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Dispersed Phantom Scatterer Technique Reveals Subtle Differences in Substrate Recognition by Phospholipase D Inactive Mutants

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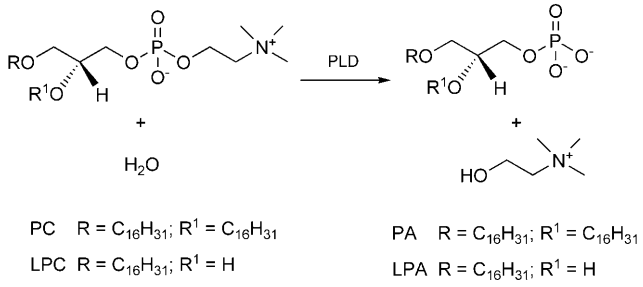
Phospholipase D (PLD; EC 3.1.4.4) catalyzes the hydrolysis of phospholipids, for example, it transforms phosphatidylcholine (PC) into phosphatidic acid (PA) and choline (Scheme 1). In the presence of alcohols, competing transphosphatidylation reactions have also been observed; this makes PLDs suitable for the synthesis of modified phospholipids of interest for the pharmaceutical and food industries.[1]



PC R = $C_{16}H_{31}$; R^1 = $C_{16}H_{31}$
LPC R = $C_{16}H_{31}$; R^1 = H
PA R = $C_{16}H_{31}$; R^1 = $C_{16}H_{31}$
LPA R = $C_{16}H_{31}$; R^1 = H

Scheme 1. The hydrolysis of phospholipids by phospholipase D (PLD).

PLDs are ubiquitous enzymes that are found in most mammalian cells as well as in plants and microorganisms. In eukaryotic cells, PLDs have been proposed to play an important role in several cellular functions,[2] and to participate in various signal-transduction cascades.[3,4] PLDs are part of a superfamily of proteins, the so-called PLD superfamily,[1,5–8] and show functional similarity and share the presence—usually in two copies—of the conserved $HxK(x)_4D$ (HKD) motif. A structural and mechanistic relationship among the members of this superfamily has been suggested.[1] Specifically, prokaryotic PLDs, which are smaller and less complex than the corresponding eukaryotic enzymes, are particularly suitable models for a more thorough investigation of the common catalytic mechanism and of the role of conserved residues in this superfamily.

The gene coding for a PLD from *Streptomyces* PMF (PLD-PMF) has been cloned and expressed in *E. coli* to afford a recombinant PLD in a soluble and active form.[9] Furthermore, the role of specific amino acids in the HKD domains for the catalytic activity of PLD-PMF has been evaluated by site-directed mutagenesis experiments.[10] Specifically, the essential role for catalysis of histidines 167 and 440 and lysines 169 and 442 of the two highly conserved HKD domains has been demonstrated by isolating and characterizing the corresponding enzyme variants PLD-H167N, PLD-K169S, PLD-H440N and PLD-K442S (numbered according to the literature[9]). The activity of these variants was completely lost even if the proteins did not present any significant structural modification.[10] X-rays analyses of PLD-PMF crystals that had been soaked in short-chain substrates and products have lately suggested a general reaction mechanism through an S_N2-type associative pathway (Scheme 2).[11] Specifically, it has been shown that the formation of the phosphohistidine intermediate (I) involves the conserved histidine of the N-terminal HKD domain (H167); this supports the hypothesis that H167 acts as the nucleophile by attacking the phosphorus atom of the phospholipidic substrates. It has been suggested that the conserved histidine of the C-terminal HKD domain (H440) plays a complementary role in the catalytic mechanism; it probably acts in the releasing of the leaving group (e.g., choline) and in the activation of the entering nucleophile (water or an alcohol). The role of other conserved residues, namely the two lysines 169 and 442, has not been fully clarified yet, and their role in substrate binding and transition-state stabilization has only been hypothesized.

Despite the recent advances in unraveling the catalytic mechanism of bacterial PLDs, little structural knowledge of the nature of substrate recognition in the active site of PLDs is available. Specifically, the identification of the choline-binding pocket might allow the design of new enzyme variants with altered substrate specificity.[12] To this end, selectively inactivated PLD mutants can be quite useful, because X-ray analyses of wild-type PLD-PMF crystals that had been soaked with short-chained substrates clearly indicate that released choline diffuses out of the active site.[11]

Before starting time-consuming X-ray analyses of PLD-PMF mutants in the presence of phospholipids, it would be advisable to evaluate the residual (if any) substrate-binding ability of these proteins. The interactions of substrates and their analogues with inactivated enzymes can be investigated with various analytical techniques, such as intrinsic fluorescence, NMR spectroscopy and surface plasmon resonance (SPR),[13–17] the latter has also been used to evaluate the interaction of some chimeric bacterial PLDs with PC vesicles.[18] In fact, none of

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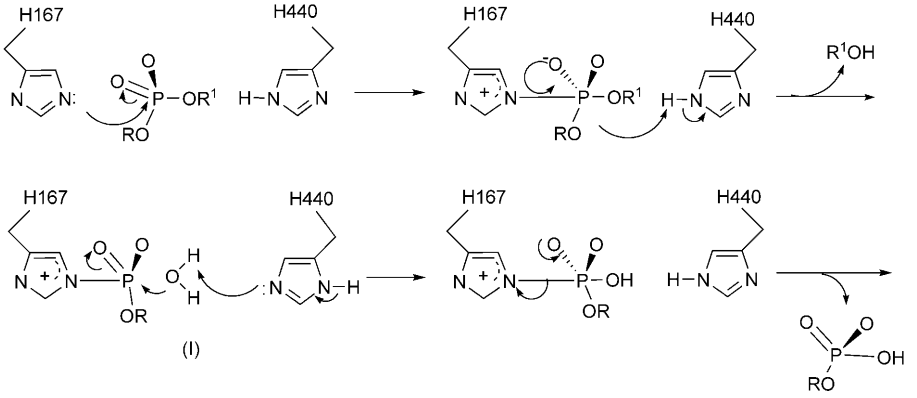
these techniques can provide a complete description of the mechanisms involved in these enzymatic processes, in terms of both quantification of the interactions and interpretation of the mechanism of recognition. For this reason, there is a continuous search for new methodologies that are capable of providing additional information on such phenomena.



Scheme 2. A general reaction mechanism through an S_N2-type associative pathway for phospholipase D-catalyzed hydrolysis of phospholipids. It has been shown that the formation of the phosphohistidine intermediate (I) involves the conserved histidine of the N-terminal HKD domain (H167); adapted from ref. [1].

In this light, we have recently developed the so-called dispersed phantom scatterer (DPS) technique, which has been successfully applied to study the interactions between different ligand–receptor systems, like antibiotics–peptides, avidin–biotinylated molecules and protein A–antibodies.[19–21] DPS exploits the variations in the intensity of the light that is scattered by a suspension of diluted colloids while molecular interactions take place at their surfaces. The keystone of the method is the use of fluoro-elastomeric nanoparticles with a radius of (39 ± 1) nm and a refractive index (n_{np}) of 1.325 at the working wavelength and temperature (1.332 is the refractive index of H_2O (n_w) under the same conditions). The scattered light of this material is at least three orders of magnitude less intense than that of ordinary commercial polystyrene latex spheres, and makes these colloids optically "phantom". The very weak observed scattering is essential to the DPS technique in that it does not mask the subtle variations in the scattering cross section due to molecular interactions. Because amphiphilic molecules in solution spontaneously adsorb onto these hydrophobic phantom nanoparticles (PNPs), these monodisperse colloids can be considered as a "substrate organizer": they favor the ordering of the molecules into a spherical monolayer that is analogous to the molecular packing of cell membranes. Once coated, PNPs can interact with other molecules ("ligands") via the molecular groups ("receptors") that the amphiphiles expose to the aqueous solution. The effects on the intensity of the scattered light (I_S) from the build up of the self-assembled amphiphilic monolayer and from the adhesion of other molecular species on top of it, can be described in the Rayleigh–Gans approximation, which is valid for scatterers that have low optical contrast with water.[22] Accordingly, I_S can be expressed as Equation (1):

$$I_S = a\ N\ (v_{PNP}\ \Delta n_{PNP,w} + v_{A1}\ \Delta n_{A1,w} + v_{A2}\ \Delta n_{A2,w})^2 + b \qquad (1)$$

where a and b are experimental constants. Parameter a depends on the specifics of the experimental setup, and b indicates the amount of spurious collected light, which is either scattered from impurities (dust, contaminants) or strays after reflections from cuvette walls; A1 and A2 are the receptor and the ligand molecules, respectively; v_{PNP}, v_{A1} and v_{A2} are the volumes of the nanoparticles and of the adsorbed species A1 and A2, respectively; $N = \phi/v_{PNP}$ is the number density of PNPs (ϕ is the volume fraction), n_{PNP} is the refractive index of the nanoparticles, n_w is the refractive index of water, and $\Delta n_{PNP,w} = n_{PNP}^2 - n_w^2$ is the optical contrast. Analogously n_{A1} and n_{A2} are the receptor and ligand refractive index, respectively, $\Delta n_{A1,w} = n_{A1}^2 - n_w^2$ and $\Delta n_{A2,w} = n_{A2}^2 - n_w^2$. Equation (1) indicates that the body of the PNP and the various molecular species adsorbed onto it contribute to I_S through the product (volume)×(optical contrast). The DPS technique is based on the fact that when $\Delta n_{PNP,w}$ of PNP is quite small, even small amounts of adsorbed molecules can affect I_S significantly. This is possible because organic molecules have a typical refractive index that is around 1.5, which is definitely higher than n_{PNP} and n_w. Hence, when anchored onto the PNPs, organic molecules effectively increase the light scattered from the particles. Therefore, measurements of I_S enable quantification of the molecular mass adsorbed on the PNPs and hence the determination of the amount of adsorbed receptors and bound ligands. The same procedure can be extended to any additional group of molecules that further increment the amount of molecules bound to the PNPs through molecular interactions. As an example, Figure 1 A shows the absorption profile of a commercial surfactant, *n*-pentaethylene glycol monododecyl ether (C12E5). In the same experiment, after completion of the C12E5 monolayer, bovine serum albumin (BSA) was added to the PNP suspension. As expected, the lack of a further increase in I_S, indicates the absence of unspecific C12E5–BSA interactions.

In this work, we have investigated the possibility of using the DPS technique to obtain qualitative and quantitative information on the molecular recognition of natural substrates by specifically inactivated PLD-PMF variants. Besides our general interest in a better definition of the role of specific active-site amino acids in the catalytic mechanism of this enzyme, there were additional reasons to consider this technique as particularly well suited for studying PLD–substrate interactions. First of all, phospholipids, the natural PLD substrates, are amphiphilic molecules and therefore it was reasonable to expect that they could efficiently adsorb onto the surface of PNPs and be

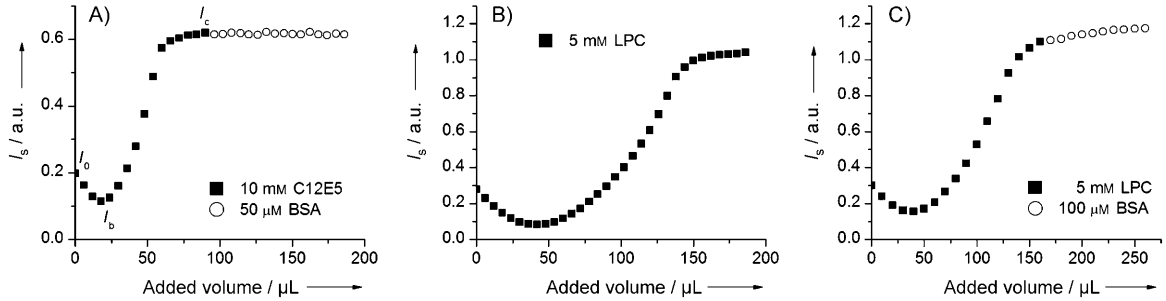


Figure 1. A) Scattered light intensity I_S was measured as the surfactant C12E5 (10 mM, ■) and BSA (50 μM, ○) were added to PNPs. I_0, I_b and I_c indicate the values of scattered light intensity for bare PNPs, the residual light collected when the amount of C12E5 coating is such that it produces perfect optical matching with water, and the value of I_S when PNPs are fully covered by C12E5, respectively. B) Adsorption profile of lysophosphatidylcholine (LPC, 5 mM, ■) on phantom nanoparticles. C) Scattered light intensity measured when BSA (100 μM, ○) was added to LPC-coated PNPs.

used as receptors. Moreover, inactive PLD-PMF variants obtained in our previous work[10] could be considered to be ideal ligands for testing the ability of this technique to discriminate extremely subtle structural differences in the enzyme active site, because the overall structures of the mutants are indistinguishable from one another and from the wild-type enzyme. Previously reported SPR analysis of the effects of specific mutations in other bacterial PLDs on substrate recognition showed variances in the shape of the association/dissociation curves that could not be easily rationalized.[18,23] This has often hampered the exact determination of the corresponding binding constants, and thus gives only "semiquantitative" information on the mutants' affinities for phospholipids, for example, the respective maximal response values expressed as SPR resonance units.

Because PC has poor solubility in water, in the experiments described here we used the water-soluble lysophosphatidylcholine (LPC, Scheme 1), a compound that is usually recognized by bacterial PLDs with similar K_M values to PC.[24] Once added to the nanoparticle suspension, LPC was quickly adsorbed on the PNPs and created a monolayer that was potentially capable of specific interactions with PLD (Figure 1B). In a control experiment, in which BSA was added after LPC coating, only a very moderate increase in I_S was observed (Figure 1C); this indicates that unspecific interactions were weak enough so as not to interfere with the analysis of PLD-PMF mutants. As a final preliminary test, the recognition of PNP-adsorbed LPC by wild-type PLD-PMF was evaluated by measuring the amount of choline released after the addition of the active enzyme by using a coupled choline oxidase–peroxidase assay (see the Experimental Section for details) and compared with the results obtained in the presence of free LPC. In both cases, more than 70% of the LPC (initial concentration 0.5 mM both for fully covered PNPs and in solution) was hydrolyzed in less than 2 min; this is a reasonable result when one considers the specific activity of PLD-PMF for this substrate (about 4.2 U mg^{-1}).

DPS measurements of binding affinity must be performed at the equilibrium and the affinity constants can be extracted from the measured fraction of ligands associated to receptors at various concentrations. The sensitivity of the method is optimized when the total concentration of receptors on PNPs is of the order of the binding constant that characterizes the investigated interaction. The binding constants of PLDs for their substrates are usually in the sub-μM range,[17,18] a concentration at which LPC would cover only a small portion of the total nanoparticles' surface. In order not to leave the PNP surfaces bare, particles were fully coated with LPC-C12E5 mixtures. The concentrations were chosen so that the coated PNPs carried a total of approximately 0.8 μM LPC, while the large majority of the particle surface was coated by a layer of the noninteracting C12E5 surfactant (see the Experimental Section for details). PNPs prepared in this way were then used in binding experiments with the inactive mutants PLD-H167N, PLD-K169S, PLD-H440N and PLD-K442S to qualitatively evaluate binding phenomena and, if possible, to extrapolate the corresponding binding constants of these proteins for LPC. As an example, Figure 2A shows the adsorption curve of PLD-H440N on LPC/C12E5-coated PNPs. Protein addition resulted first in a marked increase in I_S, which later leveled off to a saturated value corresponding to the saturation of available LPC molecules on the PNP surface. The observed curve agreed with the notion that the PLD-H440N–LPC recognition event is a first-order process. This was demonstrated by the good match of first-order Langmuir isotherm to the data (Figure 2B). Through the fitting procedure, a dissociation constant (K_d) value of ~52 nM was determined for the receptor–ligand complex formation.

Similar binding experiments allowed us to determine the dissociation constants of the other inactivated enzyme variants (Figure 2C). Although a limited number of mutants were used in this study, the observed exquisite sensitivity of the DPS technique in response to subtle active-site modifications was remarkable. The PLD-H440N and PLD-K169S variants showed a stronger affinity for LPC in comparison with the PLD-K442S mutant; their K_d values were one order of magnitude lower than the one estimated for the latter protein. Surprisingly, a similar stable interaction between LPC–PNPs and the mutant PLD-H167N was not observed at all (Figure 2C and Table 1).

As previously discussed, X-ray analysis of substrate-soaked PLD-PMF crystals[11] allowed the identification of H167 as the nucleophile in a catalytic mechanism that invokes the formation of a covalent intermediate between the substrate and the wild-type enzyme (Scheme 2). The observed lack of binding of PLD-H167N to LPC-PNPs indicates that this amino acidic substi-

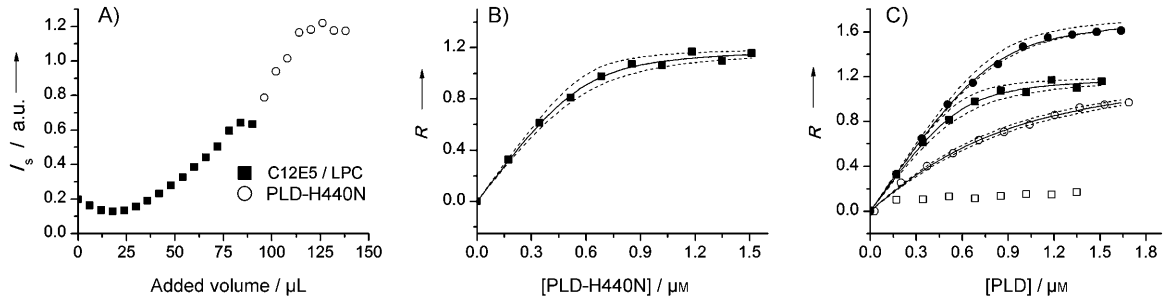


Figure 2. A) Interaction between the mutant PLD-H440N and LPC dispersed in a layer of noninteracting surfactant C12E5 (see the Experimental Section for details). B) Langmuir isotherms of the interaction between PLD-H440N and LPC/C12E5-coated PNPs. The continuous lines represent the best fit with a first-order binding process. Dashed lines are obtained by setting K_d equal to the extrema of the confidence interval given by the fitting procedure (Table 1). C) Langmuir isotherms for the interactions between the various PLD mutants with lysophosphatidylcholine. ●: PLD-K169S; ■: PLD-H440N; ○: PLD-K442S; □: PLD-H167N.

Table 1. Dissociation constants between different PLD mutants and LPC/C12E5-coated PNPs.

Enzyme variant	K_d [M]
PLD-H440N	$5.2 \pm 1.6 \times 10^{-8}$
PLD-H167N	no binding
PLD-K442S	$4.6 \pm 0.9 \times 10^{-7}$
PLD-K169S	$6.6 \pm 1.6 \times 10^{-8}$

tution affects not only enzyme activity but also substrate recognition. On the other hand, the measured tight substrate binding by mutants PLD-K169S, PLD-H440N and PLD-K442S raised the question of whether the formation of a covalent intermediate by nucleophilic attack of the H167 residue to the substrate still occurred. Because all three mutants were completely inactive from a catalytic point of view, this event should have been followed by a dead-end state in which phosphatidic acid remained bound to the protein while a stoichiometric amount of choline was released. To verify this hypothesis, incubation of LPC-PNPs with mutants PLD-K169S, PLD-H440N and PLD-K442S was repeated, and after concentration of the water phase by ultrafiltration and lyophilization, the possible release of a stoichiometric amount of choline was tested by using the choline oxidase–peroxidase assay. Choline was not detected in any of these samples; this suggests that the observed binding of the three enzyme variants with the LPC-PNPs was exclusively mediated by noncovalent interactions. At this stage it is not clear if the inability to form the covalent intermediate by the PLD-K169S, PLD-H440N and PLD-K442S mutants should be ascribed to the specific amino acids introduced by site-directed mutagenesis, that is, asparagine in position 440 or serine in position 169 or 442, or if the wild-type residues are essential in these positions to coordinate the nucleophilic attack of H167 to give a covalent intermediate.

In summary, we have described a new application of the dispersed phantom scatterer (DPS) technique, which allowed us to qualitatively and quantitatively evaluate substrate recognition by inactivated PLD-PMF mutants. Significant differences in substrate binding were measured—a result that could not be predicted a priori; this highlights the capability of the DPS technique to "see" interaction events at a molecular level. Specifically, the use of DPS seems to have some distinct advantages over other methods in the case of phospholipid-acting enzymes. In fact, the substrate (or a substrate analogue) can be organized onto the surface of PNPs at a desired concentration with optimal display of the polar head group, while the hydrophobic chains are packed into the surfactant monolayer, which limits the occurrence of unspecific interactions. We can surmise that more detailed information on the PLD-PMF substrate-recognition process might come from binding studies of additional enzyme variants (generated from inactive mutants with high substrate affinity, i.e., PLD-H440N or PLD-K169S) with phospholipid decorated PNPs.

Experimental Section

Materials: Penta(ethylene glycol)monododecyl ether (C12E5) was purchased from Fluka. Lysophosphatidylcholine was purchased from Avanti-Lipids. Water was deionized and ultrafiltered by a MilliQ apparatus from Millipore Corporation. Perfluorinated spherical latex particles with a radius of (39 ± 1) nm and refractive index (n_{np}) 1.325 were supplied by Solvay Solexis (Bollate, Italy).

Preparation of PLDs: Wild-type and mutant PLD-PMF variants were produced and purified as previously described.[9,10] Briefly, the wild-type expression vector, pETPLD, was produced by in-frame cloning of the DNA fragment that encoded the mature phospholipase D from *Streptomyces* sp. strain PMF (PLD-PMF) downstream of the *Erwinia carotovora* pelB leader sequence of pET27b(+) (Novagen, Merck KGaA, Darmstadt, Germany) and site-directed mutagenesis was performed by using the QuikChange II® XL Site-Directed Mutagenesis Kit (Qiagen) and pETPLD as a template. Freshly transformed *E. coli* BL21(DE3)pLysE(+) (Novagen, Merck KGaA, Darmstadt, Germany) harboring either pETPLD or mutant plasmids were cultured in LB medium (50 mL) that contained kanamycin (50 µg mL^{-1}) and chloramphenicol (25 µg mL^{-1}) and incubated at 37 °C and 220 rpm until $OD_{600} = 1$ was reached. The cultures were used to inoculate fresh medium (500 mL) and growth of recombinant *E. coli* was carried out at 30 °C and 220 rpm until $OD_{600} = 1$ was reached. Isopropyl-β-thiogalactopyranoside (IPTG) was then added (final concentration 0.1 mM) and the culture was incubated at 25 °C for 24 h. Secreted enzyme variants were purified to electrophoretic homogeneity from culture supernatant by ammonium sulfate precipitation and ion-exchange chromatography on Fractogel TSK CM-650 (M) and Fractogel EMD DEAE-650 (S; both from

Merck) as previously described. SDS-PAGE was performed with polyacrylamide gels (10%, *w/v*) according to Laemmli,[25] and proteins were stained with Coomassie blue R-250 solution (0.25%, *w/v*) in EtOH (50%, *v/v*) and acetic acid (10%, *v/v*). Protein concentration was determined with the Bio-Rad protein assay, according to Bradford,[26] by using BSA as a standard.

Enzyme and choline release assays: Homogenous PLDs were assayed by using the natural substrates phosphatidylcholine (PC) and lysophosphatidylcholine (LPC) in the spectrophotometric choline oxidase–peroxidase coupled assay described previously.[27] Choline release after interaction of LPC-PNPs with PLD variants was determined by using an adapted protocol. Samples were prepared by separating LPC-PNPs and proteins from soluble fractions with a Nanosep® 10K Omega centrifugal device (MWCO 10000 Da, Pall Life Science, New York, USA), and then lyophilized and resuspended in deionized H_2O (100 μL). A mixture of choline oxidase solution (15 μL; 50 U mL^{-1} in 0.1 M Tris-HCl buffer, pH 8.0), peroxidase solution (50 μL; 100 U mL^{-1} in 0.1 M Tris-HCl buffer, pH 8.0) and 4-aminoantipyrine (4-APA)/phenol reagent (700 μL) was added to the sample. The sample was then prepared to a final volume of 1 mL with Tris-HCl buffer (0.1 M, pH 8.0), mixed and incubated at 37 °C for 30 min. The 4-APA/phenol reagent was freshly prepared by mixing phenol solution (2 mg mL^{-1}; 2 mL) in Tris-HCl buffer (0.1 M, pH 8.0), 4-APA solution (3 mg mL^{-1}; 2 mL) in Tris-HCl buffer (0.1 M, pH 8.0), and Tris-HCl buffer (0.1 M, pH 8.0; 21 mL). The amount of released choline was estimated spectrophotometrically at 550 nm in comparison with a linear calibration curve obtained with choline (0–0.1 mM) under the same assay conditions.

Elastic light-scattering experiment: PNPs produced in aqueous dispersions were dialyzed before use to remove undesired physisorbed byproducts. All experiments were carried out in phosphate buffer (2 mM, pH 7.0). Each experiment used a suspension at a PNP volume fraction $\phi = 10^{-3}$ (1.5 mL). PNP suspensions were stable in H_2O because the particles bore a net negative charge. PNP concentration could be simply determined by measuring the density of the dispersions, given the high density ($\rho \approx 2.1$ g cm^{-3}) of the bulk fluoroelastomeric material from which they were made. Experiments were performed by repeatedly adding aliquots (6 μL) of a mixture of surfactants (10 mM C12E5/14 μM LPC) and measuring I_S until no more increment of scattered light was observed. The I_S measurements were performed by taking sets of 80 independent intensity acquisitions after each addition and analyzing the data to eliminate intensity spikes due to dust impurities. Typically, full coating of the PNPs surface was achieved after the addition of 90 μL of the LPC/C12E5 mixture. This yielded a final LPC concentration on the nanoparticle surfaces of about 0.8 μM. LPC–PLD binding experiments were performed by repeatedly adding aliquots (6 μL) of a PLD solution (50 μM) to the LPC/C12E5-coated PNP suspensions and measuring I_S after each addition as previously described. From the measured I_s, it was possible to extract parameter R, which is proportional to the adsorbed volume v_{PLD} of the ligand [Eq. (2)]:

$$R = \frac{\sqrt{I_s - I_b}}{\sqrt{I_0 - I_b}} - \frac{\sqrt{I_c - I_b}}{\sqrt{I_0 - I_b}} = \frac{v_{PLD}\Delta n_{PLD,w}}{v_{PNP}\Delta n_{PNP,w}} \quad (2)$$

where I_0, I_b and I_c are the light scattered from the bare particles, the background light measured in the conditions of best optical matching between PNP and H_2O (Figure 1 A, I_b) and the light scattered by PNPs fully coated with LPC/C12E5, respectively. The total amount of PLD adsorbed on the particles, and hence the parameter R, can be well approximated by a Langmuir isotherm [Eq. (3)]:

$$R = R_0 \frac{[A2]_t + K_d + [S_0] - \sqrt{([A2]_t + K_d + [S_0])^2 - 4[A2]_t[S_0]}}{2} \quad (3)$$

where $[S_0]$ is the molar concentration of the binding sites, K_d is the dissociation constant and R_0 is an experimental parameter that sets the saturated value of R at the limit of large ligand concentration. By fitting the Langmuir isotherm to the data, we determined K_d and R_0. In the case of mutant PLD-K442S, the dissociation constant was higher than that observed with the other enzymes. In this situation, an accurate measurement would have required higher concentrations of ligand, and hence too much material. In this case, we held R_0 fixed to a value that was obtained by averaging those from fittings to the data from mutants PLD-H440N and PLD-K169S.

Elastic light-scattering experiment: The 90° angle polarized scattered light from a HeNe (5 mW, 632 nm) laser beam was collected and measured by using a RCA 931B photomultiplier. A conventional spectrophotometer cuvette (4 mL) held in a suitably designed cell holder and equipped with a ministirrer and a water flow circuit to control the working temperature (30 °C), which was measured by a thermistor, was used. All proteins and surfactants were injected by using motorized commercial pumps (Ismatec Reglo piston precision pump and Kent Scientific Genie syringe pump; Torrington, CT, USA). Stirring, temperature and injections were controlled by a computer, through a suitable interface that was designed ad hoc to program DPS experiments. Experiments were performed with a programmed sequence of injection, stirring and data acquisition.

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Keywords: enzymes · light scattering · mutagenesis · nanomaterials · phospholipase D

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