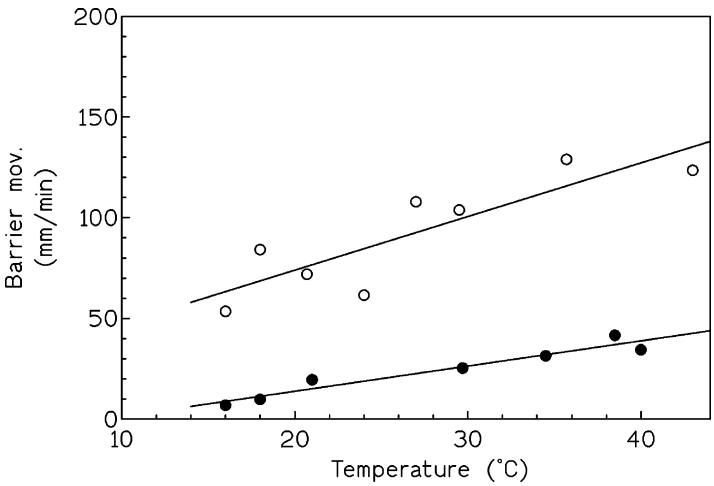

Fig. 3. Effect of temperature on the rates of hydrolysis at the steady-state for TlLW89 (open circles) and TlLW89L (closed circles), for the hydrolysis of 1,2-dicaprin in monolayers at constant surface pressure of 17 mN/m. Data is obtained from reaction progress curves in a zero-order trough.

and TlLW89L increased linearly with temperature. Activities were lower for the TlLW89L mutant, as expected because Trp89 in the α-helical lid is important for enzyme activity [23,24]. This behavior is not surprising, since it is anticipated that upon increasing the temperature, the rotational freedom of the protein residues will increase as the protein chain is stretched out due to thermal excitation, and this may favor lid opening. The thermal stability of TlL and mutants has been described [30], and differential scanning calorimetry reveals that unfolding of any of these enzymes does not occur below 62 °C. In that work, Zhu et al. [30] demonstrated that TlL and mutants become less compact with increasing temperature, and suggested that the amplitudes of conformational fluctuations increased. This was interpreted as an increase in the fraction of enzyme molecules in a catalytically active conformation below the unfolding temperature.

3.3. Binding of TlL mutants to DMPG and DMPC vesicles

TlL binds with high affinity to vesicles of POPG and POPC, as it has been demonstrated by tryptophan fluorescence and RET experiments [22]. Enzyme binding to DMPG and DMPC vesicles was also demonstrated by RET, using Trp(s) in the enzyme as fluorescence donors, and NBD groups in the vesicles as acceptors. TlL has four Trp residues, and the mutants have three Trp (TlLW89L) or one Trp (iTlLW89), thus they are suitable for RET studies. Binding of iTlLW89 to DMPG-SUVs is shown in Fig. 4; Trp fluorescence of the lipase decreased upon binding to DMPG-SUVs labeled with 2% NBD-PE (top graph in Fig. 4). Subsequent addition of excess unlabeled DMPC-vesicles did not result in any additional change on RET, indicating that the enzyme binds strongly to the anionic interface and does not exchange. Addition of labeled DMPC-SUVs to the lipase also resulted in a decrease in Trp fluorescence, although of less magnitude (lower curve in Fig. 4). This is expected because binding to zwitterionic interfaces involves another domain of the enzyme with no penetration of the lid in the membrane [22], therefore the RET efficiency is expected to be lower. Addition of excess DMPG vesicles did not modify the RET efficiency, thus suggesting that the bound enzyme did not leave the PC vesicles. Similar results were obtained with the iTlL mutant (not shown).

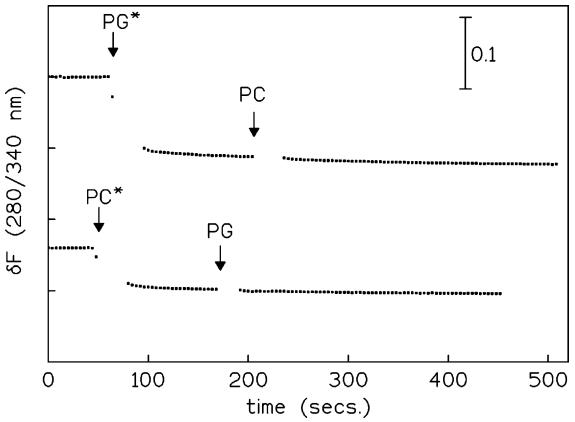

Fig. 4. Binding of iTlLW89 to SUV vesicles determined by RET. Tryptophan fluorescence was continuously monitored (excitation 280 nm, emission 340 nm). Enzyme was diluted in the cuvette with 10 mM Tris, pH = 8.0–2.47 μM, and when a stable reading was achieved, vesicles labeled with 2% NBD-PE were added (0.4 mM lipid). After a stable reading was again achieved, a second aliquot of unlabeled vesicles was added (0.8 mM lipid). Lipid composition as indicated, PG = DMPG-SUVs, PC = DMPC-SUVs, vesicles with NBD-PE indicated with an asterisk.

3.4. Effect of TlL mutants on membrane lipid order

Results described so far suggest that the physical properties of the non-substrate phospholipid interface play a fundamental role on the activity of TlL. As a consequence, it is of interest to determine the effect of the bound enzyme on the mobility and the phase transition of the lipids. Three different fluorescent probes, DPH, TMA-DPH, and NBD-PE, were incorporated in the membrane of the liposomes in order to dissect the effect of the enzymes on the dynamics of the lipids at different regions of the bilayer. DPH is a hydrophobic molecule that is oriented predominantly parallel to the fatty acid chains, and the distance of the shallow end of the group to the bilayer center in PC vesicles is around 13 Å [31]. It reports on the order of the lipid fatty acyl chains in the core region of the bilayer. TMA-DPH is anchored at the water/lipid interface, because of its additional charged trimethylammonium group [32], and the distance of the shallow end of the DPH group to the center of PC bilayers is 16.5 Å. Finally, NBD-PE is an indicator of the mobility of the lipid headgroup region

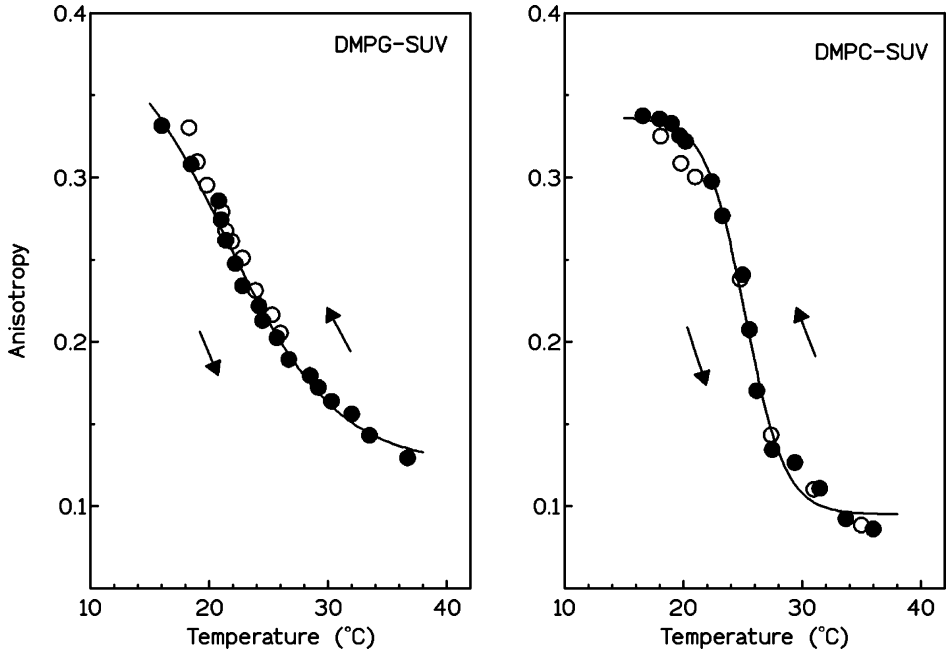

Fig. 5. Temperature dependence of the steady-state fluorescence anisotropy of DPH-labeled SUVs of DMPG (left) or DMPC (right). Vesicles (133 μM) were cooled in 10 mM Tris at pH 8.0 to the starting temperature, and then anisotropies were measured at the desired temperatures (closed circles). After the heating cycle was complete, samples were cooled to the starting temperature (open circles). Excitation 365 nm, emission 425 nm. The data are representative of at least three independent experiments (±0.002).

of the membranes [33]. Since fluorescence anisotropy is highly dependent upon the viscosity of the solution in which the fluorophore is dissolved, it can be used to obtain information on the microviscosity of lipid membranes where the fluorophore partitions.

The ordering of the lipid acyl chains in DMPG-SUVs was affected by TlL mutants. The effect of lipase binding on the dynamics of the fatty acyl chains in the hydrophobic core region of the bilayers, reported by the DPH probe, is shown in Figs. 5 and 6. DPH is the most widely used probe for estimating membrane microviscosity. It is a symmetric molecule, and probably has isotropic depolarizing rotations, since the rapid rotations along its long axis are not depolarizing [34]. The phase transitions of membranes are dramatically revealed by DPH, in such a way that it is possible to accurately estimate the temperature of the phase transition (T_m). As shown in Fig. 5 for DMPG and DMPC vesicles, anisotropy is high in the gel state of the lipid. For example, $r = 0.33$ at 16 °C, a value that is close to the anisotropy of DPH in a vitrified dilute solution (r_0) [34], thus suggesting a highly restricted rotation for the probe in these environments. Increasing the temperature resulted in a decrease of anisotropy, due to rotational depolarization as the microviscosity of the membranes decreases. When the gel-to-liquid crystal transition begins, the anisotropy decreases dramatically, and the T_m can be estimated as the inflexion point of the anisotropy versus $\log T$ curves, as described elsewhere [35]. With this method, a T_m of 23 ± 1 °C was calculated for the DMPG and DMPC vesicles, in good agreement with previously reported values for these lipids obtained by DSC [36]. After a heating cycle, samples were cooled to the initial temperature (Fig. 5), and r values were approximately the same (no hysteresis in the liquid crystal-to-gel transition). All three TlL mutants under study inserted between the lipid molecules at the hydrophobic core of the DMPG-SUVs, increasing DPH anisotropy values (Fig. 6, left). iTlL, with the four Trp(s) of the wild type enzyme, had the smallest ordering effect, and the mutants that contained less Trp residues had a more pronounced influence in lipid motion, strongly diminishing acyl chain fluidity.

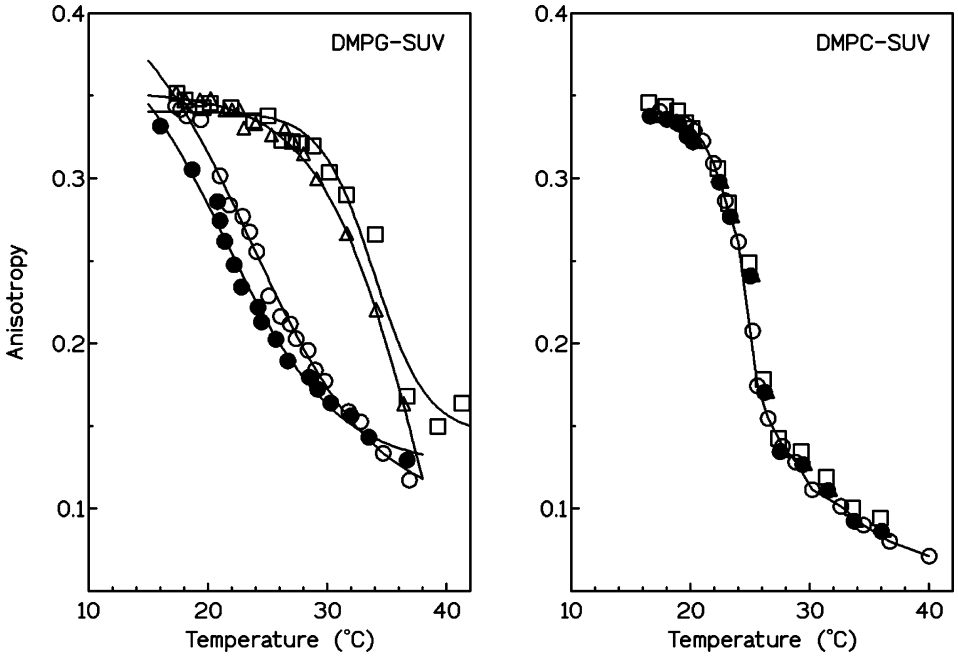

Fig. 6. Temperature dependence of the steady-state fluorescence anisotropy of DPH-labeled SUVs of DMPG (left) or DMPC (right). Vesicles alone (●); vesicles in the presence of: iTlL (○), iTlLW89 (△), TlLW89L (□). Lipid concentration 133 μM; enzyme concentration 2.47 μM; other conditions as in Fig. 5.

For example in the gel state (16 °C), anisotropy was $r = 0.332$ for pure DMPG, compared to $r = 0.344$ for DMPG/iTlL, and $r = 0.353$ for DMPG/TlLW89L and DMPG/iTlLW89. The effect of the enzymes on lipid dynamics were higher in the liquid crystal state, for example at 37 °C the anisotropy of DMPG was $r = 0.117$, and increased a 10% for iTlL, compared to approximately 40% increase for the two tryptophan mutants. The gel-to-liquid crystal phase transition temperatures (T_m), calculated from the logarithmic plots, also increased significantly in the presence of TlL mutants. The inactive enzyme induced an increase of more than 2 °C, from 23.0 to 25.3 °C, whereas the Trp-mutants induced increases of 11.0 °C. All the depolarization curves (heating cycles) were reproducible in the cooling cycles (not shown for clarity), indicating that the enzyme is tightly bound to the interface. The ordering effect was depending on the enzyme concentration, as shown in Fig. 7 for iTlL, both below and above the T_m. Binding to zwitterionic DMPC-SUVs had no effect on lipid mobility at the hydrophobic core of the bilayers, as also shown in Fig. 6 (right) indicating that the enzyme does not penetrate at this level of the membrane. Results with 100 nm LUVs are summarized in Fig. 8. Binding to anionic DMPG-LUVs did not affect lipid dynamics at the core region of the bilayer, and the same was true for DMPC-LUVs. It is worth noting that LUVs, as well as the zwitterionic SUVs, do not trigger formation of the active or open lipase form, as demonstrated by kinetic experiments (Fig. 1).

The probe that reports on the anisotropy closest to the glycerol backbone region (TMA-DPH) also showed a lipase-induced decrease in chain mobility in the DMPG-SUV vesicles (Fig. 9, left). This increase in chain order was induced both in the gel and in the liquid crystal states of the lipid. All three TlL mutants studied had the same ordering effect in this region of the membrane. Experiments at a fixed temperature showed that the rigidifying effect depended on the enzyme concentration, as shown in Fig. 10 for iTlL. In contrast, binding of TlL mutants to DMPC vesicles occurred without modification of the TMA-DPH anisotropy (Fig. 9, right), thus supporting the conclusion that the enzyme molecules did not insert between the lipid acyl chains.

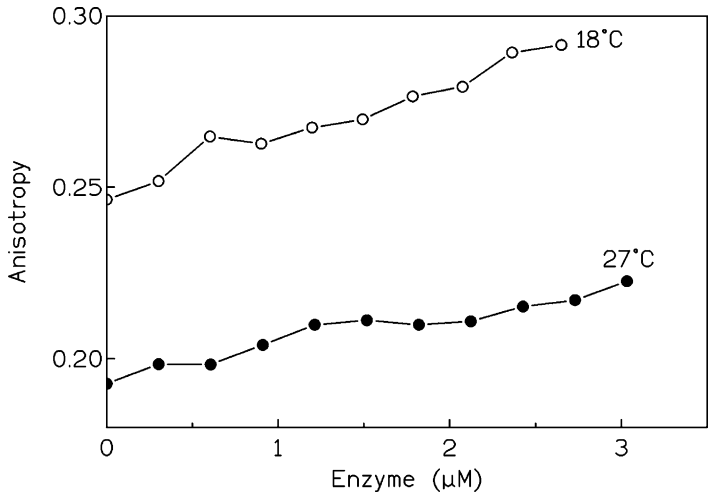

Fig. 7. Steady-state fluorescence anisotropy of DPH in DMPG-SUVs (133 μM) as a function of the concentration of iTlL, at constant temperature: below (18 °C) and above (27 °C) the T_m for the lipid.

As shown in Fig. 11 (left), TlL mutants decreased the mobility at the headgroup region of DMPG-SUVs, both below and above the T_m (23 °C), with a significant increase of the NBD anisotropy. The inactive mutant iTlL, with four Trp residues, showed the highest rigidifying effect, followed by TlLW89L, where the Trp89 that is on the lid region of the native enzyme has been mutated. Finally, iTlLW89 mutant, with only

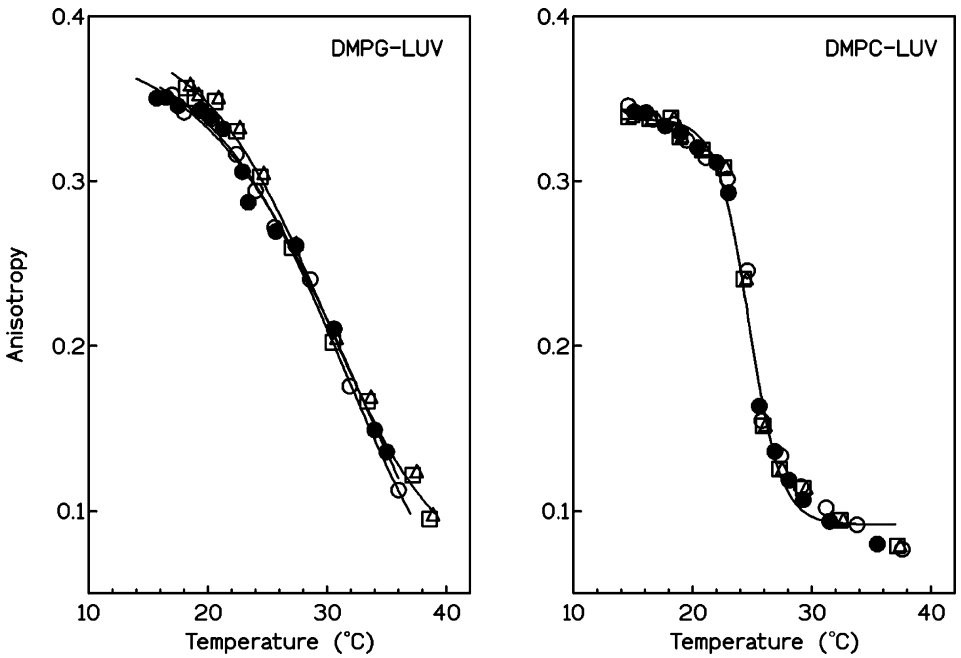

Fig. 8. Temperature dependence of the steady-state fluorescence anisotropy of DPH-labeled LUVs of DMPG (left) or DMPC (right). Vesicles alone (●); vesicles in the presence of: iTlL (○), iTlLW89 (△), TlLW89L (□). Lipid concentration 133 μM; enzyme concentration 2.47 μM; other conditions as in Fig. 5.

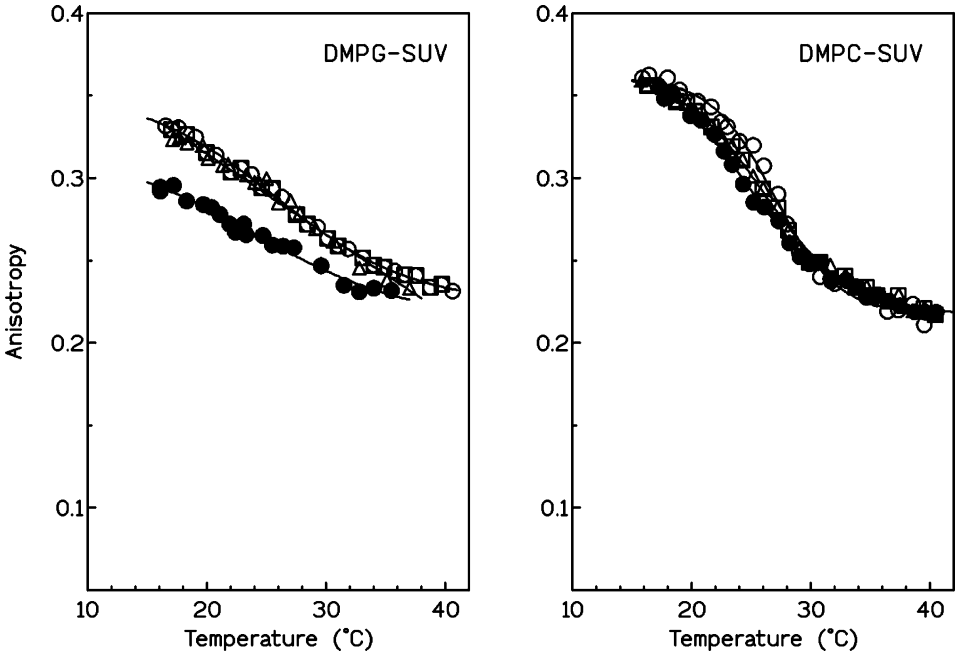

Fig. 9. Temperature dependence of the steady-state fluorescence anisotropy of TMA-DPH-labeled SUVs of DMPG (left) or DMPC (right). Vesicles alone (●); vesicles in the presence of: iTlL (○), iTlLW89 (△), TlLW89L (□). Lipid concentration 133 μM; enzyme concentration 2.47 μM; other conditions as in Fig. 5.

the Trp on the lid, had the smallest effect in lipid microviscosity. These results suggest that TlL strongly interacts with the headgroup region of DMPG-SUVs, decreasing lipid mobility, and that modification of any of the Trp residues reduces the interaction. Cooling cycles, shown only for pure vesicles in Fig. 11, did not have significant hysteresis, even for the active mutant TlLW89L (not shown), indicating that the enzymes did

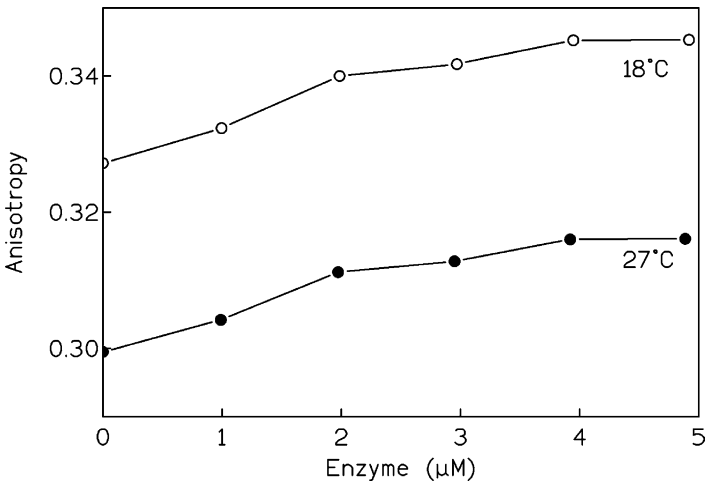

Fig. 10. Steady-state fluorescence anisotropy of TMA-DPH in DMPG-SUVs (133 μM) as a function of the concentration of iTlL, at constant temperature: below (18 °C) and above (27 °C) the T_m for the lipid.

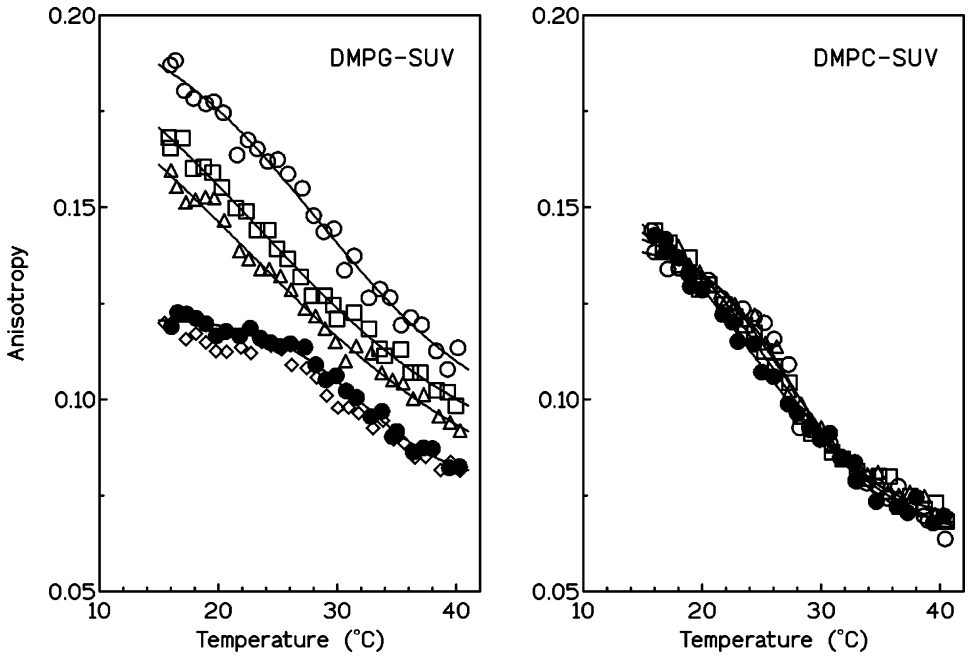

Fig. 11. Temperature dependence of the steady-state fluorescence anisotropy of NBD-labeled SUVs of DMPG (left) or DMPC (right). Vesicles alone (●); vesicles in the presence of: iTlL (○), iTlLW89 (△), TlLW89L (□). Lipid concentration 133 μM; enzyme concentration 2.47 μM; all curves correspond to heating cycles except (◇) cooling cycle of DMPG vesicles alone. Excitation 460 nm, emission 534 nm.

not alter the chemical structure of the lipids during the time of the experiments. The absence of hysteresis is also a clear indication that the bound enzyme did not leave the interface when the lipids adopt the less compact state, above the T_m. Control experiments with TlL wild type rendered a heating cycle comparable to that of the inactive mutant iTlL, but an important hysteresis was observed in the cooling cycle, so the use of inactive mutants was preferred when available. Binding to zwitterionic DMPC vesicles did not affect significantly the dynamics of the lipid headgroups for any of the mutants (Fig. 11, right), indicating that binding occurs without insertion of the enzyme.

Finally, it is also interesting to note that although both DMPG and DMPC vesicles displayed a cooperative transition, the anisotropy amplitude, and hence the transition enthalpy value, was smaller for DMPG-SUVs, whereas the same amplitudes were obtained for the thermal transitions of DMPC-SUVs or LUVs, and for DMPG-LUVs (Figs. 5 and 8). This was true for all three probes used, but especially for the interfacial and headgroup labels (TMA-DPH and NBD-PE) (Figs. 9 and 11), and suggests that the highly curved surface or the small vesicles together with the anionic charges of the phospholipid headgroups define a particular type of interface, probably due to an increase in hydrophobic interactions between the acyl chains.

4. Conclusions

Kinetic experiments using PNPB below its critical micellar concentration as substrate, partitioned in DMPG-SUVs (40 nm unilamellar vesicles), show that the catalytic activities of TlL wild type and the Trp-mutants TlLW89 and TlLW89L increase dramatically compared to the same concentration of substrate in the absence of vesicles. This interfacial activation of the fungal lipase has also been described in anionic SUVs of different compositions [20–22], and is attributed to the opening of the lid that covers the active site of TlL in solution, in what is called the open or active conformation of the lipase. In this conformation,

TlL modifies membrane lipid dynamics, as revealed by anisotropy of the membrane probes DPH, TMA-DPH, and NBD-PE. Experiments with iTlL, the inactive mutant of TlL that maintains the four Trp residues of the wild type enzyme, show that the open form of the enzyme has a rigidifying effect on the DMPG-SUV interface, and that the increase in lipid order is more important in the headgroup and water/lipid interfacial regions than at the hydrophobic core. The decrease in fluidity occurs both in the gel and the liquid crystal states of the lipid, from 15 to 42 °C, and it is probably due to the insertion of the amphipathic lid in the membrane, close to the anionic headgroups of PG. In the small vesicles used in these experiments, the lipid headgroups are spread apart due to the strong positive curvature, thus exposing more of the hydrophobic carbon chains [37] and favoring hydrophobic interactions with the lid. Lid insertion will ameliorate curvature stress, as also described for the glycosilated hydroquinone arbutin inserted in PC vesicles containing cone shaped lipids [38]. Our results are consistent with the current hypothesis that electrostatic interactions also play a major role in lid opening, probably involving cationic residues in both hinge regions of the lid (e.g. Arg84, Arg81, Lys98). These residues can act as lockers that stabilize the open structure by electrostatic attraction to the anionic PG groups [17,20,22]. This is supported by recent X-ray experiments where crystals of TlL in the closed and open form have been obtained simultaneously, under exactly the same physicochemical conditions, and that clearly show the role of Arg84 in stabilizing the active conformation of TlL by a mechanism called “arginine switch” [17]. Mutation of Trp89, located in the lid, or of the structural residues, Trp117, Trp221, and Trp260, results in a decrease of catalytic activity and a deeper insertion in the DMPG-SUV interface, with important reductions of lipid dynamics at the hydrophobic core and increases of the T_m up to 11 °C. The hypothesis that a combination of electrostatic and hydrophobic interactions triggers lipase activation at the lipid interface is supported by the fact that interfacial activation does not occur in zwitterionic PC-vesicles, due to the lack of the necessary anionic charge, or in large vesicles (LUVs of 100 nm), where the smaller curvature does not allow exposure of the hydrophobic acyl chains. In these vesicles, TlL binds in a closed or inactive form, without insertion of the lid, and thus without modification of lipid dynamics or phase transition. In conclusion, the data presented in this paper are strong evidence that interfacial activation of TlL not only affects the conformation of the enzyme, but also influences the physical properties of the neutral diluent interface, providing therefore more insight into the understanding of the structural interplay occurring on the lipid–enzyme interface.

Acknowledgements

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