Interfacial Control of Lid Opening in *Thermomyces lanuginosa* Lipase†

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Received August 18, 1999; Revised Manuscript Received October 28, 1999

ABSTRACT: Small unilamelar vesicles of anionic phospholipids (SUV), such as 1-palmitoyl-2-oleoylglycero-*sn*-3-phosphoglycerol (POPG), provide an interface where *Thermomyces lanuginosa* triglyceride lipase (TlL) binds and adopts a catalytically active conformation for the hydrolysis of substrate partitioned in the interface, such as tributyrin or *p*-nitrophenylbutyrate, with an increase in catalytic rate of more than 100-fold for the same concentration of substrate [Berg et al. (1998) *Biochemistry 37,* 6615−6627.]. This interfacial activation is not seen with large unilamelar vesicles (LUV) of the same composition, or with vesicles of zwitterionic phospholipids such as 1-palmitoyl-2-oleoylglycero-*sn*-3-phosphocholine (POPC), independently of the vesicle size. Tryptophan fluorescence experiments show that lipase binds to all those types of vesicles with similar affinity, but it adopts different forms that can be correlated with the enzyme catalytic activity. The spectral change on binding to anionic SUV corresponds to the catalytically active, or "open" form of the enzyme, and it is not modified in the presence of substrate partitioned in the vesicles, as demonstrated with inactive mutants. This indicates that the displacement of the lid characteristic of lipase interfacial activation is induced by the anionic phospholipid interface without blocking the accessibility of the active site to the substrate. Experiments with a mutant containing only Trp89 in the lid show that most of the spectral changes on binding to POPG-SUVs take place in the lid region that covers the active site; an increase in Trp anisotropy indicates that the lid becomes less flexible in the active form, and quenching experiments show that it is significantly buried from the aqueous phase. On the other hand, results with a mutant where Trp89 is changed to Leu show that the environment of the structural tryptophans in positions 117, 221, and 260 is somehow altered on binding, although their mobility and solvent accessibility remains the same as in the inactive form in solution. The form of TlL bound to POPC-SUV or -LUV vesicles as well as to LUV vesicles of POPG has the same spectral signatures and corresponds to an inactive or "closed" form of the enzyme. In these interfaces, the lid is highly flexible, and Trp89 remains accessible to solvent. Resonance energy transfer experiments show that the orientation of TlL in the interface is different in the active and inactive forms. A model of interaction consistent with these data and the available X-ray structures is proposed. This is a unique system where the composition and physical properties of the lipid interface control the enzyme activity.

Lipases (triacylglyceride esther hydrolase, EC 3.1.1.3) are enzymes that hydrolyze triglycerides at a lipid−water interface and have interesting industrial and medical applications (*1*−*3*). They differ from classic esterases in that their activity increases dramatically upon binding to the lipid surface formed by their water-insoluble substrates (*4*). This phenomena of interfacial activation is observed in lipases of different origin, including microbial lipases, and was attributed to a conformational change in the enzyme leading to an increase in activity (*5*), as supported by X-ray crystallography studies. The three-dimensional structures of several lipases have been reported (*6*−*11*), some of them complexed with inhibitors or cocrystallized with micelles (*12*−*16*). All lipases have in common the $\alpha-\beta$ hydrolase fold (*17*) and a catalytic triad composed of a nucleophilic serine activated by a hydrogen bond in relay with histidine and aspartate or glutamate (*6*, *7*). In addition, the crystal structures of many uncomplexed lipases had the active site covered by a helical surface loop or "lid" that renders it inaccessible to substrate. This is referred to as closed conformation and corresponds to the lipase in aqueous solution, in the absence of an interface or organic solvent, and has a very low lipolytic activity. On the other hand, crystal structures of lipases complexed with inhibitors show a large rearrangement of the lid, rendering the active site accessible for substrate binding, in what is assumed to be the open conformation of the enzyme at the lipid interface. In this conformation, lid movement not only opens access to the active site, but in addition it exposes a large hydrophobic patch at the same time that a previously exposed hydrophilic domain becomes buried in the protein (*18*, *19*). Although no direct structural data exists to show that activation of the enzyme at the lipid−water interface occurs by the same conformational rearrangement, biophysical data indicates that lipase adsorbs to the interface by the hydrophobic domain around the active site, and that the am-

† Financial support from the E. C. (BIO4-97-2365) is gratefully acknowledged. Y.C. is supported by the Ministry for Science of Spain.

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10.1021/bi991927i CCC: $19.00
Published on Web 12/14/1999

phiphilic lid interacts with both the lipid interface and the surface of the enzyme molecule (*20*, *21*). Fungal lipase from *Thermomyces lanuginosa* (TlL)[1] hydrolyzes substrate partitioned in the interface of anionic sonicated vesicles, such as POPG vesicles, at a rate that is severalfold higher than that for monodispersed substrate at the same concentration (*22*). In this paper, we show that the interfacial activation of TlL takes place only in small anionic unilamelar vesicles with a diameter around 40 nm, but not in larger vesicles of the same composition. Also, no activation is seen with zwitterionic vesicles, such as POPC or DMPC. To explain these differences, the spectroscopic properties of TlL native lipase and several mutants, both in solution and bound to the different lipid interfaces, are studied. TlL has four tryptophan residues in positions 89, 117, 221, and 260 of the chain. Residues Trp117, Trp221, and Trp260 are located in regions not directly involved in the interfacial activation process, whereas Trp89 is common to other pancreatic and fungal lipases and is located in the central part of the lid (*23*). 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 (*13*) and is important for enzyme activity (*24*). By the use of tryptophan fluorescence, anisotropy, and resonance energy transfer techniques, we study the binding of TlL to the different interfaces. Mutants containing only Trp89, or only the structural tryptophans 117, 221, and 260, allow to dissect the contribution of the lid region or other regions of the enzyme on binding, and results are compared to the native enzyme. By the use of inactive mutants, the effect of substrate partitioned in the lipid interface can be studied without interference from product formation. Results show that TlL binds to POPC and POPG vesicles in different forms that have different activity. In the active form obtained in POPG-SUVs, the lid becomes buried in the membrane and loses its motional freedom, leaving the entrance of the active site free for the binding and hydrolysis of substrate partitioned at the lipid interface. In POPC vesicles and also in large anionic vesicles (POPG-LUV), lipase binds in a different form or with a different orientation, which is inactive and where the lid remains highly flexible and accessible from the aqueous phase. RET experiments show that binding to the interface takes place through a different side of the enzyme. Results suggest that the open lid conformation of lipase is achieved by a combination of electrostatic stabilization of the lid and hydrophobic interactions with a highly curved anionic interface.

MATERIALS AND METHODS

Enzymes used were TlL wild-type, and mutants: iTlL, in which the catalytic Ser146 is changed to Ala; TlLw89, with mutations Trp117-Phe, Trp221-His, and Trp260-His; iTlLw89, with mutations Ser146-Ala, Trp117-Phe, Trp221-His, and Trp260-His; TlLw89l, with Trp89-Leu. All enzymes were obtained from Novo Nordisk A/S. The gene from *T. lanuginosa* encoding TlL was cloned, sequenced, and expressed as described elsewhere (*25*). Site-directed mutagenesis in the lipase expression plasmid and purification of the produced wild-type and variants were conducted as described (*1*). Protein concentration was determined spectrophotometrically at 280 nm; for TlL and iTlL, a molar extinction coefficient of 43 000 M^{-1} cm^{-1} with M_W 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).

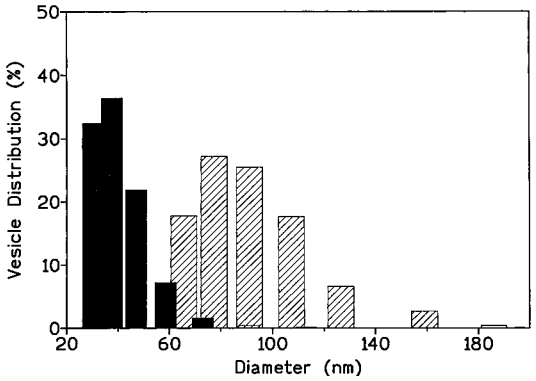

FIGURE 1: Vesicle size distribution measured by dynamic light scattering. (Full)POPG-SUV vesicles; (Diagonal lines) POPG-LUV vesicles.

PNPB, PNPL, and TB were from Sigma. DPPC, DPPG, NBD-PE, POPC, and POPG were from Avanti Polar Lipids (Alabaster, AL); DPH and TMA-DPH were purchased from Molecular Probes (Eugene, OR). Dithionite (sodium hypodisulfite) was from Fluka.

Vesicle Preparation and Characterization. Small unilamelar vesicles (SUVs) of POPG, POPC, DPPG, or DPPC, alone or with substrate PNPB or PNPL, or with the fluorescent probes NBD-PE, DPH, or TMA-DPH, were prepared by evaporation of a mixture of the lipids in $CHCl_3$/CH_3OH (2:1 v/v), except PNPL and PNPB that were added from stock solutions in tetrahydrofuran. The dried film was hydrated for a 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 (LUVs) of the same composition, the hydrated lipid film was submitted to five freeze−thaw cycles to ensure homogeneous mixture of the multilamellar vesicle suspensions (MLVs). The MLVs were extruded 8 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. In this paper, we show that both the charge and the size of the vesicles are important for the control of the lipase activity, thus the parameters are controlled for the different vesicle preparations. Vesicle size was measured by dynamic light scattering with a Malvern II-C autosizer. As shown in Figure 1, SUV vesicles have a mean diameter of 40 nm, and a narrow size distribution (polydispersity < 0.1), whereas LUVs mean diameter is 105

[1] Abbreviations: TlL, *Thermomyces lanuginosa* lipase; TlLw89, mutant with only Trp89; iTlL, inactive mutant (Ser146Ala); iTlLw89, inactive mutant with only Trp89; TlLw89l, mutant with Trp89 mutated to Leu; DMPM, 1,2-dimiristoyl-*sn*-3-phosphomethanol; DPH, 1,6-diphenylhexa-1,3,5-triene; DPPC, 1,2-dipalmitoylglycero-*sn*-3-phosphocholine; DPPG, 1,2-dipalmitoylglycero-*sn*-3-phosphoglycerol; K_{SV}, Stern−Volmer quenching constant; LUV, large unilamelar vesicles; NBD-PE, *N*-(7-nitro-2-1,3-benzoxadiazol-4-yl) dioleoylphosphatidylethanolamine; PNPB, *p*-nitrophenyl-butyrate; PNPL, *p*-nitrophenyl-laurate; POPC, 1-palmitoyl-2-oleoylglycero-*sn*-3-phosphocholine; POPG, 1-palmitoyl-2-oleoylglycero-*sn*-3-phosphoglycerol; RET, resonance energy transfer; SUV, small unilamelar vesicles; TB, tributyrin; TMA-DPH, 1-[4-(trimethylammonium) phenyl]-6-phenyl-1,3,5-hexatriene *p*-toluenesulfonate.

nm. The percentage of lipid present in the outer monolayer of the vesicles was calculated by the dithionite method (*27*, *28*) with some modifications (*29*). Briefly, an aliquot of vesicles (SUVs or LUVs) of POPC or POPG containing 0.6% NBD-PE was added to a cuvette containing 1.5 mL of 10 mM Tris, pH 8.0, saturated with nitrogen, with constant stirring (lipid concentration 110 μM). When a stable baseline was obtained, the reaction was initiated by adding dithionite from a stock solution to a final concentration of 10 mM, and NBD-fluorescence was monitored with time (excitation 460 nm, emission 535 nm). Dithionite selectively reduces the NBD groups present in the outer monolayer of the vesicles, eliminating the fluorescence signal. Stock solutions of dithionite were freshly prepared in 0.5 M Na_2CO_3 buffer, pH 11, saturated with nitrogen, and stored in ice for a maximum of 1 h. Total loss of fluorescence was achieved by addition of deoxycholate. The percentage of lipid present in the outer monolayer was calculated as described elsewhere (*30*, *31*) and was in the range of 62−68% for sonicated vesicles (SUV), and 48−50% for extruded vesicles (LUV); both values are expected for unilamelar vesicles of 40 and 100 nm diameter, respectively.

Kinetic Protocols. The kinetics of hydrolysis of PNPB or PNPL 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 (*22*). 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. Vesicles of POPG or POPC were added to the cuvette followed by PNPB from a stock solution in tetrahydrofuran, and the reaction was initiated by addition of the lipase and gently mixing of the cuvette. Also in some cases, vesicles were prepared containing 19% of PNPB or 10% PNPL incorporated in the lipid film. In the experiments done without vesicles, special care was taken in the stirring of the samples, since vigorous stirring creates bubbles that provide an air−water interface for the partition of substrate and enzyme, as described in detail (*22*). Kinetic measurements by the pH−stat protocol were carried out only in the presence of excess diluent vesicles, using TB as substrate in a 718 Stat Titrino unit (Metrohm, Switzerland). The reaction mixture containing 50 μM vesicles and 37 μM TB in 4 mL of 50 mM NaCl solution was equilibrated at pH 8.0 in a stream of nitrogen, and the reaction was initiated by adding the enzyme from a stock solution.

Tryptophan Fluorescence and Quenching Experiments. Fluorescence measurements were carried out in 10 mM Tris at pH 8.0 and 25 °C on an AB-2 spectrofluorimeter (SLM-Aminco) with constant stirring. Tryptophan fluorescence spectra were recorded with an excitation wavelength of 280 nm over an emission range of 295−450 nm, with 4 nm slit widths. The enzyme was added from a stock solution in water to a final concentration of 2.47 μM and titrated with POPG or POPC vesicles, either SUVs or LUVs, and TB was added at the end. The sensitivity (PMT voltage) was adjusted to 1% for the Raman peak from the buffer blank at the same excitation wavelength. Spectra of vesicles alone at the same lipid concentrations were obtained and subtracted, although the contribution from scattering due to the vesicles was always less than 3%. Quenching of tryptophan fluorescence of the enzymes by iodide and acrylamide was recorded at 320 nm (excitation 280 nm). Appropriate amounts of POPG or POPC vesicles were added to a solution of 1.1 μM enzyme and aliquots of quencher were added with continuous stirring. Acrylamide was added in increasing amounts from a 3.3 M stock solution in water, and potassium iodide was added from a 2 M stock solution containing 0.25 mM $Na_2S_2O_3$ to avoid I_3^- formation. Final quencher concentration ranged from 0 to 350 mM. Quenching results were analyzed according to Stern−Volmer equation for collisional quenching:

$$F_o/F = 1 + K_{SV}[Q]$$

where F_o and F are the fluorescence intensities in the absence and presence of quencher, [Q] is the molar concentration of quencher, and K_{SV} is the Stern−Volmer quenching constant. At this range of concentrations, there is no deviation from linearity ($r^2 = 0.99$).

Binding Stoichiometry. Binding of lipase enzymes to POPG and POPC vesicles containing 2.5% of NBD-PE was determined as the increase of the resonance energy transfer (RET) signal from Trp residues in the enzyme to the labeled phospholipid in the interface at 535 nm (excitation 285 nm). Vesicles in buffer (26.7 μM lipid) were titrated with enzyme from a stock solution 23.2 μM in water, and stoichiometry was determined directly from the plot of δF vs lipid−enzyme (mol:mol) as the cross-point between the line formed by the points at low-enzyme concentration, and the line defined by the points at high-enzyme concentration where the RET signal reaches saturation. The relative change in fluorescence, δF, is defined as $(F-F_o)/F_o$ where F_o and F are the intensities without and with enzyme, respectively. Since the lipid concentration was very low, the contribution from light scattering was negligible.

Resonance Energy Transfer Experiments. A solution 2.5 μM of lipase in 10 mM Tris at pH 8.0 was titrated with aliquots of POPG or POPC vesicles labeled with 2.5% of the fluorescent probe TMA-DPH or DPH. Excitation wavelength was at 280 nm and fluorescence emission was measured with excitation and emission slit widths of 4 nm each. The relative decrease in fluorescence emission intensity of the enzyme (δF) at 340 nm was plotted as a function of lipid concentration. These experiments give information on the depth of penetration in the membrane of different Trp residues of the enzyme.

Fluorescence Anisotropy. Steady-state tryptophan fluorescence anisotropy measurements were carried out on an AB-2 spectrofluorimeter (SLM-Aminco), with L-format fluorescence polarizers. Excitation wavelength was set at 285 nm, and the emission at 340 nm with excitation and emission slit widths at 4 nm. Titrations were carried out at 25 °C, adding aliquots of POPG or POPC vesicles and/or TB to a solution 2.47 μM of lipase in 10 mM Tris pH 8.0. All solutions were stirred continuously during the measurements. The fluorescence anisotropy (r) was calculated automatically by the software provided with the instrument, according to

$$r = (I_{Vv} - I_{Vh})/(I_{Vv} + 2I_{Vh})$$

where I_{Vv} and I_{Vh} are the intensity of the emitted polarized light with the emission polarizer parallel or perpendicular

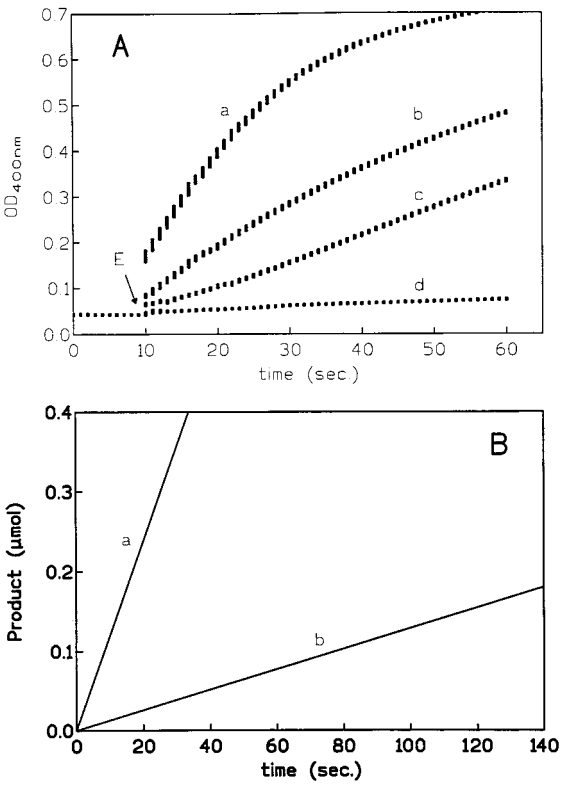

FIGURE 2: (A) Reaction progress curves monitored as change in optical density at 400 nm, for the hydrolysis of 0.312 mM PNPB in 10 mM Tris, pH 8.0, in the presence of 0.054 mM POPG-SUVs by 4 pmol of (*a*) TlL; (*b*) TlLw89; (*c*) TlLw89l; (*d*) TlLw89 without vesicles or with 0.054 mM POPC SUVs, POPG-LUVs, or DPPG-LUVs. (B) Reaction progress monitored by pH stat, for the hydrolysis of TB (0.037 mM) by TlL (7.8 pmol) in the presence of 0.050 mM vesicles of (*a*) POPG-SUVs or (*b*) POPC−SUVs or POPG-LUVs. Reaction was at pH 8.0 in a 4 mL of 50 mM NaCl. In all cases, vesicles were added first, followed by substrate and reaction was then initiated by addition of enzyme.

to the excitation polarizer. Anisotropy values were automatically corrected for dependencies in the detection system (G-factor correction). Changes in anisotropy were represented as $(r - r_o)/r_o$ where r_o and r are the anisotropy values before and after addition of vesicles, respectively. All measurements were done in triplicate.

RESULTS

Catalytic Activity of TlL, TlLw89, and TlLw89l is Enhanced by POPG-SUVs but not by POPG LUVs or by POPC Vesicles. The rate of hydrolysis of PNPB below its solubility limit by TlL is very low (<10 s^{-1}) as expected because this interfacial enzyme is not active in monodispersed substrates. The rate of hydrolysis of PNPB below its solubility limit increases dramatically in the presence of small vesicles of anionic phospholipids, such as POPG, because the vesicles act as a diluent interface to which substrate partitions, and TlL binds in an active form for the catalytic turnover (*22*). The same is true for the TlLw89 mutant, where all Trp residues except Trp89 have been mutated, and the TlLw89l mutant, in which Trp89 is mutated to Leu. In Figure 2A, enzyme activity is shown as OD change; activity using 0.312 mM PNPB (curve d) is very low because the substrate is below the solubility limit, but is greatly enhanced in the presence of POPG-SUVs for all three enzymes (curves a−c), although TlLw89 and TlLw89l mutants are less active than TlL. This effect is also seen with anionic vesicles of different compositions such as DPPG or DMPM (not shown). As also shown in this figure, the same concentration of DPPG or POPG but in the form of vesicles obtained by extrusion through 100 nm filters (LUVs) does not have any effect on the rate of hydrolysis in any of the three enzymes. In addition, unilamelar vesicles of zwitterionic phospholipids, such as POPC or DPPC, either sonicated or extruded, do not have any effect of the rate of hydrolysis of PNPB (same as curve d). Increasing the vesicle concentration does not increase the rate of hydrolysis, except in the case of anionic SUVs. As controls, mutants iTlL and iTlLw89 do not show hydrolysis even in the presence of anionic SUVs, confirming that we are monitoring lipase hydrolysis by the consensus catalytic triad Ser146-Asp201-His258, and not some unspecific hydrolytic activity. PNPB in the aqueous phase is in equilibrium with PNPB partitioned at the lipid interface, with a dissociation constant $K'_s = 2$ mM for POPG-SUV (*22*). The effect of vesicles on the rate of hydrolysis by TlL of long-chain substrate, such as PNPL, incorporated in the lipid film is useful to avoid artifacts related to substrate partitioning in the different vesicles. POPG-SUV vesicles containing 10% PNPL incorporated during vesicle preparation activate TlL-catalyzed hydrolysis, for example the rate increased around 60-fold for 0.4 mM POPG-PNPL vesicles (39 μM PNPL) compared to the same concentration of PNPL alone; also, no activation was observed for POPC-SUV vesicles.

Essentially, the same results are obtained by the pH−stat titration method (Figure 2B), using as substrate 37 μM TB, well below its solubility limit of 0.8 mM. In this assay, the rate of hydrolysis is much higher in the presence of POPG-SUV as compared to POPG-LUV, or POPC-SUVs. However, in the absence of any vesicles rates are irreproducible and significantly higher that the ones obtained by the UV technique, probably due to hydrolysis at interfaces offered by the reaction vessel or the air bubbles formed due to vigorous mechanical stirring.

The possibility that some small percentage of lipid products of hydrolysis or oxidation generated in the process of sonication were responsible for the activating effect was ruled out because extruded vesicles prepared from a sample of sonicated vesicles submitted to several cycles of freeze and thawing did not induce the enzyme activation. Likewise, sonicated vesicles prepared from a sample of extruded vesicles did induce the expected activation.

Spectral Signatures of TlL Active and Inactive Forms are Different. The fluorescence emission spectra of TlL is due to its four Trp residues in positions 89, 117, 221, and 260 of the chain, and it has a maximum at 335 nm upon excitation at 280 nm (Figure 3 curve a), which is in the range of 330−340 nm expected for semi-exposed tryptophans (*31*). TlL and the majority of lipases of different origin bind to lipid interfaces with the side that surrounds the catalytic site directed toward the lipid, and the catalytically competent, open conformations, show a distinctive characteristic, that is the movement of the loop of amino acids that covers the active site to expose it and leave it available for substrate hydrolysis. This loop, usually referred to as "lid", is an amphipathic α-helix, and in TlL is formed by residues 84−93, including one tryptophan residue in position 89. Since most of the changes upon activation at the interface are believed to take place in this lipid-contact region, the spectral

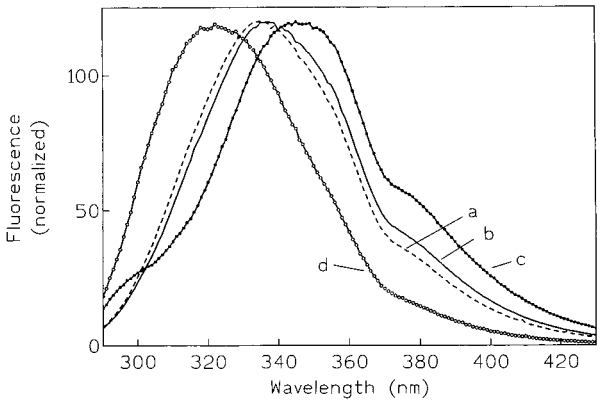

FIGURE 3: Normalized fluorescence emission spectra of *T. lanuginosa* wild-type lipase and mutants in solution. (*a*) TlL; (*b*) iTlL; (*c*) iTlLw89; (*d*) TlLw89l. Enzyme concentration 2.47 μM in 10 mM Tris pH 8.0. Excitation 280 nm.

signatures of iTlLw89 mutant, containing only Trp89, as well as those of TlLw89l mutant, in which Trp89 is substituted by Leu, were obtained and compared to the spectra of iTlL. The mutants used for the spectroscopic studies are inactive, (Ser146 in the catalytic triad mutated to Ala), so that substrate could be added without interference from product formation, except for TlLw89l, where no substrate was added. As shown in Figure 3 (curve b), the spectra of iTlL has an emission maximum at 337 nm, slightly different from the wild-type due to the modification in the environment of Trp89 when Ser146 was changed by Ala. This is consistent with molecular dynamics simulations, where the lid in the wild-type enzyme proved to be more flexible than in the inactive mutant (*32*). iTlLw89 emission spectra is shifted to 344 nm (curve c), suggesting that Trp89 is a more exposed to the bulk aqueous phase (peripheral tryptophans have an emission maximum from 340 to 350 nm), in agreement with the high flexibility predicted for the lid in the inactive or closed form of lipase in aqueous solution (*1*, *32*). In the case of TlLw89l, emission maximum at 323 nm (curve d) indicates that the structural tryptophan residues 117, 221, and 260 are essentially buried in the protein. Fluorescence properties of the different enzymes are summarized in Table 1.

The spectral changes on binding of the three enzymes to POPG-SUV vesicles (322 μM lipid, 2.47 μM enzyme) are shown in Figure 4A as difference spectra, and the change in fluorescence intensity at 340 nm at lipid-to-lipase mole ration of 185:1 are in Table 1. For iTlL, binding to POPG-SUVs results in a characteristic spectral change with an increase in the emission intensity at 320 nm and a decrease at 360 nm (curve a). The difference spectra for iTlLw89 in the same conditions (curve b) shows the high contribution of Trp89, with a decrease in intensity at 354 nm and a small increase at 310 nm. Finally, changes in fluorescence emission for

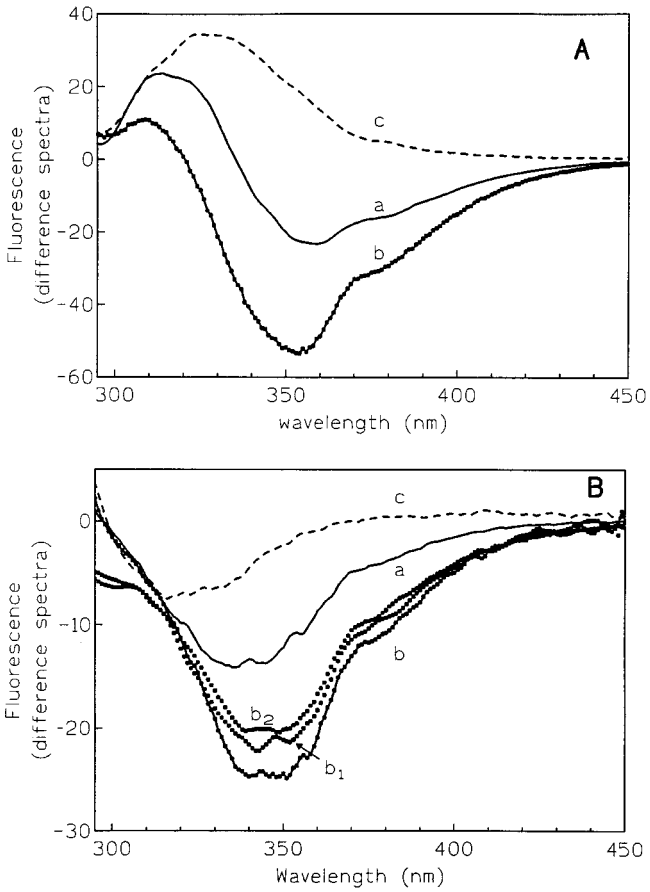

FIGURE 4: Change in the fluorescence emission spectra of *T. lanuginosa* lipase mutants bound to the lipid interface. (A) POPG-SUV vesicles, (*a*) iTlL, (*b*) iTlLw89, (*c*) TlLw89l; (B) POPC-SUV vesicles with (*a*) iTlL, (*b*) iTlLw89, (*c*) TlLw89l; (b_1) POPG-LUVs with iTlLw89; (b_2) POPC-LUV with iTlLw89. Enzyme concentration 2.47 μM; lipid concentration 322 mM. Other conditions as in Figure 3.

TlLw89l mutant on binding are very different, characterized by an increase in fluorescence at 326 nm, with no decreases at higher wavelengths (curve c). In all cases, the magnitude of the fluorescence change depends on the lipid concentration, and a well-defined isosbestic point suggests that the fluorescence change is due to a one-step equilibrium between two forms of the enzyme, the free form in solution and the form bound to the interface (for example, see refs. *22*, *33*). At the end of the titration of the two inactive mutants with POPG-SUVs, 0.2 mM TB was added and the spectra was exactly the same as in the absence of substrate. The same results were obtained when vesicles were prepared containing 19 mol % PNPB included during vesicle preparation (not shown). Also, some experiments without added substrate were done with TlL (wild-type), and essentially the same spectral changes were obtained, indicating that the Ser146-

Table 1: Tryptophan Fluorescence Properties of iTlL, iTlLw89, and TlLw89l Mutants

	λ_{em} (nm)[a]			δF (340 nm)[a]			K_d (mM)[b]			anisotropy (r)[c]		
conditions	iTlL	iTlLw89	TlLw89l	iTlL	iTlLw89	TlLw89l	iTlL	iTlLw89	TlLw89l	ITlL	iTlLw89	TlLw89l
buffer	337	344	323							0.053 ± 0.002	0.045 ± 0.007	0.090 ± 0.011
POPG-SUV	331	334	323	−0.087	−0.404	+0.308	0.065 ± 0.014	0.438 ± 0.026	0.331 ± 0.020	0.120 ± 0.007	0.124 ± 0.007	0.108 ± 0.009
POPG-LUV	337	344		−0.197	−0.134		0.367 ± 0.025	0.432 ± 0.032		0.075 ± 0.004	0.069 ± 0.008	
POPC-SUV	337	344	323	−0.130	−0.174	−0.082	0.308 ± 0.037	0.555 ± 0.034	0.498 ± 0.083	0.061 ± 0.007	0.058 ± 0.007	0.130 ± 0.018

[a,c] Lipid-to-lipase mole ratio 185:1, lipase 2.47 μM. [b] Values obtained from hyperbolic fitting of data of the type in Figure 4, excitation 280 nm, emission 340 nm. [c] Tryptophan anisotropy at 285/340 nm.

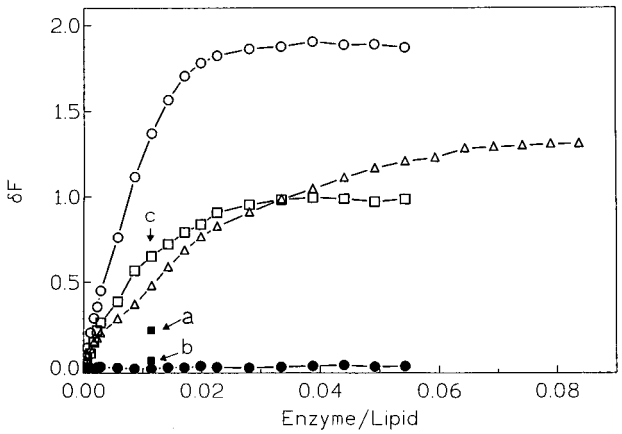

FIGURE 5: Change in the resonance energy transfer intensity at 535 nm (excitation at 285 nm) resulting from the addition of (○) iTlL, (△) iTlLw89, (□) TlLw89l to 26.7 μM POPG-SUVs containing 2.5% of NBD-PE. (●) TlLw89l added to POPC-SUV vesicles. (■) POPG-SUV (26.6 μM) added to a premixed sample containing 0.31 μM TlLw89l and POPC-SUV 26.6 μM (a) or 133 μM (b); the value in the absence of PC vesicles is (c).

Ala mutation does not affect the spectral changes in lipase on binding to lipid vesicles.

Taken together, the activity and spectroscopic results (Figures 2 and 4) suggest that TlL lipase bound to POPG-SUV vesicles is in a catalytically competent or open form, even in the absence of substrate. The spectral changes for the three mutants in the presence of 322 μM POPC-SUV vesicles, which do not support hydrolysis by TlL (Figure 2), are shown in Figure 4B. These spectra are very different from the ones obtained with POPG-SUVs; in all cases, they show a decrease in fluorescence intensity with the lipid concentration and no shift on the position of the emission maximum. Interestingly, essentially the same spectra were obtained on binding to POPG-LUV or POPC-LUV vesicles (see for example spectra b_1 and b_2 for iTlLw89 in Figure 4B), two lipid interfaces where TlL, TlLw89, and TlLw89l are not active (Figure 2). This indicates that lipase binds to the vesicles, at least partially, but it is in a different form that in POPG-SUVs, and that this form corresponds to a catalyticaly incompetent enzyme. From the experiments shown in Figure 4 at different lipid concentrations, the dissociation constants (K_d) were calculated by hyperbolic fitting and are summarized in Table 1. K_d values shown here correspond to the total number of lipid molecules, both inner and outer monolayers, thus the real value is about half the estimated number. Binding affinity for POPG-SUVs is higher for the iTlL mutant, whereas iTlLw89, with only one Trp residue, as well as TlLw89l, without the Trp in the lid, have lower binding affinities. These results suggest that residues Trp89, Trp117, Trp221, and Trp260 somehow influence binding. Binding affinities for large PG (phosphoglycerol) vesicles and small PC (phosphocholine) vesicles are of the same order in all the enzymes.

Binding Stoichiometry. The stoichiometry for high-affinity binding of iTlL, iTlLw89, and TlLw89l to the vesicles of POPG or POPC containing 2.5% of NBD-PE was determined by the increase of resonance energy transfer (RET) from the Trp donor(s) of the mutants to the acceptor NBD at the interface. As shown in Figure 5, the RET intensity at 535 nm increases with the amount of protein added, and the intensity reaches a maximum when the surface is essentially covered with the protein. The higher increase in NBD fluorescence observed for the iTlL mutant is due to the additivity of the effect of the four Trp donors in this molecule, whereas the other mutants have either one (Trp89, which has a higher quantum yield) or three (Trp117, 221, and 260). From the plot, a stoichiometry of around 80 POPG molecules per enzyme was calculated for both iTlL and TlLw89l, compared to 40 POPG per molecule of iTlLw89. If we consider the size of the SUV vesicles, with a diameter of 40 nm, then the number of lipid molecules that form each vesicle is approximately 8000; according to this calculation, the stoichiometry is 96 lipase molecules bound per vesicle for iTlL and TlLw89l, and 200 molecules of iTlLw89 per vesicle. Thus, the mutation of the structural tryptophans W117, W221, and W260, affects binding to the vesicles, in agreement with the higher K_d for this mutant calculated from the change in Trp fluorescence signal on binding (Table 1).

A surprising observation is that on binding of TlL, iTlL, iTlLw89, and TlLw89l enzymes to POPG-LUVs or POPC-SUVs, there was a very weak or no RET signal to NBD groups in the vesicle interface (Figure 5, closed circles). The possibility that the lipase does not bind to these vesicles is ruled out from the results described previously, where the Trp fluorescence emission spectra of all the enzymes changes in the presence of these vesicles in a concentration-dependent manner. In addition, when the RET experiments were done with vesicles labeled with 2.5% TMA-DPH, energy transfer was very efficient for binding of iTlL and iTlLw89 to POPG-SUV, and to POPC-SUVs, with fluorescence changes at 430 nm of $\delta F = 1.93$ and $\delta F = 0.91$ respectively at an enzyme-to-lipid mole ratio of 0.09 for iTlL. This differences between NBD and DPH acceptors cannot be analyzed, since many factors are at play including the different overlap between the tryptophan emission and the excitation of the probes (RET efficiency is smaller for the Trp/NBD pair, due to a smaller overlap), and the different location in the bilayer, where NBD is exposed and DPH is in the hydrophobic core of the membrane, but the main conclusion to be drawn is that lipase binds to the anionic and the zwitterionic interfaces. Moreover, direct evidence for binding under the conditions of Figure 5 was obtained from the same experiment; when POPG-SUV labeled vesicles were added to a premixed sample containing any of the mutants with POPG-LUVs, or POPC-SUVs, there was no increase in the RET signal to NBD, as it would be expected if lipase was free in solution. An example is shown in Figure 5, where we added POPG-SUV (26.6 μM) to a mixture of POPC-SUV (26.6 μM) with TlLw89l (0.31 μM); in this case, δF was 0.23 (point a), compared to 0.62 in the absence of the PC vesicles; increasing the amount of PC vesicles to 133 μM resulted in $\delta F = 0.04$ (point b), indicating that the mutant was effectively bound to the PC vesicles. Similar results were obtained with the other mutants. Thus, we can conclude that iTlL and iTlLw89 are bound to SUV and LUV vesicles in a different form, and the absence of a RET signal to NBD in the POPC-SUV or POPG-LUV vesicles were TlL does not show catalytic activity must be related to the distance between the Trp residues and the NBD groups at the interface, so that the efficiency of the energy transfer is very low.

Tryptophan in the Lid is Shielded from Water in the Active Form. To determine if the changes that take place in the

Table 2: Stern−Volmer Quenching Constants (K_{sv}, M^{-1}) for the Quenching of Trp Fluorescence of iTlL, iTlLw89, and TlLw89l[a]

	iodide quenching			acrylamide quenching		
conditions	iTlL	iTlLw89	TlLw89l	iTlL	iTlLw89	TlLw89l
10 mM Tris, pH 8.0	1.92	3.52	0.73	10.57	10.70	9.14
POPG-SUV	0.97	1.15	0.64	9.03	7.73	8.04
POPG-LUV	1.56	3.15		9.50	8.75	
POPC-SUV	1.65 (1.68[b])	3.45	0.63	9.77 (9.77[b])	9.80	9.05

[a] Enzyme concentration 1.1 μM; lipid concentration 267 μM; quencher 0−350 mM; results are ±10%. [b] Vesicles (267 μM) with TB (40 μM).

enzyme, and particularly in the lid region, on binding to the vesicles include shielding from the aqueous phase, the accessibility of Trp to aqueous quenchers was measured. Charged quenchers, such as iodide, will be bound to the headgroups of lipid vesicles with opposite charge (*34*) but repelled from headgroups of like charge (*35*). To avoid the possibility of any artifacts due to charge effects using iodide, parallel experiments with acrylamide as neutral quencher were conducted. The Stern−Volmer quenching constants (K_{sv}) for the quenching of iTlL, iTlLw89, and TlLw89l by iodide and acrylamide are given in Table 2. In buffer, iTlLw89 has a high iodide quenching constant, $K_{sv} = 3.52$ M^{-1}, as expected due to the dynamic motion of the lid in aqueous solution, and in agreement with the red-shifted tryptophan emission spectra for this mutant (Figure 3). In TlLw89l, K_{sv} is 0.73 M^{-1}, because iodide is considered to access only surface Trp residues. This differences are significantly reduced for acrylamide in the same conditions, because this quencher has a good access to all four Trp residues; however, a higher K_{sv} is obtained for Trp89 in the lid.

On binding to POPG-SUVs, Trp89 is shielded from the aqueous phase, with K_{sv} values that are significantly lower than for the enzyme in solution for both quenchers ($K_{sv} =$ 1.15 M^{-1} for iodide, and 7.73 M^{-1} for acrylamide). Thus, the lid in the active form of the enzyme is less exposed to water, which is consistent with either penetration in the bilayer or the lid being "sandwiched" between the lipid interface and other regions of the lipid binding zone of the enzyme. The same quenching constants were obtained if the POPG-SUV vesicles containing TB, in agreement with the previous observation that no additional spectral changes for the lipases bound to POPG-SUVs were induced in the presence of substrate (TB or PNPB). Essentially, the same results were obtained for the iTlL mutant, but the relative decrease in the accessibility was smaller. This is expected if only Trp89 becomes protected from water in the active form, as also confirmed by the experiments with TlLw89l mutant, where almost no change in accessibility of Trp117, 221, and 260 to quenchers occurs after binding to the anionic vesicles.

Interestingly, Trp89 accessibility to iodide is only slightly smaller or does not change significantly in the presence of POPG-LUVs or POPC vesicles (Table 2), indicating that in these interfaces where TlL binds in a catalytically inactive form, Trp89 in the lid is less shielded from the aqueous phase than in anionic SUVs.

Lid Mobility is Restricted in Anionic SUV. Reported X-ray structures of several known lipases complexed with inhibitors (*12*−*16*) suggest that on binding to the interface formed by substrate aggregates, the lid is stabilized in the open form; in this form, the rotational freedom of the lid is likely to be reduced as compared to the enzyme in the closed form. This

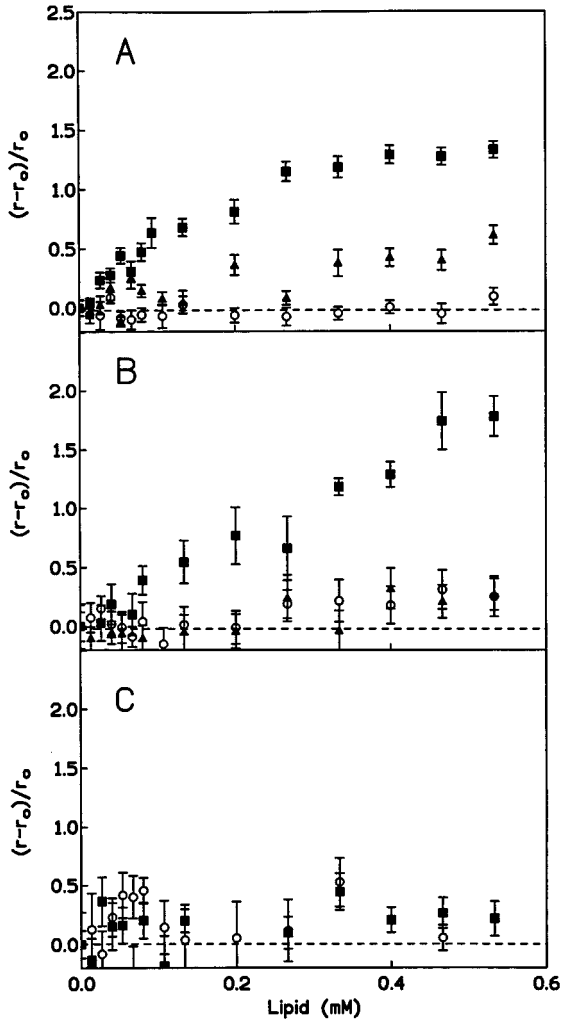

FIGURE 6: Fluorescence anisotropy change of *T. lanuginosa* lipase mutants as a function of lipid concentration (■) POPG-SUVs, (▲) POPG-LUVs, (○) POPC-SUVs. (A) iTlL; (B) iTlLw89; (C) TlLw89l. Excitation 285 nm, emission 340 nm. Titrations were carried out at 25 °C, adding aliquots of vesicles to a solution 2.47 μM of lipase in 10 mM Tris pH 8.0.

was determined by measuring the anisotropy changes in Trp residue(s) on binding; values are summarized in Table 1. Anisotropy of iTlLw89 in solution is low, $r_o = 0.045$, as expected due to the partially disordered structure of the lid under these conditions (*23*). A slightly higher value for iTlL, $r_o = 0.053$, indicates that the other Trp residues have less rotational freedom. This is confirmed with the TlLw89l mutant, with $r_o = 0.090$. Anisotropy changes for the three enzymes titrated with vesicles are shown in Figure 6. Incorporation of iTlLw89 in POPG-SUVs results in a gradual increase in fluorescence anisotropy (Figure 6B, closed squares), indicating that the tryptophan residue in the lid is localized in a motionally restricted region in the active

conformation of lipase. The relative increase in anisotropy (δr) is larger than for iTlL (Figure 6A), for example at lipid to peptide mole ratio of 185:1, δr is 1.14 for iTlL, and 1.76 for the double mutant (from values in Table 1). Also as expected, almost no changes in anisotropy take place on binding of TlLw89l to PG-SUV (Figure 6C), confirming that the mobility of structural tryptophans 117, 221, and 260 is practically the same as in solution. This reinforces our previous conclusion that most of the conformational changes on lipase activation at the interface take place in the lid domain. When the same experiment was done with POPC vesicles, or with POPG-LUVs, the change in anisotropy was very small or insignificant for iTlLw89, iTlL, and TlLw89l (Figure 6, circles and triangles, respectively). Since the possibility of a lack of binding to these vesicles is ruled out, a plausible explanation is that in the inactive form adopted by lipase at the interface of POPC or POPG-LUVs, the flexibility of the lid domain is the same as in solution. No additional anisotropy changes are induced by the presence of TB added at the end of the titration with vesicles for the inactive enzymes.

Tryptophan Depth in Membrane-Bound T. lanuginosa Lipase. Interaction of proteins with membranes can be monitored by RET (*36*). This method is based on the nonradiative transfer of the excited state energy from a donor to an acceptor molecule. The extent of energy transfer depends mainly on the extent of overlap between the emission spectrum of the donor and the absorption spectrum of the acceptor, and on the orientation and distance between them. To gain a better understanding of lipase interfacial activation in phospholipid vesicles, the penetration depth of Trp residues of the different mutants was qualitatively determined by RET. The donor molecules where the Trp residue(s) from lipases, and as acceptors for the enzyme excitation energy 2.5% of the membrane probes DPH and TMA-DPH were incorporated to POPG or POPC vesicles. This will give useful information on the depth of penetration in the bilayer of particular regions of the lipase where Trp residues are located, because of the different location of these two probes. DPH is oriented predominantly parallel to the fatty acid chains of the bilayer, and the distance of the shallow end of the group to the bilayer center in PC vesicles is around 13 Å; in TMA-DPH, the cationic TMA group is located near the membrane surface, so that the distance of the shallow end of the DPH group to the bilayer center is 16.5 Å (*37*). The RET from enzyme molecules that remain free in solution can be considered negligible, because the critical distance for RET to occur is in the range of 15−50 Å depending on the donor/acceptor pair.

As shown in Figure 7 (panels A, B), this small difference in depth results in a large difference in transfer efficiency for iTlL and iTlLw89 bound to POPG-SUVs. Whereas a high degree of energy transfer is seen for POPG-SUVs labeled with TMA-DPH (closed circles), efficiency is lower when the same vesicles where labeled with DPH (open circles). Surprisingly, no energy transfer takes place when the TlLw89l mutant was titrated with POPG-SUVs, either containing DPH or TMA-DPH probes (Figure 7C, circles). These results clearly indicate that in the form of lipase that is bound to small anionic vesicles, or open form, the lid containing Trp89 partially penetrates the membrane, and is located at a shallow interfacial region close to the polar

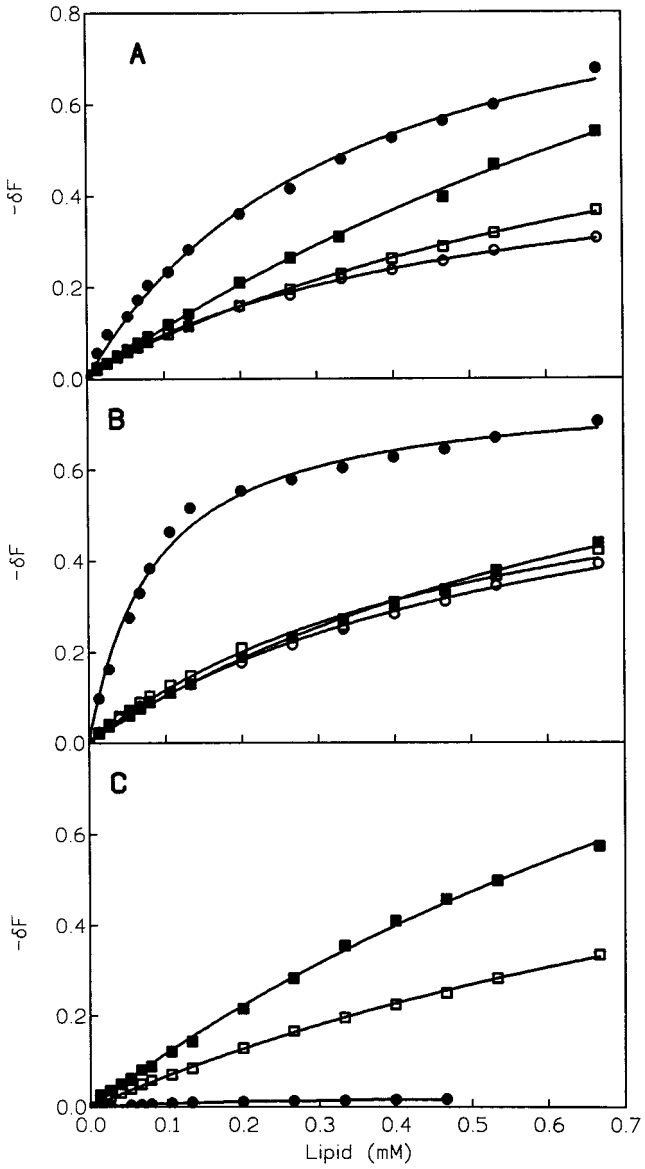

FIGURE 7: Penetration of *T. lanuginosa* mutants (2.47 μM) in the bilayer determined by RET. Enzymes were titrated with vesicles containing 2.5% of the internal label DPH (open symbols) or the interfacial label TMA-DPH (closed symbols); POPG-SUV (●, ○); POPC-SUVs (■, □). (A) iTlL; (B) iTlLw89; (C) TlLw89l. Excitation: 280 nm; emission 340 nm.

headgroups; in this form, structural tryptophans 117, 221, and 260 do not participate in the energy transfer to the DPH group, suggesting that only the lid region penetrates the membrane. Addition of TB to the inactive lipase−vesicle system did not modify the energy transfer, indicating that the presence of substrate in the active site does not change the orientation of the enzyme in the interface.

A different picture emerges from the results with POPC SUVs, as also shown in Figure 7 for the three mutants. The behavior of the iTlL mutant (Figure 7A) shows that the enzyme penetrates the membrane and also remains preferentially close to the interface, although here the differences between DPH (open squares) and TMA-DPH (closed squares) labeled vesicles are smaller than in the POPG-SUVs. One possibility is that in anionic vesicles, due to electrostatic attraction, the cationic TMA group is located closer to the anionic headgroups of the phospholipids than in zwitterionic PC, so that the attached DPH group is in a more shallow

location than in PC where the difference between DPH and TMA-DPH is only 3−4 Å (*37*). In the case of iTlLw89 (Figure 7B), no differences are seen, indicating that in this interface the structural tryptophans, and not only Trp89 in the lid are involved in the energy transfer with the membrane. This is confirmed with results from the TlLw89l mutant (Figure 7C), with high efficiency of RET to PC vesicles. These results are consistent with the interaction of the enzyme trough a different side of the protein, involving one of several structural tryptophans as well as Trp89.

DISCUSSION

In this paper, we demonstrate that not only conformational rearrangements in the enzyme, but also the molecular composition and physical properties of the lipid interface can lead to an optimized active site geometry in TlL, thus reconciling the substrate and enzyme theories for interfacial activation of lipases (*38*−*40*). The complexity of the mechanism of interfacial activation of lipolytic enzymes, and the contribution from both the enzyme and the interface to this unique behavior has been the object of elegant studies (*32*, *41*). We have previously shown TlL hydrolyzes PNPB or TB substrate partitioned in the interface of anionic small unilamelar vesicles with a mean diameter around 40 nm, such as POPG-SUVs, at a rate that is 100-fold higher than that for the monodispersed substrate or for the substrate partitioned in zwitterionic POPC-SUVs (*22*). Anionic SUVs fulfill the requirements of a neutral diluent (*42*), because they provide an interface for the partitioning of both substrate and enzyme, but at the same time do not block the enzyme's active site. In this well-defined system, the primary rates and equilibrium constants for the interfacial catalysis by TlL were obtained (*22*), in an extension to the work on phospholipase A2 catalyzed hydrolysis of micellar (*43*), or vesicular phospholipids (*44*).

In the case of TlL, there is an additional feature shared by many lipases of different origin: the presence of a loop of amino acids or lid that covers the active site when the enzyme is in solution, what is usually referred to as "closed form" of the enzyme. We suggested that the lid must move away when the enzyme is bound to the POPG interface, or displacement is induced by the substrate partitioned, so that the active site becomes accessible for the substrate. Here, we show that the interfacial activation of TlL observed in the POPG-SUV interface is shared by the TlLw89 and TlLw89l mutants, although their activity is lower, and that this activation does not take place in zwitterionic POPC vesicles, or in anionic large unilamelar vesicles of 100 nm diameter, even tough the enzyme binds to these vesicles. Results show that binding of TlL to anionic sonicated vesicles triggers lipase activation by promoting a conformational change in the lid region, that penetrates the bilayer and is stabilized in a fixed position leaving free access of the substrate to the active site. On the other hand, TlL binds to anionic LUVs or zwitterionic SUVs in a different form that is catalytically incompetent and has different spectroscopic properties.

Molecular Model for the Binding of TlL to Phosholipid Vesicles: Open and Closed Forms of the Enzyme. TlL has four Trp residues in positions 89, 117, 221, and 260 of the chain. Trp89 is in the helical lid covering the active site in the closed form, and the rest are structural residues located in different regions of the enzyme. Tryptophan fluorescence spectroscopy gives information on the molecule structure and dynamics, since it depends on the physical properties of Trp local environment, such as solvent exposure and interactions with other residues in the protein or with the lipid interface. By using mutants containing all four Trp residues (TlL, iTlL), only Trp89 (TlLw89, iTlLw89), or only the structural tryptophans (TlLw89l), we are able to dissect the domains of the enzyme that are involved more directly in the interaction with the lipid interface. Also, inactive mutants where the catalytic Ser146 is mutated to Ala allow the study of the spectroscopic changes that take place in the presence of substrate, such as TB. Tryptophan fluorescence emission spectra, anisotropy measurements and quenching experiments in aqueous solution in the absence of an interface, reveal that the lid is highly flexible and quite exposed to the solvent, in agreement with the X-ray crystals of TlL at 1.85 Å resolution (*23*). On the other hand, Trp117, 221, and 260 are more buried in the interior of the protein and have lower mobility.

Fluorescence spectroscopy shows that TlL wild-type and mutants bind with high affinity to the different vesicles studied: POPG-SUVs, POPG-LUVs, and POPC-SUVs (Table 1). However, the enzyme binds in different forms with different spectroscopic characteristics and that have different activities depending on the lipid interface. There are at least two functional forms of the bound lipase that we can distinguish: one is the form bound to anionic small vesicles, such as POPG-SUVs, which is a catalytically competent or open form of the enzyme; the other is the form bound to POPC-SUVs or to POPG-LUVs, which is a catalytically incompetent form of the enzyme. Both forms, the active or open, and the inactive or closed, have qualitatively different spectroscopic signatures. The same spectral change seen with POPG-SUVs was obtained with other anionic SUVs, such as DMPM or DPPG; this two types of vesicles are in the gel phase at room temperature as opposed to the POPG vesicles that are in the liquid crystal phase; therefore, any effect of the lipid phase in the interfacial binding and activation can be ruled out. A model shown in Figure 8 emphasizes two membrane bound forms of TlL: the closed inactive form in PC-SUVs, and the open-lid active form in PG-SUVs.

Characterization of the Open or Active Form of TlL in POPG-SUVs. In the active form of TlL bound to POPG-SUVs, the helical lid becomes partially buried in the lipid bilayer, becoming less accessible to the aqueous quenchers iodide and acrylamide (Table 2). In addition, the lid is stabilized in a fixed position even in the absence of substrate, as suggested by the increase in anisotropy of Trp89, leaving the active site free for substrate binding and hydrolysis. Structural Trp residues 117, 221, and 260, less flexible than Trp89 in the closed form in solution, do not change their rotational freedom and solvent accessibility in the active form of the lipase. RET experiments using acceptor groups located at different positions in the membrane show that in the open form, the lid penetrates partially in the POPG-SUV bilayer, and it stays close to the anionic lipid headgroups. The three structural Trp(s) are not involved in RET to acceptor groups located in the hydrophobic core of the bilayers (DPH, TMA-DPH), therefore, the distance of these residues to the interface must be higher that the efficiency distance of this pair (around 15 Å). On the basis of these results together with the X-ray

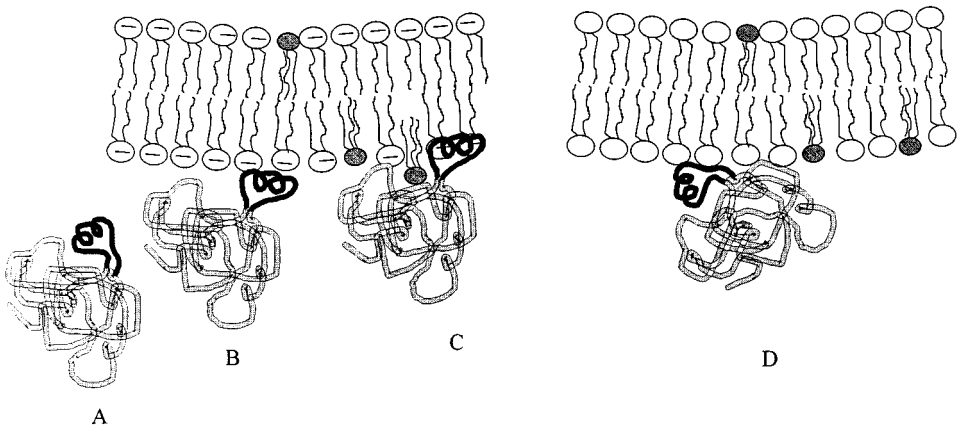

FIGURE 8: Model for the interfacial binding and activation of *T. lanuginosa* lipase. (Left) Lipase in solution is in the closed (inactive) form, with the lid (in black) covering the active site (A); Binding of TlL to anionic SUVs (B) promotes lid displacement, exposing a large hydrophobic concavity around the active site that must interact with the lipid interface, stabilizing the open active form; partitioned substrate (shown in gray) can then access the catalytic triad (C). (Right) TlL binds to zwitterionic interfaces with a different orientation and without penetration of the lid in the interface, in a catalytically incompetent form (D).

structure of the open form of another fungal enzyme, *Rhizomucor miehei* lipase, a model of binding and activation is shown in Figure 8. *R. miehei* has a virtually identical three-dimensional structure to TlL (*23*), and its open structure complexed with an inhibitor has been solved by X-ray (*12*, *45*); it shows that the lid rolls away from the active site and across the surface of the molecule moving its center of gravity 8 Å and rotating around its axis by 167°, thus exposing a large hydrophobic surface of approximately 1739 $Å^2$ formed by 12 amino acids among then Trp88 in the lid (*5*, *38*). Interfacial activation is, therefore, explained in terms of the stabilization of this newly exposed hydrophobic face surrounding the active site due to lid opening by binding to the interface (*21*). This would stabilize a completely developed oxyanion hole, which in turn stabilizes the transition state of the catalytic reaction (*13*, *46*). The three-dimensional structure of a TlL−inhibitor complex (*13*) is very similar, and it also has the hydrophobic cleft around the active site. Summarizing, our proposed model for the interfacial activation of TlL in POPG-SUVs is shown in Figure 8 (left). TlL binds to POPG-SUV interface and the lid is displaced away, partially penetrating the bilayer and exposing a large hydrophobic domain that will be interacting with the curved POPG-SUV interface to avoid exposure to the aqueous phase; furthermore, the positively charged residues present in the hinge regions of the lid, such as Arg81, Arg84, and Lys98 may act as lockers that stabilize the open structure by electrostatic attraction to anionic PG groups at the interface. In this conformation, the distance of the Trp221 and 260 to the interface will be more than 20 Å, and that of Trp117 is estimated to be around 30 Å from the crystal structure. However, an electrostatic interaction is not sufficient to explain the interfacial activation of TlL, since in POPG-LUVs the enzyme does not adopt the active form. One possibility is that the highly positively curved interface of sonicated vesicles also plays a role, causing the headgroups to be further spread apart exposing more of the hydrocarbon chains to the aqueous phase (*47*). This will favor the hydrophobic interaction with the nonpolar lipid-binding face of TlL, which has a concavity complementary to that of the interface as suggested by the X-ray structure of the complexed form. Our results suggest that this open form of TlL is attained even in the absence of substrate. Therefore, interfacial activation of TlL is the result of a delicate balance of electrostatic and hydrophobic interactions between the fungal lipase and the lipid interface. In a very different way, the influence of curvature in the activation of a lipase has also been reported for porcine pancreas lipase−colipase−micelles ternary complex, where a certain micelle size that fits in a concavity formed by colipase and the C-terminal part of the lipase is critical to yield the active form (*16*).

Characterization of the Inactive form of TlL in POPC-SUVs and POPG-LUVs. As stated above, TlL also binds to POPC vesicles, as well as to POPG-LUVs, but adopting a different form that is catalytically inefficient to hydrolyze substrate. The fluorescence emission spectra associated with this form is qualitatively different from that of the active form, and it is the same in POPC vesicles and POPG-LUVs, with or without substrate partitioned in the vesicles. In this inactive form of TlL, the accessibility of the four Trp residues to aqueous quenchers as well as the anisotropy are the same as in the free form in solution, indicating that there is no penetration of the lid or the other regions of the enzyme around the Trp residues in the lipid membrane. RET experiments from Trp in the enzyme to DPH groups located in the membrane interior show that not only Trp89 in the lid, but also at least one of the structural Trp residues are close to the lipid interface. We propose that the lid is not displaced on binding to these vesicles, and the enzyme binds to the interface with a slightly different orientation, as shown in the model in Figure 8 (right). In the surface of the closed conformation of *R. miehei*, there are two large hydrophobic patches (*48*) that together occupy about the same area as the large patch in the open form; they are located in close proximity to the lid and have also been found in *Candida rugosa* lipase (*21*). Also in TlL, there is a quite hydrophobic patch in the triangle defined by Trp89, Trp221, and Trp260, and we suggest that this is the lipid-binding face of TlL in the inactive bound form. This side will be in close proximity to the interface but without insertion.

ACKNOWLEDGMENT

Excellent technical assistance from Helena Carvajal (University of Barcelona) is gratefully acknowledged. We also thank Jesper Vind (Novo Nordisk) for preparation of lipase variants.

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BI991927I