Linking Phospholipase Mobility to Activity by Single-Molecule Wide-Field Microscopy


Many of the biological processes taking place in cells are mediated by enzymatic reactions occurring in the cell membrane. Understanding interfacial enzymatic catalysis is therefore crucial to the understanding of cellular function. Unfortunately, a full picture of the overall mechanism of interfacial enzymatic catalysis, and particularly the important diffusion processes therein, remains unresolved. Herein we demonstrate that single-molecule wide-field fluorescence microscopy can yield important new information on these processes. We image phospholipase enzymes acting upon bilayers of their natural phospholipid substrate, tracking the diffusion of thousands of individual enzymes while simultaneously visualising local structural changes to the substrate layer. We study several enzyme types with different affinities and catalytic activities towards the substrate. Analysis of the trajectories of each enzyme type allows us successfully to correlate the mobility of phospholipase with its catalytic activity at the substrate. The methods introduced herein represent a promising new approach to the study of interfacial/heterogeneous catalysis systems.

1. Introduction

Lipases and phospholipases are interfacial enzymes found in most organisms in the microbial, plant and animal kingdoms. They play a crucial role as catalysts of lipid metabolism and as mediators of cell signalling processes.[1] In industry lipolytic enzymes are gaining increasing importance, being employed in biotechnological processes occurring at solid and oil–water interfaces.

Due to the prevalence and importance of these enzymes, their mode of action has been the subject of extensive study and is considered to be a model for understanding interfacial enzymatic catalysis. Nevertheless, a full understanding of the action of soluble enzymes acting on insoluble substrates is difficult, due to the innate heterogeneity of the system. Single-molecule fluorescence spectroscopy (SMFS) is naturally suited to the study of such complex systems as it allows deeper insight into locally inhomogeneous molecular behaviour not otherwise available from ensemble measurements.[2]

In the last decade, SMFS has been successfully applied to the study of enzyme kinetics. In ground-breaking experiments, the catalytic activities of individual immobilized enzymes, containing either a fluorescent co-factor[3] or using a pro-fluorescent substrate,[4,5] were monitored by confocal microscopy. The observed temporal fluctuations in catalytic turnover were attributed to cycling between several conformations of the enzyme, each conformation having a different catalytic activity.[6] Furthermore, it was observed that at high substrate concentrations, clusters of catalytic turnover events were separated by periods of low activity. Other so-called molecular memory phenomena, such as the stepwise deactivation of chromotrypsin enzymes, have also been observed.[7] While highly informative, such confocal microscopy studies are restricted to the availability of pro-fluorescent substrates. Furthermore, the use of large, non-natural substrates and enzyme immobilization may affect the catalytic activity of the enzyme.

Wide-field SMFS has the potential to complement confocal microscopic studies as the technique allows for the study of a wide range of enzymes acting on their natural substrates, as has been elegantly demonstrated for a number of DNA interacting proteins.[8–11] Importantly the ~μm² field of view and ms temporal resolution of wide-field fluorescence microscopy makes it ideally suited to the study of molecular diffusion processes. This is particularly relevant to the study of interfacial catalysis, where the catalytic action is convolved with diffusion of the enzyme across the target substrate. The wide field of

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view also enables the study of a large number of enzymes simultaneously, allowing good statistics to be obtained from a few experiments.\[14\]

For the majority of lipolytic enzymes it is accepted that catalytic action involves the following steps: 1) diffusion and adsorption of the enzyme to the lipid surface, 2) activation of the enzyme (constituting the opening of an α-helical loop—“lid”—to expose the hydrophobic active site of the enzyme), 3) penetration of the enzyme into the lipid phase, 4) catalytic hydrolysis and 5) either scrolling of the enzyme to the next substrate molecule or enzyme desorption.\[15\] Diffusion/binding processes are therefore crucial both before and after the catalytic reaction and should not be neglected if a full picture of interfacial catalysis is to be obtained.

To date only a few investigations of enzyme diffusion on substrates using fluorescence techniques have been reported. Fluorescence recovery after photobleaching (FRAP) was employed in most of those studies.\[16,17\] This technique measures the rate at which a photobleached area becomes repopulated with fluorescent enzymes, yielding an average diffusion coefficient. The heterogeneous motions of individual enzymes are therefore not resolvable using this technique.

Such heterogeneous motion can be revealed by the use of single-particle tracking (SPT). This technique is often used in quantitative studies of lateral mobility of proteins in membranes and has been extensively applied to the study of biomolecules, namely for the motion of lipid molecules in biomembranes.\[18,19\] the infection pathways of virus molecules,\[20,21\] and tracking receptors and proteins in living cells.\[22–25\] Recently, SPT was used to detect heterogeneous motions of single quantum dot (QD) lipase complexes diffusing on a substrate surface.\[26\] In that case the QD (~25 nm in diameter) dominated the diffusion behavior of the complex compared to the lipase (~5 nm in average diameter). However, the study suggests that SPT can be a powerful tool in the study of interfacial enzymology.

While the proposed mechanism of lipase/phospholipase activation emphasizes the importance of enzyme action, the pre-existing structural ordering of the lipid and/or lipid-water interface also influences the catalytic activity. Previously, atomic force microscopy (AFM) was used to follow changes to the lipid structure during enzyme action.\[27–30\] In these studies, enzymatic activity was inferred from the breakdown of the substrate layer (indicative of product desorption after lipid hydrolysis). These studies found a relation between pre-existing structural defects on the bilayers and the observed desorption.

Herein, we illustrate the potential of wide-field fluorescence microscopy to study interfacial enzymology within biological model systems at the single-molecule level. Specifically we study a phospholipase acting on phospholipid bilayers. By tracking the heterogeneous motions of fluorescently labelled single enzymes, whilst simultaneously monitoring substrate structure, we gain significant insight into the action of phospholipase. The information gained is greatly augmented by the study of closely related mutants with different affinities and catalytic activities towards the substrate.

Using this new approach, we are able to link the mobility of a phospholipase enzyme to its activity, providing a clearer picture of the overall mechanism of a biological interfacial enzymatic catalysis.

2. Results

The active phospholipase utilised herein is a mutant of the lipase Thermomyces lanuginosus (TLL). The TLL enzyme has been studied extensively and is an excellent candidate for enzyme movement towards phospholipases. Both the lid and the C-terminal region of TLL were mutated in order to activate it to the hydrolysis of phospholipids. The resulting mutant cleaves the ester bond of the phospholipid at the sn-1 position and is therefore designated phospholipase A1 (PLA1).

An inactive phospholipase, iPLA1, was generated from TLL in the same way as for the active PLA1 (aPLA1), but with an additional mutation on the catalytic site. The active serine is replaced by an alanine residue (S146A). Although this mutation causes some changes in the interaction between the substrate molecule and the active site of the enzyme, it is often used to generate inactive mutants in enzyme/substrate binding studies.\[31–34\] The iPLA1 was studied to evaluate the activation and penetration of an enzyme into a phospholipid layer, without subsequent hydrolysis occurring.

TLL, the parent enzyme of the PLA1 mutants and a catalyst for the hydrolysis of glycerides, is a lipase with a low affinity for phospholipid bilayers.\[32–34\] TLL is therefore of interest to characterize the diffusion and adsorption of an enzyme on phospholipid bilayers, without activation and penetration of the enzyme into the phospholipid layer.

As a phospholipid substrate, supported layers of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) were used. Although cellular membranes present higher complexity (consisting of different lipid/phospholipid components, sterols, and membrane proteins), this simple model is often used for mimicking biological membranes. While phospholipid molecules with saturated chains form highly ordered bilayers, unsaturated phospholipids form more fluid bilayers.\[35\]

2.1. The Relationship between Substrate Structure/Fluidity and Substrate Breakdown by Enzyme Action

Herein we investigate the relationship between substrate structure and substrate breakdown due to enzymolysis by imaging a fluorescently labelled POPC substrate in the presence of the non-fluorescent active PLA1 (aPLA1). The dye, Dil, was evenly dispersed (0.5 mol%) in the phospholipid bilayers (Figure 1a). Different fluorescence intensities allow discrimination between a single bilayer on the mica support and a bilayer on top of another bilayer (Figures 1b–d).

After addition of the aPLA1 enzyme (~10⁻⁷ M) to a dye-labelled POPC bilayer on mica, darker regions appeared in the fluorescence image of the bilayer (Figure 2, Supporting Information, Movies 1 and 2). Upon hydrolysis by PLA1, POPC is converted into palmitic acid and lyso-oleyl-phosphocholine. Previous studies performed using phospholipase A2 (PLA2)
showed that the enzymatic breakdown of the phospholipid molecules leads to the destruction of the supported bilayer and solubilisation of up to 90% of the lipid material. The dark features observed herein are attributed to the breakdown of the POPC layer, caused by desorption of reaction products and resulting in the release of the incorporated DiI label. The fluorescence quantum yield of DiI is known to be strongly reduced upon diffusion from a lipid layer into an aqueous environment due to the increase of cis–trans isomerisation.

The observed breakdown of the labelled POPC substrates nearly always starts from existing defect regions, like the edge between two consecutive bilayers. This observation is in good agreement with previous AFM studies. Depending on the number of bilayers present, the observed desorption patterns are different (Figure 2). For a phospholipid multilayer, hydrolysis is observed as the smooth recession of the top layer (Figure 2a and Supporting Information, Movie 1). However, for a single phospholipid bilayer in contact with the mica support, the desorption process depends on the enzyme concentration (Figures 2b–d and Supporting Information, Movie 2). At a high enzyme concentration (3.7 × 10⁻⁷ M), "wave-front"-like behaviour is observed. Desorption proceeds over a large area and the layer has a smooth receding edge (Figure 2b). At an aPLA1 concentration of 1.9 × 10⁻⁷ M, the morphology of layer desorption changes. Desorption is now confined to small channels with a typical width of 1.2 μm, creating fractal patterns in the substrate layer (Figure 2c). When the enzyme concentration is decreased further (4.0 × 10⁻⁹ M), layer desorption becomes extremely localised and is observed as a "nibbling" effect at the layer edge (Figure 2d).

It is known that pre-existing ordering and packing of the substrate bilayers influences the catalytic activity of interfacial enzymes. In recent years several studies have been published concerning lipid mobility, especially on cellular membranes. In order to quantify the fluidity of the POPC bilayers used here, single-membrane dye tracking experiments were carried out.

Substrate bilayers were prepared as described above, however in this case with less than 0.01 mol% of the fluorescent probe DiI. This concentration of DiI is still high enough to allow discrimination between successive phospholipid bilayers in initial imaging experiments. After photobleaching of most of the DiI by continuous irradiation, single DiI molecules could be resolved and their diffusion in POPC multi- and single bilayers tracked over time. The trajectories described by the single DiI probes were analysed using cumulative distribution functions (CDFs), following a statistical approach published by Schütz (see Section 2.3 and the Experimental Section for details). For diffusion through the top layer of a POPC multilayer, the DiI probe molecules show one mode of motion, with a diffusion constant of (4.88 ± 0.03) × 10⁻⁴ cm² s⁻¹. This homogeneity is lost for DiI molecules in a bilayer in contact with the mica support. In a bilayer on mica, a fraction of the DiI molecules were immobilized (~30%), and the remainder diffused slowly, D = (2.46 ± 0.03) × 10⁻⁸ cm² s⁻¹ (Supporting Information, Figure S2). This value is in good agreement with that obtained in previous studies (Supporting Information, Table).
The single-membrane dye tracking experiments confirm that the fluidity of a bilayer on mica is strongly reduced compared to the fluidity of multilayers. The reduced fluidity of the bilayer on mica is attributed to interactions between the mica and the phospholipids and can account for the unusual morphology of desorption observed in such bilayers. In the top layer of a POPC multilayer, the high fluidity allows product desorption to occur immediately following enzyme hydrolysis, leading to the smooth recession of the layer. Since this is also observed in natural systems, multilayers were employed in all subsequent experiments.

2.2. The Relation between Enzyme Localization and Substrate Structure

In order to visualize the enzyme acting on the substrate, aPLA1 was labelled with a water-soluble, fluorescent perylene diimide derivate (PDI). Due to its photostability, this class of dyes has proven to be suitable for SMFS experiments.[44, 45] More importantly herein, the relatively small size of the PDI (~20 Å) means it is less likely to influence enzyme behaviour compared to other labels (e.g. quantum dots with diameter > 20 nm).

The enzyme was labelled on the primary amine groups of its 6 lysine residues and one N-terminal lysine (Supporting Information, Figure S3). Since excess PDI was used in the labelling process, the maximum possible number of PDI molecules bound to each enzyme is seven. In control experiments, the enzymatic activity of the labelled and non-labelled aPLA1 against 5-carboxyfluorescein diacetate (5-CFDA) was the same[45] (Supporting Information, Figure S4). When the labelled aPLA1 (~10^7 M) was added to a non-labelled POPC multilayer, enzymes could be visualized as bright spots and areas of high enzyme localisation could be clearly seen (Figure 3 and Supporting Information, Movie 3). Despite the fact that the substrate layer itself is not directly visualised in this experiment, it is clear that the enzyme is localised primarily at the edge region between two consecutive bilayers. The position of the top layer of the POPC multilayer can easily be inferred as it is outlined by a high concentration of the enzyme, and this area is reduced over time with enzyme action (visualised as the directed motion of the line of high enzyme concentration). The experiments also show a non-uniform distribution of the labelled aPLA1 along the layer edge. Importantly, areas with a faster retraction of the top layer coincide with areas of higher local enzyme concentration (indicated by the dashed red circle in Figure 3).

2.3. Single-Enzyme Tracking

In this set of experiments, the mobility of fluorescently labelled enzymes was studied whilst simultaneously imaging the underlying fluorescently labelled POPC multilayer. Two-colour excitation was efficiently employed to excite both the PDI label on the enzyme and a 3,3'-dioctadecyloxocarbocyanine perchlorate (DiO) label dispersed in the POPC substrate (Experimental Section and Supporting Information, Figure S5). By choosing an appropriate optical filter before the detection apparatus, we could detect the majority of PDI emission and block most of the DiO emission (Figure 4b). Thus, we could easily discrimi-
nate between enzyme emission and substrate emission based on their relative brightness (single enzymes appear clearly as bright spots against the background of the weakly fluorescent substrate—Figure 4 a). In all experiments, a low enzyme concentration (~10^{-9} M) was used to allow discrimination of single enzymes and their subsequent tracking.

We include the study of two other enzymes of interest, iPLA1 and TLL enzymes. Both enzymes were fluorescently labelled in the same way as aPLA1.

2.3.1. The Mobility of aPLA1, iPLA1 and TLL

Single aPLA1 enzymes were tracked while catalysing the hydrolysis of POPC multilayers (Supporting Information, Movie 4). Despite the lower amount of enzyme present, it is still possible to observe the recession of the top substrate layer due to enzyme action (Figures 5 a,b and Supporting Information, Movie 5). The observed substrate desorption rate is not constant over time (Figure 5 c). In confocal microscopy studies of immobilized enzymes on non-natural substrates, temporal dynamics of catalytic turnover were attributed to the existence of several enzyme conformations.[6] However, when substrate bilayers are employed, other factors such as local membrane fluidity likely play the main role. The packing of phospholipid molecules fluctuate over time during hydrolysis, influencing the efficiency of enzyme penetration through the hydrophobic phospholipid residues and subsequent catalysis.

The diffusion of aPLA1 on the substrate bilayers is a highly heterogeneous process. Both fast-diffusing and immobilized enzyme molecules were observed. For each individual enzyme trajectory we can calculate its residence time on the bilayers. Most of the enzymes (95%) stay on the substrate for less than 0.3 s [under similar conditions the PDI label has a survival time (time until irreversible photodegradation) of ~5 sec].

The behaviour of the labelled iPLA1 enzyme contrasts greatly with the behaviour of the aPLA1. When the iPLA1 was added to POPC bilayers under identical conditions as for the active enzyme, recession of the top layer due to phospholipid desorption was not observed (Figure 6 and Supporting Information, Movie 6). Nevertheless, the inactive enzymes still have a strong binding affinity for the layer edge, with apparent residence times of 3.5–9 s. As the survival time of the label is ~5 s, photobleaching of the label rather than enzyme desorption can account for the disappearance of enzyme emission. The actual residence time of iPLA1 at the layer edge is probably much longer, as after an enzyme stops emitting, no further emission is observed at that site. This suggests that no new enzymes are able to access the layer edge at this site (Figure 6).

Since TLL has a lower affinity towards phospholipid bilayers, the enzyme molecules diffusing on the layer can only be visualised when the solution has 100 times higher enzyme concentration. Even then, only fast diffusion processes are observed. As expected, the TLL molecules adsorbed poorly on the layer and diffuse without any further interaction with the phospholipid molecules (Supporting Information, Movie 6).

2.3.2. Histogram-Based Image Reconstruction of Enzyme Localisation

The affinity of the aPLA1 for the layer edge was confirmed using reconstructed images of the precise location of each enzyme detected over an 1800 frame
movie (Figure 7a). A similar method, known as point accumulation for imaging in nanoscale topography (PAINT),146 was used to obtain high-resolution images of lipid bilayers. While PAINT images reveal roughly the areas where enzymes are located frequently (Figure 7a), the affinity of aPLA1 for the layer edge is obvious when a histogram image of enzyme location is calculated (Figure 7b). Figure 7b shows an example of a histogram-PAINT (H-PAINT) image of aPLA1 with a binsize of of 200 nm (note that the resolution of H-PAINT can be improved by increasing the number of fluorescent species detected). The H-PAINT images also reveal binding hotspots along the layer edge at which frequent enzyme localisation occurs.

The H-PAINT image of iPLA1 location shows clearly that the inactive enzyme also has a strong affinity for the layer edges (Figure 7d). As expected, both the PAINT and H-PAINT images of TLL (Figures 7e and f) show a random distribution of the enzyme molecules on the layer, with no special affinity towards the edge [the position of the layer edge is indicated by (----) in Figures 7e and f].

2.3.3. Analysis of Enzyme Trajectories

As mentioned previously, a major advantage of SPT is its ability to discriminate between different modes of motion of individual molecules. In membranes it is possible to observe several modes of motion: immobile, directed, confined tethered, normal diffusion and anomalous diffusion. For homogeneous diffusion, the time dependence of the mean-square displacement (MSD) is very different for these different modes, allowing easy classification.47 However, the trajectories of single molecules often exhibit dynamic heterogeneity of their diffusion modes (Figure 8). In this case, analysis cannot be per-

![Figure 7. Histogram-based image reconstruction. a), c) and e) PAINT image showing each enzyme molecule detected during the 1800 frames, for the a) aPLA1, c) iPLA1 and e) TLL. b), d) and f) H-PAINT image showing the histogram of the spatial distribution of enzyme molecules on the substrate. The probability of enzyme localization is indicated by the color bar, for the b) aPLA1, d) iPLA1, the inset showing the lower affinity for the top of the layer—background values and f) TLL, (-----) indicating the position of the layer edge.](image)

![Figure 8. Diffusion behaviour of labelled aPLA1 molecules. a) Snapshots of a single labelled enzyme diffusing on the layer edge and its corresponding trajectory. Scale bar: 1 μm. b) Typical trajectories of individual aPLA1 molecules diffusing on the layer (blue) and on the edge (red). The background image is accumulated over 100 frames. Some heterogeneous trajectories are magnified. In trajectories 2 and 3, it is possible to distinguish the hot spots (indicated by the red circles) where diffusion is slow, probably linked to catalysis.](image)
are apparent for enzyme motion on top of the layer ($D = 5.1 \times 10^{-8}$ and $7 \times 10^{-8} \text{cm}^2\text{s}^{-1}$, Figure 9a). Enzymes localized at the layer edge are seen to diffuse ($D = 1.7 \times 10^{-8}$ and $2.3 \times 10^{-8} \text{cm}^2\text{s}^{-1}$) with 19% of trajectories also including immobilised periods (Figure 9b). In the same analysis of the iPLA1 enzyme (516 trajectories), only one mode, immobilisation, was detected at the layer edge. On top of the layer, two motions are detected with $D = 5.2 \times 10^{-8}$ and $2.3 \times 10^{-8} \text{cm}^2\text{s}^{-1}$ (Figure 9c).

For the TLL (531 trajectories) only one mode of motion is detected, irrespectively of position on the substrate ($D = 3 \times 10^{-8} \text{cm}^2\text{s}^{-1}$, Supporting Information, Figure S8). Table 1 summarises the diffusion coefficients obtained. Since the surface of TLL was mutated, the absolute value of diffusion for this enzyme is expected to be different to that of the active and inactive PLA1 enzymes.

### Table 1. Diffusion constants detected for both active and inactive PLA1.

<table>
<thead>
<tr>
<th>Diffusion Constant [$10^{-8} \text{cm}^2\text{s}^{-1}$]</th>
<th>aPLA1</th>
<th>iPLA1</th>
</tr>
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<tr>
<td>Layer Edge</td>
<td></td>
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<tr>
<td>$5.1-5.2$</td>
<td>$58%$</td>
<td>$62%$</td>
</tr>
<tr>
<td>$1.7-2.3$</td>
<td>$41%$</td>
<td>$38%$</td>
</tr>
<tr>
<td>$0.7$</td>
<td>$42%$</td>
<td></td>
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<tr>
<td>$0.07$</td>
<td>$52%$</td>
<td></td>
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<tr>
<td>$\sim 0$</td>
<td>$7%$</td>
<td>$100%$</td>
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3. Discussion

By labelling the POPC substrate bilayers with a fluorescent probe, it was possible to follow its catalytic breakdown by a non-fluorescent aPLA1 enzyme. From this first set of experiments we concluded that breakdown of the substrate by hydrolysis starts from pre-existing defects on the layer. This confirms previous AFM studies and is easily rationalised given that penetration of the enzyme into the lipid layer is much easier at defect regions where molecular packing is less dense.\(^{48,49}\) This work improves upon existing AFM studies as the perturbation of the phospholipid bilayers by contact with the AFM probe is avoided and better time resolution can be achieved (milliseconds for wide-field imaging versus seconds for AFM measurements).

While desorption occurs as a smooth retraction of the top bilayer on a POPC multilayer, for a single bilayer directly on mica, desorption occurs in fractal and ‘nibbling’ patterns depending on enzyme concentration. The interaction between the phospholipids and the mica is confirmed by single-membrane dye tracking experiments that show a reduction in the fluidity of the bilayer. In contrast, the high fluidity of the top bilayer in multilayers makes them more suitable as models for biological membranes. Since the enzyme molecules were also found to interact strongly with the mica, single phospholipid bilayers on mica are considered inappropriate as substrates for these studies (Supporting Information, Figure S9).

In the second set of experiments, fluorescently labelled aPLA1 was imaged acting on a non-fluorescent POPC multilayer. While the substrate itself was not directly imaged in these experiments, the position of the top layer of the POPC multilayer was “outlined” by the high concentration of the enzyme at the layer edge. The experiments showed a heterogeneous distribution of enzymes along the layer edge, suggesting that the packing structure of a POPC layer edge is not uniform.

Figure 9. Dependence of the cumulative distribution function coefficients for PLA1 molecules with time. a) Active enzyme diffusing on the layer (•) $D = 5.1 \times 10^{-8} \text{cm}^2\text{s}^{-1}$ and (•) $D = 7 \times 10^{-8} \text{cm}^2\text{s}^{-1}$, b) Active enzyme diffusing on the layer edge, (†) $D = 1.7 \times 10^{-8} \text{cm}^2\text{s}^{-1}$, (©) $D = 7 \times 10^{-11} \text{cm}^2\text{s}^{-1}$ and immobilized, (•); c) Inactive enzyme, (•); immobilized, (†) $D = 2.3 \times 10^{-4} \text{cm}^2\text{s}^{-1}$, and (©) $D = 5.2 \times 10^{-6} \text{cm}^2\text{s}^{-1}$. Fitting is performed for as long as the data has statistical value.
Specific local defects along the layer edge or layer curvature can facilitate the formation of the enzyme–substrate complex and therefore the phospholipid hydrolysis. These experiments strengthen the link between the substrate breakdown and enzyme activity and provide an indication of the heterogeneous structure of the layer edge region.

In the final set of experiments, single labelled enzymes and labelled substrate bilayers were visualised simultaneously. Comparison of the motion of three different enzyme types allows us to correlate enzyme diffusion behaviour with specific stages of the catalytic process. Although single-enzyme emission was observed as diffusion-limited spots, the centre of mass can be localised with sub-diffraction limit resolution. The construction of histogram images of single enzyme location over time (H-Paint), whilst simultaneously monitoring substrate structure, was especially helpful in revealing the distinct behaviours of each enzyme.

TLL, an enzyme which cannot interact strongly with phospholipid bilayers, was found to diffuse quickly on the POPC multilayers (D = 3.0 × 10⁻⁸ cm² s⁻¹) with no specific preference for the edge or the top of the layer. The motion detected is most likely associated with weak adsorption and desorption of the enzyme on the layer, since the diffusion constant is 100 times slower than that expected for free diffusion in solution. These motions correspond to parts A and B of the catalytic cycle shown schematically in Figure 10.

Similarly, fast diffusive motions (D = 5.1–5.2 × 10⁻⁸ cm² s⁻¹) were observed for both the aPLA1 and iPLA1 diffusing on the POPC multilayers (Figure 8b). The surface of the TLL and PLA1 enzymes are different, and thus their diffusion constants cannot be compared directly. Nevertheless, we attribute the fast motions of the aPLA1 and iPLA1 on top of the layer to weak adsorption/desorption events (Figure 10A and B).

An additional motion was observed for both the PLA1 and aPLA1 on top of the layer (D = 0.7 × 10⁻⁸ cm² s⁻¹ for the active enzyme and D = 2.3 × 10⁻⁸ cm² s⁻¹ for the inactive enzyme). This motion may be that of enzymes that have been activated (i.e., are in the lid-open conformation) attempting to penetrate into the lipid phase, as seen in Figure 10C and D. The different speed of this motion for the two forms of the PLA1 enzymes can be attributed to the slight structural differences between them. Studies show that the replacement of the active serine by an alanine residue results in higher mobility of the lid and consequently to a lower binding affinity of the inactive mutant.

Unlike the TLL, both the active and inactive forms of PLA1 show periods of immobilization at the POPC layer edge. However, the residence time is much greater for the inactive enzyme. Enzymatic activity is thus not a prerequisite for strong enzyme intercalation at the layer edge, but is clearly a prerequisite for efficient desorption of the enzyme from the layer edge. The products of the hydrolysis reaction cause considerable reorganisation and solubilization of the supported bilayer and either effect could trigger desorption of the active enzyme.

In a previous study performed by FRAP comparison of the diffusion constants of active and inactive forms of TLL were made. In those ensemble measurements, the diffusion constant of the inactive form was ~4 times slower than that of the active form. In light of our results, the lower diffusion constant measured for the inactive lipase in the FRAP experiments is more likely due to the contribution of a higher percentage of immobilized enzymes. This is a clear example of the extra information that can be provided by single-molecule studies of heterogeneous systems.

A detailed look at the individual trajectories of the aPLA1 enzyme reveals that 64% of 1444 trajectories display changing diffusion behaviour (Supporting Information, Figure S10). These enzymes show periods of slow diffusion at different points on the layer edge being interspersed with faster diffusive motions along the edge. Trajectories 2 and 3 in Figure 8b are just two representative examples of hundreds of the aPLA1 enzymes monitored that show this varied diffusion behaviour. The heterogeneous motion is consistent with the notion of an active enzyme diffusing to and docking at the layer edge, hydrolysing the substrate, and then undergoing desorption or scrolling to another point on the layer edge.

In some cases (33% of the enzyme molecules diffusing on the edge) it is also possible to observe aPLA1 enzymes with trajectories that present only slow components of diffusion (D = 0.07 × 10⁻⁸ cm² s⁻¹ and immobilization). Importantly, all the aPLA1 trajectories exhibiting immobilized periods present at least one other mode of diffusion. Once again, this result is in agreement with the proposed model of lipase/phospholipase action: before efficient docking occurs, the enzyme needs to approach the substrate and undergo interfacial activation. When such docking does not occur, the enzyme molecule diffuses faster on the layer edge, and the trajectory described displays no “binding hot spots” (trajectory 1 in Figure 8b).

Figure 10. Proposed catalytic cycle for the mutants used. While in solution, the enzyme remains in the closed form (E), with the lid covering the active site. A) Binding of PLA1 to the surface (B) promotes lid displacement exposing hydrophobic residues that interact with the phospholipid interface (C), stabilizing the open form (E*). Partitioned substrate can then access the active site (D), forming the enzyme–substrate complex of the interface-bound form, (E*S). Hydrolysis leads to the formation (E) and subsequent desorption of the products (P). Following product release, the enzyme E* can diffuse on the substrate or into solution.
4. Conclusion

Herein, wide-field SMFS was used to gain deeper insight into the action of phospholipases acting on their natural substrate. We were able to establish for the first time the direct relationship between the local structural characteristics of the membrane and the activity of the enzyme. More importantly, we were also able to establish a direct relationship between enzyme mobility and enzyme activity at the single-molecule level. By comparing the behaviour of enzymes with different activities, diffusive motions specifically associated with different steps of the overall catalytic process could be distinguished. The results obtained are in agreement with, and contribute significant new understanding towards, the proposed model for catalytic action of lipases/phospholipases. The success of this approach suggests that our methods can be applied to the study of other important interfacial enzymes as well as other enzymatic processes that occur on membrane interfaces, such as signalling cascades or active transport. The methods reported herein should also be applicable to the study of a wide range of other heterogeneous systems.[52,53]

Experimental Section

Bi/multilayer Preparation: Phospholipid layers were prepared by the dehydration method.[54] Briefly, a POPC solution in chloroform (10 mg mL⁻¹, Avanti Polar Lipids) containing dye (1,1'-dioctadecyl-3,3,3',3'-tetrachlorofluorescein diacetate, DiOC₁₀, Molecular Probes) was spin-coated onto freshly cleaved mica (3000 rpm for 40 s). After drying under vacuum for 2 h, the samples were hydrated by immersion in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 7.5). The solution was then incubated at 4 °C for 3 h. In order to reduce the optical aberration effect caused by the use of mica, it was cleaved until it became almost transparent. To avoid damaging this extremely thin support, the mica was glued to a glass coverslip.

To study the influence of mica on enzyme activity, the use of mica was avoided by cleaving it until it became almost transparent. The cleaved mica was then glued onto a glass coverslip. To avoid damaging this extremely thin support, the mica was glued to a glass coverslip.

Protein engineering: The mutations were performed through recombinant DNA technologies using the Fusarium oxysporum phospholipase gene as template. Variants of TLL were generated and purified essentially as described previously.[55] The differences between the mutated PLA₁ and TLL sequences are shown in a sequence alignment (Supporting Information, Figure S1). A model structure was made using the homology program from Accelrys (www.Accelrys.com).

HEPES buffer solution: Enzyme solutions were prepared in a buffer containing 0.01 M HEPES (Fluka, 99.5%), 30 μM calcium chloride (Sigma 99%), 10 μM EDTA (Sigma 95%) and 0.15 M sodium chloride (Sigma–Aldrich 99.99%). The pH of the buffer solution was adjusted to 8 using sodium hydroxide (Sigma–Aldrich 99.998%).

Enzyme labelling: The N-hydroxysuccinimide perylene derivatized PDI-NHS (PDI-NHS) was synthesized and purified as described previously.[56] PDI-NHS was added in 20-fold excess to an enzyme solution in carbonate buffer (pH 7.5). The solution was then incubated at 4 °C for one hour to produce the dye-labelled enzyme. The PDI-NHS bond formed through the amino groups of the lysine residues. Removal of the dye from the enzyme solution was accomplished by the addition of a solid support. A support consisting of polystyrene beads with low degree of cross-linking, onto which was grafted a polystyrene glycol derivative with free terminal amino groups. The reacted dye was captured at the surface of the beads after shaking for five minutes. The solid support was separated from the enzyme solution by filtration. Gel electrophoresis studies confirmed that the reacted dye was efficiently removed from the enzyme solution.

Bulk activity measurements: The bulk enzymatic activity of each species was tested by means of a pro-fluorescent substrate. Specifically, hydrolysis of non-fluorescent 5-carboxyfluorescein diacetate (5-CFDA, Molecular Probes) yields the fluorescent product 5-carboxyfluorescein (5-FAM, Molecular Probes). Fluorescence intensity as a function of time was measured using a Spex Fluorolog 1500 (Spex Industries, Meuchen, NJ). The time base scan experiments were performed with 1 Hz acquisition rate. The fluorescent product was excited at 500 nm and the fluorescent emission was detected at 520 nm.

Concentration analysis: After labelling, the enzyme concentration was calculated by amino acid analysis. The protein was hydrolysed in a 18.5% HCl/0.1% phenol aqueous solution and incubated at 110 °C for 16 h. The mixture was dried and resolved in loading buffer (0.2 M NaCitrate, pH 2.2), filtered and loaded onto a Biochrom 20 Plus amino acid analyser. The measured amino acid content was fitted to the protein’s theoretical amino acid composition and the enzyme concentration calculated.

Wide-field microscopy: Imaging of layers and individually labelled enzymes on the multilayers was performed using an inverted epifluorescence microscope (IX71, Olympus) equipped with a 60x air objective (for Figures 1–2) or TIRF (total internal reflection fluorescence) objective (for Figures 3–6) and a cooled Electron Multiply-CCD (Cascade 512B, Princeton Instruments, Inc.). The labelled enzyme and labelled bilayers were excited with 1–5 kW cm⁻² of 532 nm (CDPS 532M-50, JDS Uniphase Co.) and 1–100 W cm⁻² of the 488 nm line from an Ar⁺ laser (Stabilite 2017, Spectra-Physics), respectively. Both laser lines were guided onto the sample through the same dichroic mirror (z532rdc, Chroma Technology, Inc.) and aligned in TIRF mode. The emission from both the enzyme and the membrane were observed through the same 545 nm longpass filter (HQS45 LP, Chroma Technology, Inc.). Most of the fluorescence from the bilayers is rejected by the long-pass filter such that the enzyme fluorescence can be detected with sufficient S/N ratio. Movies were recorded with 50 ms integration time at 20 Hz for single-enzyme tracking and with 500 ms integration time at 2 Hz for the visualization of layer retraction in Figure 2.

Spatial resolution and tracking: The determination of single-enzyme trajectories was performed using a home-developed routine in Matlab. The enzyme can be located with a precision of ~100 nm for slowly diffusing enzymes and ~200 nm for quickly diffusing enzymes. The layer edge is determined with a precision of 50–100 nm.

Determination of diffusion constants: As described elsewhere,[56] the mean-square displacement MSD(r) for individual enzyme molecules is the average over the complete trajectory of all measurement intervals nΔt, as given by Equation (1):

\[
\text{MSD}(r = n\Delta t) = \frac{1}{N-n} \sum_{i=1}^{n-1} \left[ (x_{i+n} - x_i)^2 + (y_{i+n} - y_i)^2 \right]
\]

where Δt (0.05 s herein) is the frame interval and N is the total number of frames for the trajectory. x and y give the position of the detected particle at t = i. For each n the sum runs over all possible intervals of length t = nΔt. For a particle diffusing randomly...
In two dimensions, the diffusion constant can be calculated using \( \langle r^2(t) \rangle = 4Dt \).

Instead of analyzing the trajectories individually, track sets at each region can be accumulated and analyzed with cumulative distribution functions (CDFs), following a method proposed by Schütz et al.\(^\text{40}\) CDFs \( \langle r^2(t) \rangle \) were constructed for every timelag \( t \) by counting the number of square displacements with value \( \leq r^2(t) \) and normalized by the total number of data points. For lateral diffusion, the CDF is characterized by Equation (2):

\[
P(r^2, t) = 1 - \exp \left[ -\frac{r^2(t)}{\langle r^2(t) \rangle} \right]
\]

(2)

\( P(r^2, t) \) describes the probability that a particle starting at an initial position will be found within a circle of radius \( r \) at time \( t \). It is important to note that the data can be analyzed in terms of the probability density function\(^\text{17}\) or the CDF\(^\text{40}\). The advantage of CDF analysis is that the summation integrates out much of the noise. For trajectories with a high degree of heterogeneity, the data can be analyzed assuming the existence of two or even three populations with different diffusion coefficients.

For a two-component model Equation (2) is modified to Equation (3):

\[
P(r^2, t) = 1 - \left\{ \alpha \exp \left[ -\frac{r^2(t)}{\langle r^2(t) \rangle} \right] + (1 - \alpha) \exp \left[ -\frac{r^2(t)}{\langle r^2(t) \rangle} \right] \right\}
\]

(3)

and for a three-component model Equation (2) is modified to Equation (4):

\[
P(r^2, t) = 1 - \left\{ \alpha_1 \exp \left[ -\frac{r^2(t)}{\langle r^2(t) \rangle} \right] + \alpha_2 \exp \left[ -\frac{r^2(t)}{\langle r^2(t) \rangle} \right] + \alpha_3 \exp \left[ -\frac{r^2(t)}{\langle r^2(t) \rangle} \right] \right\}
\]

(4)

where \( \langle r^2(t) \rangle \) and \( \alpha_i \) (\( i = 1, 2, \ldots \)) are the MSDs(\( t \)) and the relative contribution of each motion, respectively.

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