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Surface Dilution Kinetics of Phospholipase A₂ Catalyzed Lipid-Bilayer Hydrolysis

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Abstract

Phospholipase A₂ (PLA₂) enzymes catalyze hydrolysis of phospholipids in membranes. Elucidation of the kinetics of interfacial enzymatic activity is best accomplished by investigating the interface substrate concentration dependence of the activity, for which appropriate diluents are required. PLA₂ is stereo selective toward the L-enantiomers of phospholipids. A novel approach employing D-phospholipids as diluents to perform surface dilution kinetic studies of PLA₂ is presented. Activity of bee-venom PLA₂ at mixed L+D-DPPC (dipalmitoylphosphatidylcholine) bilayer interfaces was measured as functions of substrate L-DPPC mole fraction and vesicle concentration, using a sensitive fluorescence assay. A model for interface enzymatic activity based on the three-step kinetic scheme of: (i) binding of PLA₂ to the bilayer interface; (ii) binding of a lipid to PLA₂ at the interface; and (iii) hydrolysis, was applied to the hydrolysis data. Activity profiles showed that D-enantiomers also bind to the enzyme but resist hydrolysis. Activity dependences on vesicle and substrate concentrations could be disentangled, bringing resolution to an outstanding problem in membrane hydrolysis, of separating the effects of the three steps. Individual values of the kinetic parameters of the model including the vesicle-PLA₂ equilibrium dissociation constant of step (i), interface Michaelis-Menten-Henri constant for L and D-DPPC of step (ii), and the rate constant for interface hydrolysis, step (iii) were obtained as solutions to equations resulting from fitting the model to the data.

Keywords

Kinetics; Phospholipase A₂; Phosphatidylcholine; Membranes/Physical chemistry; Interfacial Enzymology

INTRODUCTION

Investigations of protein-lipid bilayer interactions and their interfacial reaction kinetics need a medium in which the substrate lipid concentration can be varied so as to observe the variation of the reaction velocity as a function of the interface substrate concentration. Bilayer hydrolysis is of significance to membrane biochemistry because the hydrolysis products perform several crucial physiological functions¹. An immediate problem that arises in lipid bilayer surface kinetics is the inability to vary interface substrate concentration because bilayers are normally unimolar comprising a single type or mixtures of lipids; all of which are substrates. Micelle forming detergents disrupt membrane structure and therefore are not suitable as neutral diluents^{2,3}. This has been a bottleneck to progress in testing kinetic models of membrane lipid hydrolysis⁴. The problem is overcome in the case of those enzymes that are stereo-selective toward their substrates by a novel use of mixtures of

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substrate and non-substrate enantiomers in various proportions to actualize a variable concentration reaction medium. Lipases and phospholipases are a super family of enzymes that exhibit this selectivity⁵. Phospholipase A₂ (PLA₂) is selective toward the L-phospholipid and catalyzes hydrolysis of L-phospholipids at the *sn*-2 bond. The concept of surface dilution kinetics in bilayers, using D-phospholipids as diluents, is demonstrated and a Michaelis-Menten-Henri type of kinetic model for membranes is tested, with the example of phospholipase A₂ (PLA₂) catalyzed lipid bilayer hydrolysis. Kinetic parameters of the model for membrane hydrolysis are derived. The focus of this work is on the elucidation of the interfacial kinetic scheme. Several excellent kinetic modeling and investigations of PLA₂ have been reported^{6,7}. However, the various steps of the kinetic scheme can be dissected experimentally only through surface dilution studies. The parameters of interfacial kinetics result from the physicochemical properties of the membrane. PLA₂ enzymes are activated, inhibited, modulated by biophysical behaviors of membranes such as domain formation, phase transitions, rafts etc. which affect any or all of the steps in the kinetics. Membrane biophysical properties as well as direct drug-enzyme binding contribute to inactivation⁸. Some drug molecules inhibit activity by preventing PLA₂ binding to the bilayer interface⁹. This work is of significance to the biophysical chemistry of interfacial kinetics of PLA₂ because the effect of membrane properties on the different kinetic steps can be distinguished.

The kinetics of bee-venom PLA₂ catalyzed hydrolysis of L-dipalmitoylphosphatidylcholine (DPPC) in small unilamellar vesicles (SUV) was investigated using D-DPPC as the diluent. Measurements of interface enzymatic activity, defined as the initial reaction velocity per mg of enzyme, were conducted as a function of the mole fraction of the substrate L-DPPC.

Surface dilution employing D-enantiomers was first applied to mixed micelles of bile salts and phospholipids where the lipid portion of the micelle was a mixture of D and L-phospholipids¹⁰. Activities at micellar interfaces are high enough for measurement by standard pH-Stat methods so that surface dilution kinetics is observable for lipids dispersed in detergent micelles. The low levels of the more biologically relevant bilayer hydrolysis on the other hand are beyond the precision of pH-Stat.

Recent development of a sensitive fluorescence assay, using the acrylodan labeled rat-intestinal fatty acid binding protein (ADIFAB), permitted the present measurements of the low levels of activity at bilayer interfaces¹¹. Together with D-lipids as diluents, observation of surface dilution kinetics in lipid bilayers is realized.

MATERIALS AND METHODS

Materials

L-DPPC was obtained from Avanti Polar Lipids as lyophilized powders. D-DPPC and bee-venom PLA₂ were obtained from Sigma. PLA₂ was purified by dialysis against 0.05M Hepes buffer at pH 7.4 for three days, changing the buffer every 8 hours¹². Protein concentration was determined by the extinction coefficient method¹². The dialyzed enzyme was stored at 4°C. The fluorescence probe, ADIFAB, was obtained from FFA Sciences (San Diego, CA).

Kinetic Model for Membrane Hydrolysis

The activity, A , as a function of the substrate lipid mole fraction, X_L , for a mixed bilayer vesicle of L and D-phospholipids, where the D-enantiomer is hydrolysis-resistant but binds to the enzyme is¹⁰,

$$A = \frac{X_L}{a_1 - a_2 X_L}, \quad (1)$$

where

$$a_1 = \frac{K_S K_{MS}}{k_3 n [\text{vesicles}] [L+D_DPPC]_S} + \frac{K_{MS}}{k_3 K_{DS}} + \frac{K_{MS}}{k_3 [L+D_DPPC]_S} \quad (2)$$

$$a_2 = \frac{1}{k_3} \left(\frac{K_{MS}}{K_{DS}} - 1 \right), \quad (3)$$

based on the three step kinetic scheme (Fig. 1):

(i) enzyme- interface binding, with association and dissociation rate constants k_1 and k_{-1} respectively, and equilibrium dissociation constant denoted by $K_S (= \frac{k_{-1}}{k_1})$, to form the interface-bound enzyme E^* ; (ii) enzyme-substrate lipid binding at the active site to form the interfacial complex E^*L or E^*D with association and dissociation rate constants k_{2L} and k_{-2L} or k_{2D} and k_{-2D} respectively and $K_{DS} = \frac{k_{-2D}}{k_{2D}}$ is the interfacial equilibrium D -enantiomer-enzyme dissociation constant and (iii) substrate lipid hydrolysis with rate constant k_3 ¹³. [vesicles] is the concentration of vesicles in solution; n is the number of binding sites per vesicle. The subscript S on the concentrations refers to interface or surface concentrations. $[L+D_DPPC]_S$ is the constant total lipid surface concentration, given by the outer monolayer surface concentration,

$$[L+D_DPPC]_S = \frac{N}{2A_{\text{ves}}N_0} = \frac{N}{8\pi R_{\text{ves}}^2 N_0} \quad (4)$$

where N , A_{ves} , and R_{ves} are the vesicle lipid aggregation number, area and radius of DPPC SUV, respectively. The outer monolayer aggregation number is taken to be $N/2$. N_0 is Avogadro's number. The interface substrate concentration is $[L_DPPC]_S = X_L [L + D_DPPC]_S$. Because $[L+D_DPPC]_S$ is constant, the molar fraction X_L is used to express the variable substrate concentration.

$$K_{MS} = \frac{k_{-2L} + k_3}{k_{2L}}, \quad (5)$$

is the interface Michaelis-Menten-Henri parameter defined by the interface reaction rate constants k_3 , k_{2L} and k_{-2L} .

In the limit $K_{DS} \rightarrow \infty$ for non-binding diluents, the classic equation of Deems et al. for lipid-detergent mixed micellar interfaces is recovered¹³. Furthermore, if the hydrolysis-resistant non-substrate component binds to the enzyme, then it is functionally similar to a competitive inhibitor. The present eq. 1 is identical to the equation derived by Berg et al. for activity in the presence of competitive inhibitors (eq. 1 in the reference cited)¹⁴.

The activity, according to the model in eq.1, depends on the substrate concentration quantified by the L_DPPC mole fraction and the concentration of vesicles given by

$$[\text{vesicles}] = \frac{[L + D_DPPC]}{N}, \quad (6)$$

where N is the number of lipid molecules in a vesicle.

Experimental Methods

Activity, A, as a function of X_L for each of several vesicle concentrations obtained by varying the total lipid concentration (eq.6) was measured. Fitting of the data of A vs. X_L to eq. 1 yields the values of a_1 and a_2 . The vesicle concentration dependence of a_1 was then fit to eq. 2. Results of the fits of a_1 vs. [vesicles] together with a_2 form a system of equations which was solved for the individual values of the kinetic parameters, K_S , K_{MS} , K_{DS} , and k_3 . Software of Kaleidagraph 4.1 was used to perform non-linear curve fits based on an iterative Levenberg-Marquardt algorithm.

PLA₂ activity measurements were conducted at 37 °C for eight different total lipid ([L + D_DPPC]) concentrations from 10 μM to 400 μM and for various mole fractions, X_L , of the substrate L_DPPC at each of these concentrations. Sample temperature was maintained by a circulating water bath. Activity was measured using a new modified assay reported in recent work¹¹. The activity measurement assay relies on the shift in the ADIFAB fluorescence, excited at 386 nm, from 432 to 505 nm upon binding of ADIFAB to the hydrolysis products that partition into the aqueous phase from the bilayer¹⁵. The ADIFAB fluorescence response, quantified by the generalized polarization, GP, given by^{16,17}

$$GP = \frac{I_{505} - I_{432}}{I_{505} + I_{432}}, \quad (7)$$

where I_{505} and I_{432} are the fluorescence emission intensities at 505 nm and 432 nm respectively, is linearly proportional to the concentration of hydrolysis products¹¹.

Fluorescence emission intensities of ADIFAB, included in the reaction mixture of lipid vesicles + 1 mM of CaCl₂ in Hepes buffer, at 432 and 505 nm were recorded continuously following addition of enzyme, using the Multi option mode of a Fluoromax-4 Spectrometer (Horiba Scientific). In this mode of operation, the spectrometer grating swivels between two or more wavelength positions to measure the fluorescence intensities at user selected wavelengths at appropriately specified slew rates. In the present study the two wavelengths were 432 nm and 505 nm. The time to swivel between the two wavelengths was about 500 μs and intensities were recorded at both of these wavelengths every 6 seconds. The rapid swiveling speeds makes the measurements almost simultaneous and continuous kinetic data are obtained.

The GP calculated from these intensities, using eq. 7, as a function of time represents the time course of hydrolysis. The initial temporal rate of GP variation was converted to activity in units of μM of product/min/mg of enzyme using the slope of the calibration curve of GP vs. known amounts of equimolar mixtures of hydrolysis products dispersed in DPPC at the same total lipid concentration¹¹. Calibration measurements were conducted at every single DPPC concentration for which activity was measured¹⁸. The calibration measurements also give the partition coefficient of the LPPC / PA mixtures in DPPC. These results were reported recently¹⁸. Conductance of the ADIFAB fluorescence assay to determine PLA₂ activity was described in detail in a previous publication^{11,12}.

Dynamic Light scattering measurements (DLS) were conducted to determine the hydrodynamic radii of the mixed vesicles of L_ and D_DPPC vesicles on DynaPro Nanostar

Model WDPN06 (Wyatt Technologies), equipped with GaAs laser (120mW) operating at a nominal wavelength of 658nm. The scattered light was collected at 90° by a solid state Single Photon Counting Module (SPCM) detector. The sampling time was set to an optimum value to obtain a fully decaying intensity correlation function (ICF), which was typically 10 seconds. The ICF's were single exponential decays with baselines that were unity within the precision of the measurements. The exponential fit to ICF yielded the translational diffusion coefficient, D_t , of the particles in the sample. The hydrodynamic radius was then derived from D_t using the Stokes-Einstein equation¹⁹. All sample solutions were filtered through 0.2 μm Whatman nylon syringe filters. The temperature of the sample solutions was controlled by an internal Peltier effect heat pump with an accuracy of $\pm 0.01^\circ\text{C}$.

The surface area of vesicle (A_{ves}) was calculated from the average radius of DPPC SUV measured by DLS. The vesicle lipid aggregation number, N , was calculated assuming the phospholipids to form the spherical shell of a sphere of radius given by DLS. The shell thickness was set equal to twice the length of the lipid molecule. Dividing the shell volume by the lipid molecular volume gave the number N . The phospholipid length was obtained from ChemDraw 3D as 30.9 Å and volume was calculated from the Vander Waals radii to be 796.4 Å³.²⁰

Sample Preparation

The vesicles for enzymatic activity were prepared by first dissolving calculated amounts of L_ and D_phospholipids in ethanol. The ethanol solution was vortexed thoroughly to produce a clear solution, which was then dried under dry N_2 flux to produce a film of lipid. Required amount of the Hepes buffer (pH=7.4) was then added to the dry film to achieve the final desired lipid concentration. Small unilamellar vesicles (SUV) of DPPC were prepared by vortexing the DPPC solution for 5 min followed by sonication in an ultrasonic bath (model no. G112SP1G from Laboratory Supplies Inc., NY) for 20–25 minutes. The ultrasound power output was 80 watts.

RESULTS

Figure 2 shows the static fluorescence spectra of ADIFAB in Hepes buffer and in SUVs of DPPC before and one hour after addition of PLA₂. The appearance of the peak at 505 nm is a signature of the presence of the hydrolysis reaction products.

Reaction progress curves of hydrolysis of L_DPPC, are represented by the time variation of the GP. The GP calculated from the values of the continuously monitored fluorescence intensities at 432 nm and 505 nm, are shown for a few representative vesicle and substrate (L_DPPC) mole fractions (with total lipid concentration, $[L + D_DPPC] = 50 \mu\text{M}$) in Fig. 3a and Fig. 3b respectively.

PLA₂ activity, derived from the initial slope and the calibration curves (as described in *Methods*) as a function of the substrate mole fraction, X_L , is shown in Fig. 4, for each of the different total lipid or vesicle concentrations. The error bars, typically $\pm 8\%$ are standard deviations from several repeated measurements at a few of the concentrations.

The activity vs. X_L profile in Fig. 4 is different from the usually observed occurrence of saturation at high concentrations in reaction kinetics. The observed behavior results when the D_DPPC also binds to the enzyme but resists hydrolysis, as shown by the model derived for kinetics in the presence of diluents like D_DPPC and described briefly below¹⁰.

The negative sign in the denominator in eq. 1 is a consequence of the presence of a competitively binding but hydrolysis-resistant component which leads to a continuous non-saturating increase in activity with X_L . If the D_enantiomer were to not bind, then $K_{DS} \rightarrow \infty$, leading to $a_2 = -1/k_3$. Activity vs. X_L would then exhibit the well-known saturation behavior. The observed behavior in Fig. 4 therefore shows that the D_enantiomer binds to the enzyme but resists hydrolysis.

Fitting of eq. 2 to the data in Fig. 4 for each of the concentration yields a_1 and a_2 for each of the eight total lipid concentration investigated. a_2 (eq. 3) is a constant that depends on the kinetic parameters only and not on sample properties of X_L and [vesicles]. The numerical value of a_2 and its uncertainty obtained from the fit was

$$a_2 = \frac{1}{k_3} \left(\frac{K_{MS}}{K_{DS}} - 1 \right) = 0.0031 \pm 0.002 \quad (8)$$

a_1 on the other hand depends on [vesicles] according to eq. 2. Figure 5 illustrates the dependence of a_1 on [vesicles]. The vesicle concentrations were calculated as described in *Methods* (eq. 6). Fitting the data of Fig. 5 with eq. 2 gave the following two equations:

$$\frac{K_{MS}}{k_3 K_{DS}} + \frac{K_{MS}}{k_3 [L+D_DPPC]_s} = 0.0064 \pm 0.0019 \quad (9)$$

and

$$\frac{K_S K_{MS}}{k_3 n [L+D_DPPC]_s} = (4.55 \pm 0.32) \times 10^{-12} \quad (10)$$

The system of three equations from eq. 8–10 contains the four unknown parameters k_3 , K_{MS} , K_{DS} , and K_S/n . K_S and the number of binding sites per vesicle, n , occur grouped together in the equations and therefore the quantity K_S/n is solved for as one entity. The numerical value of the surface lipid concentration $[L+D_DPPC]_s$ required for solving the equations was calculated using eq. 4 with the vesicle radius, $R_{ves} = 42$ nm obtained as an average of DLS measurements on several samples of varying L to D_DPPC ratios and total lipid concentrations. The solutions for the parameters were solved for iteratively as described below:

In mixed bilayer interfaces, the enzymatic activity is highest when $X_{L_DPPC} = 1$. For $X_{L_DPPC} = 1$, eq. 1 can be written as

$$A = \frac{B[\text{vesicles}]}{C + [\text{vesicles}]} \quad (11)$$

where

$$B = \frac{k_3 [L_DPPC]_s}{K_{MS} + [L_DPPC]_s}$$

and

$$C = \frac{K_S K_{MS}}{n(K_{MS} + [L_DPPC]_S)}$$

The activity vs. [vesicles] data were fit to eq. 11. The fit gives the values of B and C. The data and the fit are shown in Fig. 6. The saturation value of the activity is B and its value from the fit was,

$$B = \frac{k_3 [L_DPPC]_S}{K_{MS} + [L_DPPC]_S} = 333 \pm 30 \quad (12)$$

The errors quoted in the numerical results of the fit in eq. 8–10 and eq. 12 are the uncertainties given by the fitting routine. The starting value of k_3 in the iteration procedure was set to B, which means that $[L_DPPC]_S \gg K_{MS}$ in the first approximation. Substitution of $k_3 = 333$ in eq. 3 yields the first value of K_{MS}/K_{DS} . These values of k_3 and K_{MS}/K_{DS} were then used in eq. 9 and 10 to obtain the first values of K_{MS} and K_S/n , respectively. K_{MS} was then substituted back in eq. 12 to recalculate the next approximation of k_3 . The surface lipid concentration $[L_DPPC]_S$ is required for the second and higher approximations of k_3 . The second iteration with this value of k_3 gives the second set of K_{MS} , K_{DS} and K_S/n . This process was repeated until the values converged. The final values are given in Table 1.

The number of binding sites, n , is required to determine K_S from K_S/n . Following Deems et al., the value of n was calculated from the area per binding site which was set to be the area of about five phospholipid head groups¹³. Accordingly, the number of binding sites n can be calculated as

$$n = \frac{A_{ves}}{5A_{lipid}}, \quad (13)$$

where A_{ves} and A_{lipid} are the surface area of vesicle and phospholipid head group, respectively. A_{lipid} was taken to be $85 \text{ \AA}^2$ ¹³ and A_{ves} was calculated from the average radius of DPPC SUV measured by DLS. The errors in the kinetic parameters are the calculated propagated errors due to the uncertainties in the fitted values shown in eq. 8–10 and eq. 12. Contributions to these errors from uncertainties in the aggregation number resulting from the spread in the vesicle radius (data given in the next section) are less than the quoted error values.

DISCUSSION

D₂-phospholipids as diluents made possible an investigation of interface substrate concentration dependence of PLA₂ activity in bilayers and derivation of the individual kinetic parameters of the various kinetic steps; thus bringing resolution to an outstanding problem in membrane hydrolysis of disentangling the effects of the coupled steps present in interface kinetics. The present data as in Fig. 4 and 6 vividly capture the features of the kinetic model based on the Michaelis-Menten-Henri scheme. The profiles in Fig. 4 show that D₂-enantiomers are hydrolysis-resistant but bind to the enzyme. The data in Fig. 6 show dependence of activity on [vesicles] which is a signature of the presence of enzyme hopping between vesicles.

Jain et al. introduced 2-hexadecyl-sn-glycero-3-phosphocholine (2H-GPC), a micelle forming amphiphile, as a neutral diluent and used it to study kinetics in the scooting mode with 1,2-dimyristoyl-sn-glycerophosphomethanol (DMPM) vesicles⁶. The method used in that work was restricted to the scooting mode and cannot separate and yield the different kinetic parameters. More importantly, the use of micelle forming detergents as diluents in bilayer as well as in micellar kinetics is problematic.

A basic requirement a diluent must meet is that their presence must not alter the properties of the kinetic medium. 2H-GPC like other detergent diluents does not satisfy this criterion. DMPM vesicles with 2H-GPC at mole fraction > 30 %, break down⁶. Use of detergents as diluents dates back to early experiments of PLA₂ kinetic studies on phospholipids dispersed in micelles. Kinetic assays of phospholipase activity have been conducted mostly with phospholipids dispersed in micelles of detergents like triton-X100, bile salts, and zwitterionic detergents^{12,13,21}. The substrate concentration was varied by varying the lipid to detergent ratio. This also changes the micelle aggregation number and thereby the micelle concentration. Even at constant lipid to detergent ratio, micelle size changes with concentration^{10,22}. Whether a diluent binds to the enzyme or not is not important (of course it must not have a much greater affinity than the substrate, such that it precludes activity). The mathematical model can easily include diluent-enzyme binding as done in this work.

D_lipids present the best solution for conducting surface dilution studies and, to the best of our knowledge, we have used them for the first time in this role to observe interface substrate concentration dependence of PLA₂ activity in membranes over a wide range. Using D_lipids as diluents has many advantages and is most appropriate. Experiments conducted show that key vesicle properties do not change with L to D_lipid ratio. ESR experiments showed that the membrane properties of polarity and microviscosity remained unchanged. The gel to liquid phase transition temperatures, which are leading indicators of intermolecular interactions, of D_DPPC, L_DPPC and their mixtures in various proportions were verified to be identical at 41°C¹⁰. The vesicle size does not change with vesicle concentration or with substrate to diluent ratio. This was verified by DLS measurements. The vesicle radii did not show any remarkable dependence on the L and D_DPPC mixture proportions and also on total lipid concentration (Fig. 7). In any case, substrate concentration in vesicles is affected only by the mole fraction of L_DPPC and not by the size, because of the large radius to bilayer shell thickness in vesicles. In addition, effects of polydispersity in vesicle radii on substrate concentration are mitigated in vesicles but not so for micelles.

Another outstanding problem in PLA₂ enzymology is the existence of isozyme-lipid specificity²³. Bilayer surface dilution kinetic investigations can be of value in this area. Interface microstructure plays a key role in PLA₂ activity. The phenomenon, referred to as “interface quality effects” is known to exist, but lacks complete mechanistic understanding^{4,12,24,25}. Elucidation of the micro structural origin of these effects becomes possible if kinetic models can be tested and the derived kinetic parameters shown to be specific to the type of lipid, enzyme, and interface; with the rationale being that such effects are expressed through the kinetic parameters. The present example of bee-venom PLA₂-DPPC bilayers is an illustration. a_2 is an order of magnitude smaller than a_1 at the higher vesicle concentration (above total lipid concentrations of 50 μ M), making the activity vs. substrate concentration appear linear. The smallness of the parameter a_2 results when $K_{MS} \approx K_{DS}$. Physically this means that the interfacial equilibrium binding constant of the enzyme to either D or L_DPPC is the same and that the rate constant, k_3 , of the chemical step is < the interfacial enzyme-lipid dissociation rate constant, k_{-2L} for bee-venom PLA₂ at DPPC SUV interfaces.

The model investigated in this work is for the substrate concentration dependence of the activity (slope) in the initial linear or steady state period, where product accumulation, substrate depletion, and activation or inhibition by products are as yet not pertinent. Effects are specific to the type of products generated by the particular phospholipase. Lysolipids generated by PLA₂ inhibit the enzyme²⁶. Product induced effects and substrate depletion manifest as non-linearity at longer times in the product time release curves^{14,27} and therefore not of consequence to the present data on the initial reaction velocities.

CONCLUSIONS

Interface substrate concentration dependence of phospholipase A₂ activity on lipid bilayers using D₂-phospholipids as diluents was investigated. The observed interfacial PLA₂ activity dependence on bilayer substrate and vesicle concentrations was described by a modified Michaelis-Menten-Henri model for interfacial kinetics. Kinetic parameters were extracted from fitting the model to the data. D₂-lipid diluents together with the sensitive fluorescence assay provide a method for investigating any lipid system and any kinetic model. The PLA₂ studied here is a representative of lipolytic enzymes. The experiments and modeling have direct relevance to delineating the kinetics of lipolytic enzymes in general, because other lipolytic enzymes also act on L-phospholipids at bilayer interfaces. Without surface dilution kinetics, the different steps in a multi-step process cannot be disentangled. The ability to perform bilayer surface dilution kinetics, separate the effects of interface binding and lipid-enzyme binding and derive the individual values of the kinetic parameters is a step forward in membrane enzymology and kinetics.

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FIGURE 2. Fluorescence emission spectra of free and hydrolysis-product bound ADIFAB.

FIGURE 3.

Reaction progress curves, of PLA₂ catalyzed hydrolysis of L+D_DPPC SUVs, represented by the time dependence of the GP of ADIFAB fluorescence for various (a) lipid or vesicle concentrations and (b) substrate mole fraction at a constant total lipid concentration [L + D_DPPC] = 50 μM. The lines are fits to the data in the initial linear region.

FIGURE 4.

Bee-venom PLA₂ activity dependence on interface substrate concentration, expressed in mole fraction of the substrate L_DPPC, in L+D_DPPC mixed vesicles, for various vesicle concentrations obtained by varying the total lipid concentration denoted by [DPPC]. The curves are fits to eq. 1, which gives a_1 and a_2 for each of the data sets.

FIGURE 5.

Dependence of a_1 , obtained from fitting eq. 1 to each of the data sets in Fig. 4, on [vesicles]. The curve is a fit to eq. 2, which gives eq. 9 and 10.

FIGURE 6.

Bee-Venom PLA₂ activity dependence on vesicle concentration, for $X_{L_DPPC} = 1$. The curve is the fit to eq. 11, which gives the values of B and C.

FIGURE 7. Dynamic Light Scattering profiles of (a) [L+D_DPPC] vesicle solutions for various mole fractions of L_DPPC and (b) [L_DPPC] vesicles for various concentrations. Peak positions give the hydrodynamic radii of aggregates.

Table 1

Numerical values for the kinetic parameters of bee-venom PLA₂ activity on DPPC vesicles.

$K_{MS}/10^{-10}$ (Mcm ⁻²)	$K_{DS}/10^{-10}$ (Mcm ⁻²)	$K_S/n/10^{-9}$ (M)	$K_S/10^{-5}$ (M) ^a	k_3 (μM/min/mg)
1.4±0.4	0.6±0.02	6.8±1.4	3.6±0.3	375±34

^a Number of binding sites n, required to calculate K_S, was estimated using eq. 13.