Spectroscopic studies of the interfacial binding of *Humicola lanuginosa* lipase†

The Analyst

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The interaction of *Humicola lanuginosa* lipase (HLL) with small unilamellar vesicles of 1-palmitoyl-2-oleoylglycero-*sn*-3-phosphoglycerol (POPG) and in the presence of tributyrin (TB) as a substrate was studied by the use of steady-state fluorescence techniques. An inactive mutant with the serine from the catalytic triad changed by alanine (S146A) was used in experiments with TB to avoid interferences from product formation. HLL binds to POPG vesicles in an active or open form for the catalytic turnover, and therefore POPG provides a suitable system for studying the conformational changes involving the movement of the loop of amino acids that covers the active site of the enzyme in solution. Tryptophan (Trp) fluorescence shows that HLL binding to POPG occurs with a change in the environment of Trp residue(s) and that there is only one type of bound form, even in the presence of TB. Accessibility to aqueous quenchers indicates shielding of Trp in the membrane. Fluorescence anisotropy of the enzyme increases on binding to the vesicles, indicating restricted rotational freedom for the Trp due to penetration in the bilayer. Resonance energy transfer experiments using an interfacial membrane probe, 1-[4-(trimethylammonio)phenyl]-6-phenylhexa-1,3,5-triene *p*-toluenesulfonate (TMA-DPH), and an internal membrane probe, 1,6-diphenylhexa-1,3,5-triene (DPH), indicate that HLL does not penetrate very deeply in the hydrophobic core of the membrane, but preferentially stays close to the lipid interface. Addition of substrate (TB) does not result in any additional changes in the spectroscopic properties of HLL. It is suggested that the observed changes are due to the 'opening of the lid' on binding to POPG vesicles, leaving the active site accessible for the substrate to bind.

Introduction

Lipase from *Humicola lanuginosa* is a hydrolytic enzyme which breaks down triacylglycerols into free fatty acids and glycerols. Lipase activity is depressed in monomeric substrates, but it is greatly increased in the lipid–water interface, a phenomenon known as interfacial activation.[1] The majority of the known structures of lipase have the catalytic center buried beneath a surface loop or "lid" that renders it inaccessible to solvent and substrate molecules. The lid changes its conformation on binding to a lipid–water interface, exposing the active site and creating a non-polar surface which can stabilize the contact between the enzyme and the lipid interface.[2] A better understanding of the movement of the surface loop is important because this is believed to be one of the main events in the interfacial activation process.

A high-resolution (1.8 Å) three-dimensional structure of *Humicola lanuginosa* lipase has been determined by X-ray crystallography.[3] The structure shows the α/β hydrolase fold characteristic of all lipases, and the consensus catalytic triad Ser–His–Asp forming the active site, which is covered by a lid of nine amino acids with an α-helical structure. This form of the enzyme is catalytically inactive and is referred to as the closed conformation of the enzyme. This lid must move to allow access of the substrate to the active site, forming the open enzyme conformation. In some lipases, such a molecular change was described in molecular detail from the X-ray structure of lipase–inhibitor complexes, for example for *Rhizomucor miehei*[4,5] and more recently for *Humicola lanuginosa.*[6] However, although these crystallographic data are a first step towards elucidating the molecular mechanism underlying lipase activation, the inhibitors used for crystallography are usually very different structurally from the natural substrates. For this and other reasons, the structure of lipase in these complexes cannot be considered equivalent to the active form generated in the lipid–water interface under physiological conditions. The actual process of interfacial activation involving the displacement of the lid when bound to the lipid surface is very complex and it has not been fully elucidated.

In this paper, the conformational changes that take place in *Humicola lanuginosa* lipase (HLL) upon binding to the lipid vesicles of 1-palmitoyl-2-oleoylglycero-*sn*-3-phosphoglycerol, and also in the presence of tributyrin as a substrate, were studied by fluorescence techniques. The catalytic activity of the enzyme under the same conditions as used for fluorescence was measured spectroscopically by UV spectrophotometry using *p*-nitrophenylbutyrate as a substrate. An inactive mutant, with active site Ser in position 146 of the chain mutated by Ala (S146A HLL), was used in the fluorescence experiments to avoid interference from product formation. Changes in the fluorescence emission of the protein on binding to the vesicles and accessibility to aqueous quenchers iodide and acrylamide were used to study the interaction with the bilayer. Fluorescence anisotropy and resonance energy tranfer measurements were used to demonstrate the incorporation of the lipase in the vesicle membrane.

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Experimental

Chemicals

Humicola lanuginosa wild type (HLL), and its mutant in which the catalytic serine is changed to alanine (S146A HLL), were obtained from Novo Nordisk (Denmark). For the calculations of enzyme concentration, a molar absorptivity of 43 000 l mol^{-1} cm^{-1} with an M_r of 32 kDa were used.[7] *p*-Nitrophenylbutyrate (PNPB) and tributyrin (TB) were obtained from Sigma (St. Louis, MO, USA). *N*-(7-Nitro-2,1,3-benzoxadiazol-4-yl)dioleoylphosphatidylethanolamine (NBD-PE) and 1-palmitoyl-2-oleoylglycero-*sn*-3-phosphoglycerol (POPG) were purchased from Avanti Polar Lipids (Alabaster, AL, USA) and 1,6-diphenylhexa-1,3,5-triene (DPH) and 1-[4-(trimethylammonio)phenyl]-6-phenylhexa-1,3,5-triene *p*-toluenesulfonate (TMA-DPH) from Molecular Probes (Eugene, OR, USA).

Vesicle preparation

Small unilamellar vesicles (SUVs) of POPG alone or with the fluorescent probes NBD-PE, TMA-DPH or DPH were prepared by evaporation of a mixture of the lipids in $CHCl_3$–CH_3OH (2 + 1 v/v). The dried film was hydrated for a lipid concentration of 20 mM, and then sonicated in a G112SPIT bath type sonicator (Laboratory Supplies, Hicksville, NY, USA) above the gel–fluid transition temperature until a clear dispersion was obtained (typically 2–4 min). Vesicles were annealed for 1 h above their transition temperature before use.

Kinetic protocols

The kinetics of hydrolysis of PNPB were monitored as the change in the absorbance at 400 nm, corresponding to the absorption maximum of the *p*-nitrophenolate anion with a molar absorptivity of 14 000 l mol^{-1} cm^{-1}, as described elsewhere.[8] Data acquisition and manipulation were carried out in a Hewlett-Packard (Avondale, PA, USA) diode array spectrophotometer with a 0.5 s acquisition time. Measurements were made in 0.7 ml of 10 mM TRIS buffer (pH 8.0) at 25 °C in a quartz cuvette. Vesicles of POPG 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 gentle mixing of the cuvette. The rate of hydrolysis was calculated from the slope of the initial zero-order phase of the progress curve, and expressed as turnover number per second.

Tryptophan (Trp) fluorescence and quenching experiments

Fluorescence measurements were carried out in 10 mM TRIS buffer (pH 8.0) at 25 °C on an AB-2 spectrofluorimeter (SLM-Aminco, Urbana, IL, USA) with constant stirring. Tryptophan fluorescence spectra were recorded with an excitation wavelength of 280 nm over an emission range 295–450 nm, with 4 nm slitwidths. S146A was added from a stock solution to a final concentration of 2.47 μM and titrated with vesicles and TB. The sensitivity (PMT voltage) was adjusted to 1% for the Raman peak from the buffer blank at the same excitation wavelength. The contribution from vesicles to the signal was negligible. Quenching of tryptophan fluorescence of S146A by iodide and acrylamide was recorded at 320 nm (excitation at 280 nm). Appropriate amounts of POPG 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. The final quencher concentration ranged from 0 to 350 mM. Quenching results were analyzed according to the Stern–Volmer equation for collisional quenching:

$$F_0/F = 1 + K_{SV}[Q]$$

where F_0 and F are the fluorescence intensities in the absence and presence of quencher, respectively, [Q] is the molar concentration of quencher and K_{SV} is the Stern–Volmer quenching constant. In this range of concentrations there is no deviation from linearity ($r^2 = 0.99$).

Binding isotherms

The binding of S146A to POPG vesicles containing 2.5% of NBD-PE was determined as the increase in the resonance energy transfer (RET) signal from Trp residues in the enzyme to the labeled phospholipid in the interface at 535 nm (excitation at 285 nm). Vesicles in buffer were titrated with S146A HLL from a stock solution 23.2 μM in water, and the stoichiometry was determined directly from the plot of δ*F versus* lipid/enzyme (mol/mol). The relative change in fluorescence, δ*F*, is defined as $(F-F_0)/F_0$, where F_0 and F are the intensities without and with enzyme, respectively. Since the lipid concentration was very low (26.7 μM), the contribution from light scattering was less than 3%.

Fluorescence anisotropy

Steady-state fluorescence anisotropy measurements were carried out on an AB-2 spectrofluorimeter, with L-format fluorescence polarizers. The excitation wavelength was set at 280 nm and the emission at 340 nm with excitation and emission slitwidths at 4 nm. Titrations were carried out at 25 °C, adding aliquots of POPG vesicles and/or TB to a solution 2.47 μM S146A HLL in 10 mM TRIS buffer (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 to the excitation polarizer, respectively. Anisotropy values were automatically corrected for dependences in the detection system (G-factor correction). Changes in anisotropy were represented as $(r - r_0)/r_0$, where r_0 and r are the anisotropy values before and after addition of vesicles, respectively. All measurements were carried out in triplicate.

Penetration in the membrane by resonance energy transfer (RET) measurements

A solution 2.5 μM S146A HLL in 10 mM TRIS buffer (pH 8.0) was titrated with aliquots of POPG SUVs labeled with 2.5% of the fluorescent probe TMA-DPH or DPH. The excitation wavelength was 280 nm and fluorescence emission was measured with excitation and emission slitwidths of 4 nm each. The relative decrease in fluorescence emission intensity of the enzyme (δ*F*) at 340 nm was plotted.

Results and discussion

Activity measurements of *Humicola lanuginosa* lipase (HLL) in POPG vesicles as interfaces

Substrates for HLL are di- and tri-glycerides, molecules that when suspended in water at concentrations above their

solubility limit form droplets or aggregated forms of uncontrolled dispersity, or adsorb on the available surfaces. It is therefore almost impossible to generate well defined and controlled structures of known surface area. We have recently reported a new strategy to analyze steady-state kinetics for the hydrolysis by HLL[8] of a soluble substrate (*e.g.*, TB or PNPB) partitioned into the interface provided by POPG vesicles. As shown in Fig. 1, HLL hydrolyzes PNPB partitioned in the interface of POPG SUVs at a rate that is more than 100-fold higher than that for the monodisperse substrate (less than 20 s^{-1} for PNPB in solution and 1200 s^{-1} in the presence of 0.054 mM POPG). POPG is not a substrate for lipase, but POPG vesicles act as a neutral diluent interface to which the lipase binds and probably adopts its open or active conformation, in which its active site is accessible for the substrate.[8] Therefore, POPG provides a well controlled interface to study the changes that take place in HLL lipase involving the opening of the lid when the enzyme adopts its fully open conformation.

Changes in Trp fluorescence on binding to POPG vesicles

The fluorescence of Trp is dependent on the polarity of its environment. This effect can be used to study the interaction of the HLL lipase in POPG vesicles. HLL has four Trp residues; one of them, Trp-89, is located in the lid that covers the active site in the closed or inactive form of the enzyme in solution. The major changes in the enzyme conformation on binding to the lipid–water interface are associated with the lipid contact zone region, which includes the lid.[2] Therefore, changes in Trp fluorescence on binding to the POPG interface, where the enzyme adopts its open conformation, can give valuable information on the environment of the lid. As shown by the difference spectra (Fig. 2) as a function of POPG concentration, the binding of S146A HLL is accompanied by an increase in the emission intensity at 320 nm and a decrease at 360 nm. A well defined isosbestic point suggests that the fluorescence change is due to a one-step equilibrium between two forms of the enzyme, namely the free form in solution and the form bound to the interface. Addition of TB at the end of the titration with POPG does not result in additional changes in fluorescence; therefore, we can speculate that the lipase bound to POPG vesicles is already in the open form, even in the absence of substrate. The same experiment but without addition of TB was carried out with HLL wild type, and the changes in fluorescence were comparable (not shown).

Fluorescence quenching of S146A HLL by iodide or acrylamide

Iodide and acrylamide were used as aqueous-phase quenchers of the Trp fluorescence. The Stern–Volmer plots of the quenching of Trp fluorescence by iodide and acrylamide are shown in Fig. 3. In the presence of POPG vesicles, the Trp residues of S146A HLL become less accessible to the quenchers, as compared with the enzyme in buffer; this suggests penetration of the enzyme in the lipid bilayer and shielding of Trp from the aqueous quenchers. The Stern–Volmer quenching constants (K_{SV}) for a bimolecular collisional quenching process were calculated from the apparent slopes of the plots of $F_0/F-1$ *versus* [Q] calculated by linear regression analysis, and are given in Table 1. No significant change in K_{SV} was seen on addition of TB to the POPG–enzyme system, in agreement with the absence of additional changes in Trp fluorescence under the same conditions as described previously. Comparing the two quenchers, the decrease in K_{SV} on binding to the interface was more pronounced when iodide was used. This can be explained because iodide is considered to access only the surface Trp

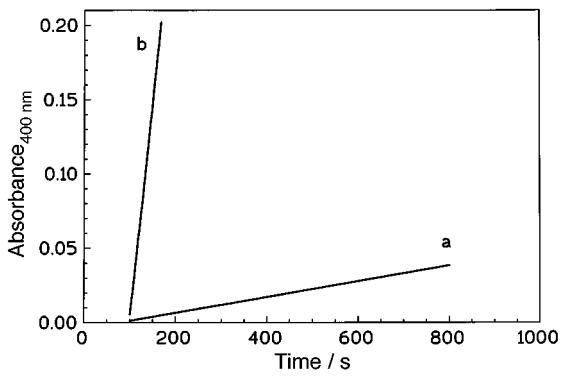

Fig. 1 Reaction progress, monotored as change in absorbance at 400 nm, for the hydrolysis of 0.07 mM PNPB by 3.9 nmol HLL. (a) PNPB alone; and (b) PNPB in the presence of 0.054 mM POPG vesicles.

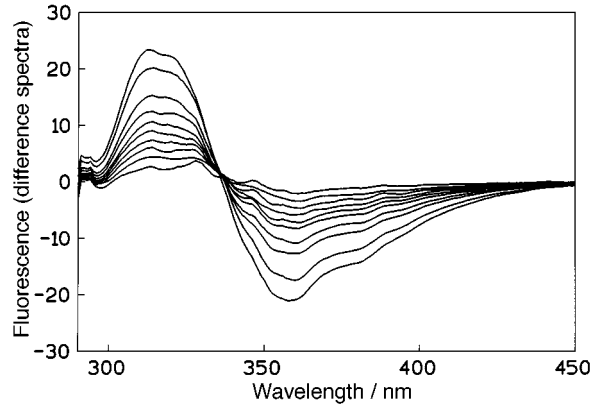

Fig. 2 Change in the intensity of fluorescence in the emission spectra of the S146A mutant of HLL (2.47 μM) as a function of POPG concentration (from 0.013 to 0.266 mM) in 10 mM TRIS buffer (pH 8.0). Excitation was set at 280 nm.

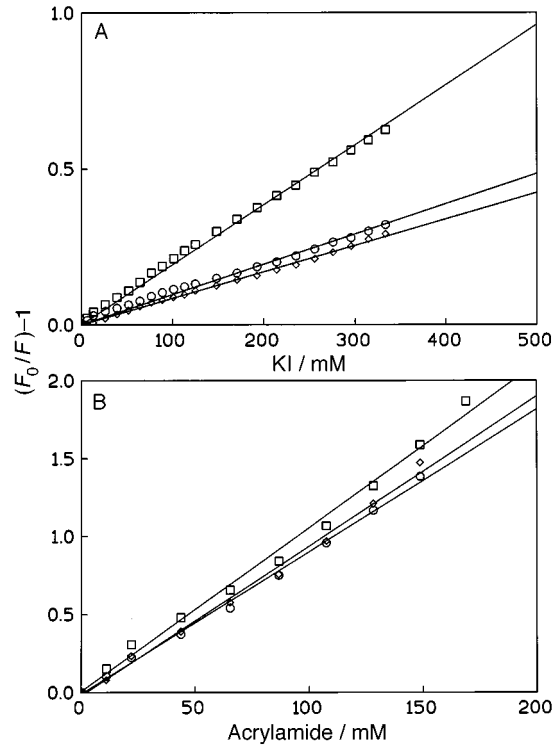

Fig. 3 Stern–Volmer plots showing the tryptophan fluorescence quenching of S146A HLL (1.1 μM) in 10 mM TRIS buffer (pH 8.0) by (A) iodide or (B) acrylamide. (□) S146A HLL in buffer; (○) S146A HLL with POPG vesicles; (◊) S146A HLL with POPG vesicles and TB. POPG concentration 267 μM; TB concentration, 40 μM. The excitation wavelength was set at 280 nm and emission was monitored at 320 nm.

residues, whereas acrylamide would have good access to all but the most highly buried Trp residues.[9] Therefore, it is possible that iodide will mainly quench the fluorescence of Trp-89 in the lid. When the enzyme binds to the vesicles, the lid possibly becomes more buried in the membrane and thus less accessible to iodide quenching. On the other hand, acrylamide may be able to access also the other more buried Trp residues of the enzyme, which most probably do not change their accessibility to the water on binding to POPG vesicles. Therefore, the small decrease in K_{SV} (from 10.57 to 9.03 l mol^{-1}) probably reflects the shielding of Trp-89. This will be further analyzed with a new mutant enzyme which contains only Trp-89. The accessibility of the Trp fluorophore to acrylamide was also determined for the enzyme bound to 2 mM TB. At this concentration, well above its solubility limit, the substrate is in an aggregated form, and therefore the enzyme will bind in the active form. In this case, K_{SV} was 8.9 l mol^{-1} (Table 1), very similar to the value in POPG vesicles, and again pointing towards the idea that in both cases we are looking at the same active enzyme form.

Fluorescence anisotropy of HLL

Steady-state fluorescence anisotropy can be used for the characterization of the incorporation process of a protein into a vesicle bilayer. The rotational freedom of the protein will be restricted upon incorporation. Consequently, the anisotropy of the enzyme will increase upon addition of the vesicles.[10,11] This phenomenon was used to monitor the lipase–vesicle interaction. For this experiment, as for all spectroscopic studies, S146A HLL mutant was used. The free enzyme exhibited an anisotropy value (r_0) of 0.052 05 ± 0.003 26, and the anisotropy value of the bound protein (r_{max}) was 0.1137 ± 0.003 52. In Fig. 4, the relative change in anisotropy is shown as a function of lipid concentration. Incorporation of the enzyme to the POPG vesicles results in an increase in anisotropy until all the enzyme is bound. An estimate of the stoichiometry of the binding can be obtained directly from this plot, which gives a POPG to enzyme ratio of 80:1 (mol/mol). At the end of the titration with POPG, TB was added to the cuvette, and no additional changes in anisotropy were seen.

Table 1 Stern–Volmer quenching constants for the quenching of S146A HLL fluorescence. Enzyme, 1.1 μM; POPG, 267 μM; TB 40, μM unless indicated otherwise

	K_{SV}/l mol^{-1}	
Conditions	Iodide quenching	Acrylamide quenching
S146A	1.92	10.57
+POPG	0.97	9.03
+POPG+TB	0.85	9.18
+TB (2 mM)	—	8.90

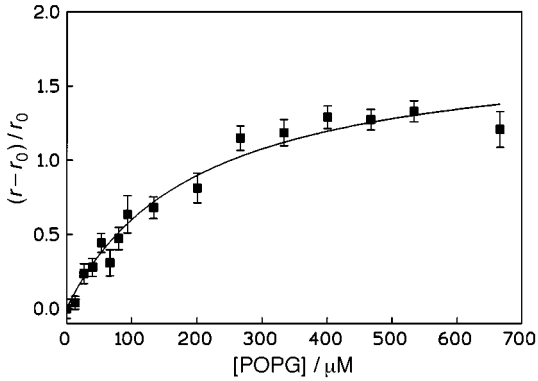

Fig. 4 Fluorescence anisotropy change of S146A HLL as a function of POPG concentration. The excitation wavelength was set at 280 nm and the emission at 340 nm with excitation and emission slitwidths of 4 nm. Titrations were carried out at 25 °C, adding aliquots of POPG vesicles to a solution 2.47 μM of S146A in 10 mM TRIS buffer (pH 8.0).

Estimation of protein-to-vesicle ratio by RET

Evidence for the interaction of S146A HLL with POPG vesicles comes from the resonance energy transfer signal resulting from the Trp donors of the mutant to the NBD–lipid acceptor at the interface, with a RET distance of about 15 Å.[12] As shown in Fig. 5, the RET intensity at 535 nm increases with increase in the amount of protein added, and the intensity reaches a maximum when the surface is essentially covered with the protein. From the plot, a stoichiometry of 80 POPG molecules per enzyme molecule was calculated, in perfect agreement with the estimate from anisotropy measurements described above. If we consider the size of the SUV vesicles, with a diameter of approximately 50 nm, the number of lipid molecules that form each vesicle is approximately 8000; according to this calculation, the stoichiometry is 100–110 lipase molecules bound per vesicle.

Measurement of penetration depth of HLL in the membrane

Interaction of proteins with membranes can be monitored by RET.[9] This method is based on the non-radiative 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 absoption spectrum of the acceptor and on the orientation and distance between them. The donor molecules used in these experiments were the Trp residues from S146A HLL, and different molecules located in the lipid membrane were used as acceptors. The RET from enzyme molecules that remain free in solution can be considered negliglible, because the critical distance for RET to occur is in the range 15–30 Å, depending on the donor–acceptor pair. As acceptors for the enzyme excitation energy, 2.5% of the membrane probes DPH and TMA-DPH were incorporated in POPG vesicles. The location of these probes in the bilayer membrane is different. DPH is deeply buried in the hydrophobic core of the membrane, whereas TMA-DPH is located more shallowly in the membrane, in order to allow the TMA group to remain near the membrane surface.[13] By the use of an internal label and a label close to the interface, information can be obtained on the penetration of the enzyme in the bilayer, as already demonstrated with *Candida cylindracea* lipase.[14]

In Fig. 6, a typical example of the change in the fluorescence spectra of S146A HLL upon titration with TMA-DPH vesicles

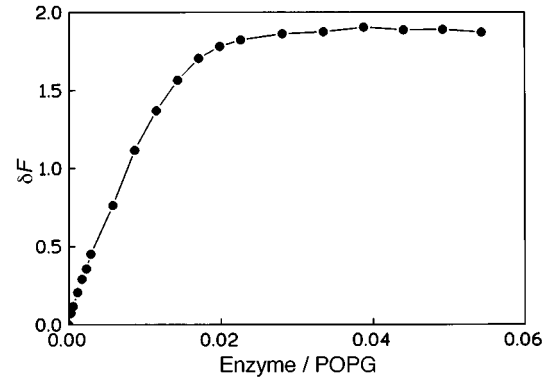

Fig. 5 Binding of S146A HLL to POPG vesicles (26.7 μM) containing 2.5% of NBD-PE. The RET intensity from Trp of enzyme to the label at the interface was measured at 535 nm (excitation at 280 nm).

is shown. The decrease in the fluorescence of the enzyme at around 330 nm coincides with an increase in the signal emanating from the label, indicating that the lipase binds to the membrane and that it is in close proximity to the interfacial probe. Similar spectra were obtained with DPH as acceptor.

In Fig. 7, the change in fluorescence of the Trp signal of S146A HLL bound to POPG vesicles with TMA-DPH or DPH are shown. The RET is more efficient with vesicles containing the shallow probe TMA-DPH, with a steep decrease in the enzyme fluorescence upon titration with the vesicles. This indicates that the enzyme is readily associated with the vesicles. The energy transfer to DPH labeled vesicles is less efficient, suggesting that the enzyme in the active form preferentially stays at the vesicle interface, with the α-helical lid possibly oriented parallel to the membrane surface and with the hydrophobic residues, such as Trp-89, slightly inserted in the hydrophobic part of the membrane but remaining close to the interface. This results are consistent with the fluorescence and quenching experiments described above. This is an important aspect in the activity of HLL, and it will be a topic for further investigation with mutants containing only one Trp residue.

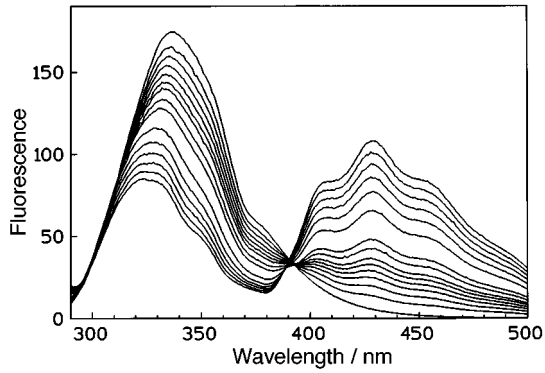

Fig. 6 Changes in the fluorescence spectrum of S146A HLL (2.5 μM) upon addition of POPG vesicles containing 2.5% TMA-DPH. Lipid concentration ranges from 0 to 666 μM. Excitation wavelength, 280 nm.

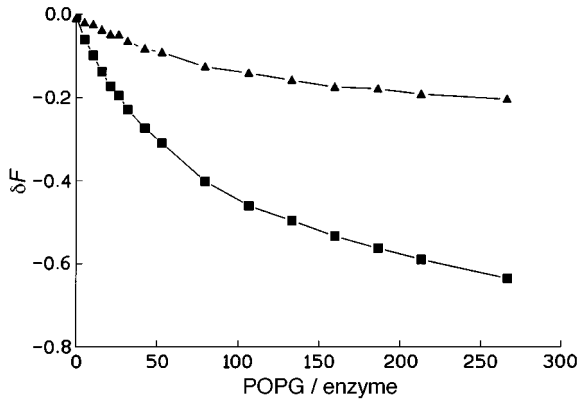

Fig. 7 Fluorescence emission at 340 nm monitored during the titration of S146A HLL (2.5 μM) with POPG vesicles containing (▲) the internal label DPH or (■) the interfacial label TMA-DPH. Excitation wavelength, 280 nm.

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