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Kinetics of binding of phospholipase A_2 to lipid/water interfaces and its relationship to interfacial activation

Mahendra Kumar Jain [a], Joe Rogers [a] and G.H. DeHaas [b]

[a] *Department of Chemistry, University of Delaware, Newark, DE (U.S.A.) and* [b] *Department of Biochemistry, Rijksuniversiteit Utrecht, Utrecht (The Netherlands)*

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The time-course of binding of phospholipase A_2 and prophospholipase A_2 to vesicles and micelles of a variety of substrate and nonhydrolyzable phospholipid analogs is obtained by monitoring the change in the fluorescence intensity of Trp-3 on the protein or of the 5-dimethylaminonaphthalene-1-sulfonyl (dansyl) chromophore on the surface of the vesicles. The time-dependent increase in the fluorescence intensity of phospholipase A_2 is observed only under conditions where catalysis and equilibrium binding are also observed. The overall kinetics of binding is described by two rate constants. A rapid second-order rate constant (k_a) for binding of both the proteins is $2 \cdot 10^7$ per s per mol expressed in terms of phospholipids as monomers, and 10^{10} per s per mol expressed in terms of vesicles. This is probably a diffusion-limited encounter of the protein with vesicles as the first step in binding. An additional first-order rate constant ($k_b = 4$ per s) was also discerned for the binding of phospholipase A_2 but not for prophospholipase A_2. The rate of desorption of the bound phospholipase A_2 in the presence of EGTA is very slow (less than 0.0002 per s), whereas the rate of desorption of the bound prophospholipase A_2 is much more rapid (2.9 per s). The mechanistic significance of these rate constants is elaborated in terms of the differences in the rates of interfacial catalytic turnover of phospholipase A_2 and prophospholipase A_2. As shown elsewhere (Jain et al. Biochim. Biophys. Acta 860, 435–447) the hydrolysis of anionic vesicles by phospholipase A_2 occurs in the scooting mode such that the bound enzyme remains on the target vesicles for several thousand catalytic turnover cycles. On the other hand, as shown in this paper, the kinetics of hydrolysis by prophospholipase A_2 is dominated by its intervesicle exchange. Therefore, interfacial catalysis by prophospholipase A_2 in the hopping mode would involve an on- and an off-step in each cycle, resulting in a catalytic turnover number of about 1.2 per s. A change from the hopping to the scooting mode of catalysis thus provides the kinetic basis for activation of interfacial catalysis by phospholipase A_2 compared to that for prophospholipase A_2.

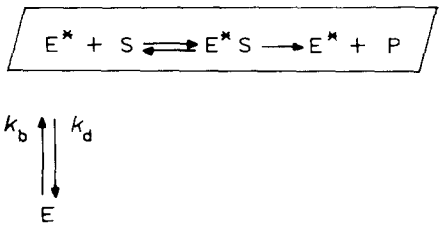
Introduction

Binding of phospholipase A_2 to the lipid/water interface is a key step in the interfacial catalysis: this step regulates the magnitude of the interfacial

Abbreviations: dansyl-, 5-dimethylaminonaphthalene-1-sulfonyl-; DMGPMe, 1,2-dimyristoyl-*sn*-glycero-3-phosphorylmethanol; GPMe, *sn*-glycero-3-phosphorylmethanol; GPC, *sn*-glycero-3-phosphorylcholine; HPC, *n*-hexadecylphosphorylcholine; HPE, *n*-hexadecylphosphorylethanolamine; HPMe, *n*-hexadecylphosphorylmethanol; PLA, phospholipase A_2 from pig pancreas; proPLA, prophospholipase A_2 from pig pancreas; Trp-3, tryptophan residue in the 3-position of phospholipase A_2; TTGPE, 1,2-ditetradecyl-*sn*-glycero-3-phosphorylethanolamine; TTGPMe, 1,2-ditetradecyl-*sn*-glycero-3-phosphorylmethanol; PC, phosphatidylcholine.

Correspondence: M.K. Jain, Department of Chemistry, University of Delaware, Newark, DE 19716, U.S.A.

activation of the enzyme by favoring interfacial catalysis in the scooting mode [1–5]. According to Scheme I and as shown experimentally for catalysis in the scooting mode, the binding of the enzyme to the interface is rapid [2], and the bound enzyme is not desorbed from the interface in between or during the catalytic turnover cycles [2,3]. On the other hand, in the hopping mode of interfacial catalysis the binding and desorption (E to E* step) of the enzyme are a part of each catalytic turnover cycle. Thus, an appreciation of the magnitudes of the rate constant for binding (k_b) and for dissociation (k_d) is critical for understanding interfacial catalysis and activation.



Scheme I. Scheme of interfacial catalysis.

In the published literature several ad hoc assumptions have been made to account for the origin of the pre-steady state lag phase in the reaction progress curves. For example, it has been assumed that the binding of phospholipase A_2 to monolayers of medium chain zwitterionic phospholipids is governed by a slow rate constant of less than 0.01 per s [6]. On the other hand, in vesicles [2], monolayers [6], and micelles [2,7] of anionic phospholipids a rapid onset of hydrolysis is observed after the addition of the enzyme. This suggests that the intrinsic rate constant for binding of phospholipase A_2 to the interface (E to E* step) is greater than 0.2 per s. Similarly, in vesicles of zwitterionic phospholipids, which have a very low affinity for phospholipase A_2, the binding equilibrium for phospholipase A_2 is shifted towards the bound form (E to E*) in the presence of the products of hydrolysis [8,9]. However, for this system it has been assumed [10] that the binding of phospholipase A_2 to vesicles is a kinetically slow step. In order to resolve such contradictory assumptions we have investigated the kinetics of binding of phospholipase A_2 to the lipid/water interface under a variety of kinetically meaningful conditions. In this communication we report the values of the kinetic rate constants for the E to E* step, i.e., for the binding and desorption of phospholipase A_2 to vesicles and micelles.

Materials and Methods

Sources of the reagents, isolation of pig pancreatic phospholipase A_2 and prophospholipase A_2, general methods for the synthesis of phospholipids, preparation of vesicles, and protocols for obtaining the equilibrium binding data by steady-state fluorescence measurements have been described elsewhere [2–5,8,9,11]. The time-course of binding of phospholipase A_2 to sonicated dispersions of substrates and their analogs were obtained on a stopped-flow mixing device equipped to monitor fluorescence emission intensity (made available to us in the laboratory of Professor Eigen at the Max Planck Institute for Biophysical Chemistry in Göttingen, F.R.G.). Excitation was set at 285 nm and a 305 nm cut-off filter was used on the emission side to reduce the contribution of scattering due to changes in the turbidity. For measurements involving dansyl-HPE excitation was set at 345 nm, and a 30 nm bandpass filter at 485 nm was used on the emission side. The dead-time of the instrument was about 4 ms. The output from the photomultiplier tube was digitized. Of the 4000 data points collected in each run, 200 were assigned for the pretrigger delay, 1800 for the fast sweep A, and the last 2000 points for the slower sweep B so as to adequately resolve the slow and the fast time components. Curve fitting and statistical analysis of the data was done on a Univac computer using a program that is routinely used in the laboratory mentioned above.

Results

As shown elsewhere [3,8], the binding of phospholipase A_2 to anionic vesicles (E to E* step) under catalytically meaningful conditions can be readily quantitated by spectroscopic methods. For example, phospholipase A_2 binds with a high affinity to 35–40 molecules of DMGPMe in the outer monolayer of the target vesicles, and the integrity of the vesicle is retained after binding of phopholipase A_2, as well as after hydrolysis of the

substrate in the outer monolayer. The binding equilibrium of the enzyme to the anionic interface is shifted by cationic lipophiles (e.g., local anesthetics) that apparently reduce the catalytic turnover number of phospholipase A_2 by lowering the concentration of the bound enzyme (E*). The kinetic studies [2] suggest that the apparent dissociation constant of the enzyme bound to anionic vesicles is less than 0.1 pM. Therefore, for all practical purposes considered in this paper the amount of free enzyme in the mixture is determined by the lipid-to-protein ratio. The lipid-to-protein stoichiometry suggests that the outer surface of a vesicle would be completely covered by the enzyme when the total lipid-to-protein ratio is about 60 because the phospholipid molecules in the inner monolayer are not accessible for binding.

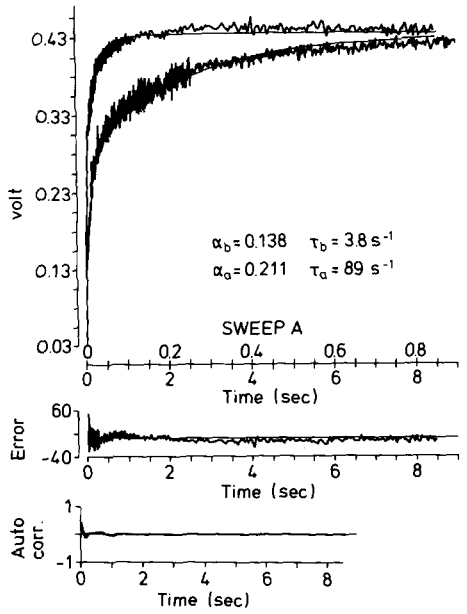


Fig. 1. The time-course of the fluorescence change of tryptophan of phospholipase A_2 (2.9 μM, given as the final concentration after mixing) on mixing with vesicles of TTGPMe (0.2 mM after mixing) at 25°C in a stopped-flow mixing device as described in the text. The reaction mixture also contained 10 mM Tris-HCl and 0.5 mM $CaCl_2$ at pH 8.0. Such a curve could be deconvoluted into two relaxation constants τ_a and τ_b, and the corresponding amplitudes α_a and α_b. One of the worst cases of the systematic deviation in the residuals was chosen for this figure. Most of the time-course curves used in this study showed a significantly better distribution of the residuals. The ordinate for the error plot is in mV.

The time-course of binding of phospholipase A_2 was recorded by monitoring the change in the fluorescence intensity of Trp-3 on phospholipase A_2. As shown in Fig. 1 the time-course of the increase in the Trp-3 fluorescence on binding of phospholipase A_2 to vesicles of TTGPMe is rapid and complex. The overall change in the intensity is about 3-fold as seen with the steady-state fluorescence measurements [4]. The binding of phospholipase A_2 to vesicles containing 5–10 mol% dansyl-HPE was monitored at 500 nm, the emission peak due to resonance energy transfer from tryptophan excited at 285 nm (see Ref. 11 for details). In both cases the time course of the

TABLE I

THE KINETIC CONSTANTS FOR THE BINDING OF PHOSPHOLIPASE A_2 (PLA) AND PROPHOSPHOLIPASE A_2 (proPLA)

All these experiment were done under the conditions described in the legend to Fig. 1: pH 8.0 in 10 mM Tris-HCl buffer at 25°C. Monitored by resonance energy transfer to dansylhexadecylphosphorylethanolamine (7 mol%); ex. 285 nm em. 480 nm with a band pass filter of 30 nm bandwidth. The final concentration of phospholipase A_2 was kept at 2.8 μM, and that of lipids was varied between 0.05 and 2 mM. The concentration of calcium was kept at 0.5 mM in all cases except for GPC analogs where the calcium concentration was 10 mM.

No.	Lipid	Enzyme (10^{10} s^{-1}·mol^{-1})	k_a (s^{-1})	k_b
1	TTGPMe	PLA	1.8	3.5
2	TTGPMe + dansyl-TTGPE	PLA	2.7	4
3	TTGPMe + dansyl-HPE	PLA	5.4	6
4	HPC	PLA	1.5	11
5	HPMe	PLA	1.6	6
6	HHGPMe	PLA	2.7	6
7	Dioctyl-GPMe	PLA		8
8	Dioctyl-GPC	PLA		2
9	DMGPMe	PLA	2.5	1.3
10	DMGPMe + dansyl-HPE	PLA	2.6	4.5
11	POGPMe	PLA	3.2	1.2
12	POGPG	PLA	3.0	3
13	1,3-DMGPMe	PLA		9
14	Dioctanoyl-GPMe	PLA	6	5
15	Products	PLA		2
16	TTGPMe	PLA	2.0	–
17	DMGPMe	PLA	2.0	–
18	Dioctyl-GPMe	PLA		20

fluorescence increase could be satisfactorily fitted to two exponentials under most conditions. As shown in Fig. 1, sometimes a systematic error of about 10% is observed in the residuals and the origin of such deviations is not known. These deviations could arise from the pressure shock to vesicles, because such systematic deviations are not observed with micelles. A systematic deviation could also arise from peculiarities in the mechanism of binding of phospholipase A_2 to vesicles. At present we do not have a model for the mechanism of binding of a protein to vesicles, and this step is not necessarily a part of the catalytic turnover cycle for interfacial catalysis. In fact, a priori there is no reason to believe that the step E to E* would obey a rate law involving a set of simple exponential functions. There are two types of experimental difficulties that do not permit resolution of such possibilities. The dead-time of our instrument is about 4 ms which means that the measurement and manipulation of a significant part of the time-course of the binding curve is not possible. This problem could not be obviated by other techniques like T-jump because the binding constant for the enzyme in the interface is very large. Also at low vesicle concentrations the rate law for binding could become very complex, as the excluded surface area effects would dominate binding of the enzyme to vesicles which already have some protein molecules on their surface. This would cause an apparent time dependence of the rate constants for the binding. A complete mathematical description of such a process would be intractable (Berg, O., personal communication; see also Ref. 12). For the purpose of discerning the trends and to minimize the contribution of the various instrumental and data processing artifacts we have kept the experimental conditions comparable for these measurements and for data processing.

The time-course for the fluorescence change during the first 10 s could be fitted to two exponentials (Fig. 1). The values of the two rate constants k_a and k_b that characterize the time-course of the total fluorescence change for phospholipase A_2 on binding to vesicles and micelles under a variety of conditions are summarized in Table I, and their significance is discussed below. For computations all the four curve-fitting variables (two rate constants and the corresponding

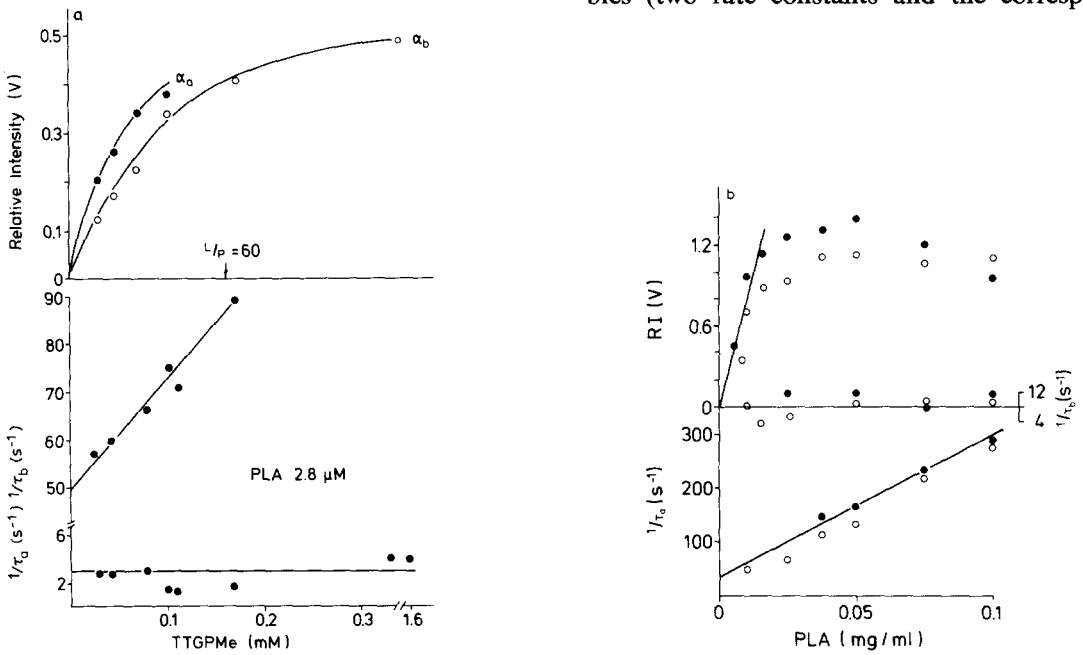


Fig. 2. Dependence of the reciprocal of the relaxation constants (bottom) τ_a and τ_b and (top) relative fluorescence intensity (RI) on the concentration of (a) TTGPMe (as sonicated vesicles) and (b) phospholipase A_2. Other conditions were as given in legend to Fig. 1. Two typical experiments are presented.

amplitudes) were allowed to 'float' freely and no attempt was made to force-fit any of the parameters.

The dependence of the two relaxation constants that describe the time-course of the Trp-3 fluorescence change is a function of phospholipase A_2 and TTGPMe concentration is summarized in Figs. 2a and 2b. The faster relaxation is seen at low vesicle-to-enzyme ratios, and it is responsible for about half of the total change in the fluorescence intensity. However, there is some uncertainty in the values of the amplitudes. The contribution from the fast relaxation is not observed at high vesicle concentrations although a significant part of the corresponding change in the fluorescence intensity is apparently completed within the dead-time of the instrument (about 4 ms). The dependence of the faster relaxation rate and its amplitude (shown in Figs 2a and 2b) on the vesicle concentration would be expected if the faster relaxation is due to a second-order rate constant, and if only the tail-end of the binding curve is recorded at relatively high concentrations of vesicles. The value of k_a obtained under a variety of conditions are summarized in Table I. The magnitude of a narrow range of values of k_a suggest that it is related to the diffusion-limited encounter of phospholipase A_2 with vesicles. The values of k_a summarized in Table I are expressed in terms of the monomer lipid concentration. It is probably more meaningful to consider the diffusion-limited process in terms of a random encounter of phospholipase A_2 with vesicles. When expressed this way the values of k_a are about 10^{10} per s per mol vesicles. Similar diffusion-limited rate constants have also been observed for binding of a variety of solutes to vesicles [13].

According to equilibrium binding studies, there is one binding site per 40 phospholipid molecules in the outer monolayer of the target vesicle [3]. Therefore, above 0.2 mM phospholipid concentration a substantial portion of the total fluorescence change occurring with the rate constant k_a would occur during the dead-time of the instrument, i.e., in the first 5 ms. Thus, it is very difficult to determine the amplitudes of the fluorescence change corresponding to each rate constant.

The value of the second rate constant, k_b (about 4 per s for TTGPMe), does not depend on the concentration of the protein or vesicles (Fig. 2), and it mediates about 35% of the total change in the fluorescence intensity. The contribution of k_b can be seen at low, as well as at high vesicle-to-enzyme ratios. These observations suggest that the underlying process is most probably not due to artifacts arising from the binding of phospholipase A_2 to the 'excluded area' on vesicles. This conclusion is further supported by the fact that the value of k_b remains relatively constant even when a significant number of the data points at the beginning of the curve are ignored for curve fitting. This would not be the case if the tail-end of the process underlying k_a (due to the excluded area effect) contributed to k_b.

As shown in Fig. 3, the two relaxation constants do not exhibit an anomalous effect as a function of temperature, i.e., the phase properties of the bilayer do not have a noticeable effect on the kinetic constants for binding. This is also consistent with the fact that k_a and k_b values remain the same for binding of phospholipase A_2 to homologous 1,2-dihexadecyl-GPME (6) whose T_m is 44°C. The apparent dissociation constant for E* on TTGPMe vesicles remains immeasurably low over the whole temperature range from 10 to 45°C (data not shown). Also, as shown in Fig. 4, the catalytic turnover constant (k_i) for the interfacial catalysis by phospholipase A_2 in the scooting mode and catalytic turnover number of prophospholipase A_2 in the hopping mode

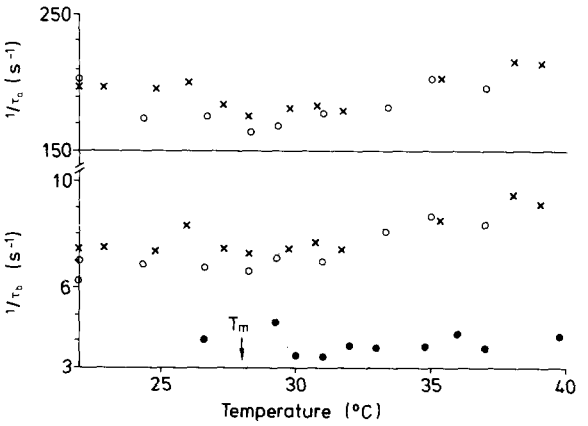


Fig. 3. Dependence of the reciprocal of the relaxation constants τ_a and τ_b on temperature. 0.3 mM TTGPMe (●) 0.06 mM TTGPMe alone (○) or with 4 μM dansyl-HPE (×). Other conditions were as given in legend to Fig. 1.

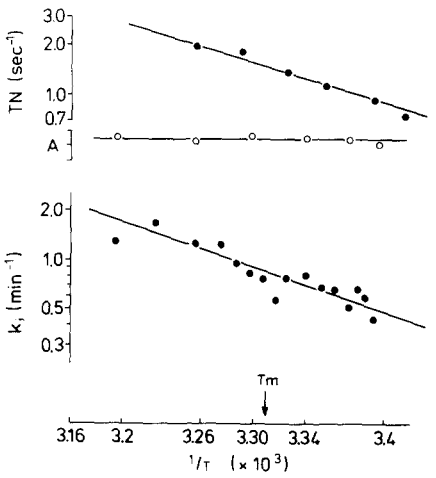


Fig. 4. Temperature dependence of the catalytic turnover number (TN) for prophospholipase A_2 (top), the extent of hydrolysis (A, middle), and the first-order catalytic constant (k_i) for the hydrolysis in the scooting mode (bottom) by phospholipase A_2. DMGPMe vesicles were used as substrates. other conditions were as given in the legend to Fig. 6 and in the text.

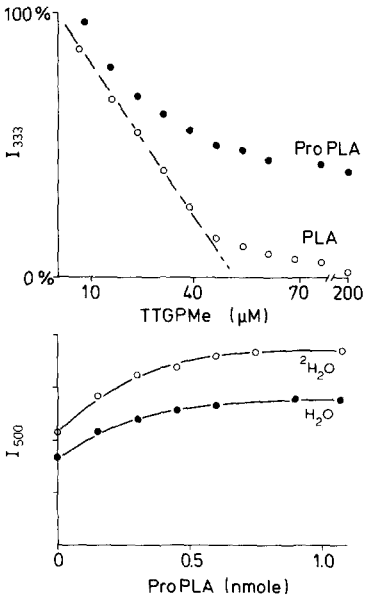


Fig. 5. (Top) The dependence of the relative fluorescence emission of tryptophan of phospholipase A_2 (PLA, ○) and prophospholipase A_2 (ProPLA, ●) at 333 nm (excitation 290 nm) as a function of the concentration of TTGPMe vesicles containing dansyl-HPMe (16 : 1 mole ratio). (Bottom) The fluorescence emisson intensity of dansyl chromophore in the presence of prophospholipase A_2 in water (●) and deuterated water (○). Other conditions were as described in legend to Fig. 1. Excitation at 290 nm and emission at 500 nm. The TTGPMe to dansyl-HPE mole ratio was kept at 16 : 1 and the probe was added to the preformed vesicles. For full significance and the appropriate controls see Ref. 11.

(elaborated below) does not exhibited any anomalous effect of temperature. These results show that the rate of binding, the equilibrium constant for binding (data not shown; see, however, Ref. 3), the catalytic parameters as well as the size of the vesicles are not noticeably influenced by the gel-to-fluid phase transition properties of the bilayer.

The time-course of binding of phospholipase (e.g., data in Figs. 2 and 3, and in Table I) was also monitored at the emission of dansyl chromophore localized in the interface of vesicles [11]. This chromophore is excited at 345 nm by the resonance energy transfer from the emission of tryptophan. As shown in Fig. 5 (top) the efficiency of the resonance energy transfer is about 97% when the surface of the vesicles is completely covered by phospholipase A_2. It should be noted that in these vesicles dansyl-HPE is added to the preformed vesicles, and it is present only in the outer monolayer. The high efficiency of the energy transfer means that the two chromophores (Trp-3 and dansyl attached to the head group on hexadecylphosphorylethanolamine) are within a distance of about 8 Å (based on the critical transfer distance of about 17 Å) when the surface of vesicles is completely covered by phospholipase A_2. The time-course of the fluorescence change from resonance energy transfer is also described by the two rate constants k_a and k_b. As summarized in Table I (Nos. 2 and 3) the values of these rate constants and their dependence upon concentration (Fig. 2) and temperature (Fig. 3) is the same as that obtained by monitoring the emission of Trp-3. It is probably significant that the values of k_a and k_b are somewhat larger (typically 20% and just outside the estimated error range) when monitored by resonance energy transfer. This small difference is apparently not due to a specific interaction of dansyl-HPE with the protein because the catalytic turnover is not influenced (data not shown). A possible interpretation in terms of an improved signal-to-noise ratio or due to an intrinsic difference in the time-course for accessibility of the two chromophores in the interface during the binding is likely.

The dependence of the rate constants k_a and

k_b on other experimental variables is summarized below.

Binding to micelles

Binding of phospholipase A_2 to micelles of hexadecylphosphorylcholine (No. 4 in Table I), hexadecylphosphorylmethanol (No. 5), as well as those of dioctyl-GPC (No. 8) and dioctyl-GPMe (No. 7) was investigated. In all cases, the time course of binding to micelles can be fitted to k_a and k_b. The slower rate constant (k_b) has values of 2–11 per s. The concentration-dependent rate constant (k_a) remains diffusion limited, and its contribution is not observed at high lipid concentrations.

Binding to substrates

According to Scheme I the binding of phospholipase A_2 to the interface is not a part of the turnover cycle in the scooting mode of catalysis. As summarized in Table I for dispersions of several substrates (Nos. 9–14), the values of k_a and k_b for binding to the substrate interface are essentially the same as those seen with the corresponding ether analogs.

Binding of prophospholipase A_2

The zymogen of phospholipase A_2 (prophospholipase A_2) does not exhibit interfacial activation [1] although it is known to bind to anionic vesicles and micelles [3,14,15]. As shown in Fig. 5a, the efficiency of resonance energy transfer from tryptophan to dansyl-HPE is about 60% for prophospholipase A_2 compared to over 97% for phospholipase A_2. This difference suggests that the nature of binding of these two proteins to TTGPMe vesicles is appreciably different, and that it is consistent with the suggestion that the Trp-3-containing face of prophospholipase A_2 remains about 15–20 Å away from the bilayer interface. Moreover, the fluorescence intensity due to resonance energy transfer from prophospholipase A_2 to dansyl-HPE is higher in deuterated water than in water (Fig. 5b). Such quenching by water is not observed with bound phospholipase A_2 [11]. Thus, differences in the resonance energy transfer characteristics of prophospholipase A_2 and phospholipase A_2 are due to the fact that the microinterface between prophospholipase A_2 and the vesicle to which it is bound is not dehydrated, whereas the microinterface is dehydrated in bound phospholipase A_2 [11].

As summarized in Table I (Nos. 16–18), the kinetics of binding of prophospholipase A_2 is described only by the diffusion-limited second-order rate constant k_a. This implies that the binding of prophospholipase A_2 is diffusion limited, and that the time-course of the fluorescence change does not involve the second slow step corresponding to the rate constant k_b. As elaborated below, the significance of such a difference in the rates of binding and desorption of phospholipase A_2 and prophospholipase A_2 can be appreciated in terms of the difference between the scooting versus the hopping modes of interfacial catalysis.

The rate-constants for desorption of the bound enzyme

Based on the kinetics of hydrolysis the apparent dissociation constant for phospholipase A_2 bound to TTGPMe vesicles is estimated to be less than 0.1 pM because the rate of intervesicle exchange is negligibly slow even when the enzyme concentration is 0.5 nM and the vesicle concentration is 50 nM in the reaction mixture [2]. This is consistent with a very slow rate of desorption (rate constant less than 0.002 per s) of the bound enzyme in the presence of EGTA (Table II). On the other hand, under comparable conditions the rate of desorption of prophospholipase A_2 is about 2.9 per s. An estimate of the lower limit for the rate of intervesicle transfer of phospholipase A_2 and prophospholipase A_2 from the dansyl-containing vesicles to TTGPMe vesicles could also be obtained by monitoring the kinetics of the decrease in the emission intensity at 500 nm (due to the resonance energy transfer from Trp-3 to dansyl-HPE). Under these conditions the rate constant for exchange is less than 0.0002 per s for phospholipase A_2 and 2.9 per s for prophospholipase A_2. A precise determination of the lower values for k_d is not possible because a slow rate of fusion of vesicles could influence these results. The results summarized in Table II also demonstrate that the rate of exchange of phospholipase A_2 is considerably more rapid in micelles (22, 23 in Table II) as well as in the vesicles of the products of hydrolysis (21).

TABLE II

THE SLOWEST RATE CONSTANT FOR DESORPTION OF PHOSPHOLIPASE A_2 OR PROPHOSPHOLIPASE FROM THE LIPID-WATER INTERFACE

The complexes were prepared by premixing appropriate solutions. During the stopped flow studies the complex was mixed with 50 mM EGTA. Final concentrations: EGTA 25 mM; phospholipase A_2 or prophospholipase A_2 2.5 μM; lipids 0.3–1.5 mM in 10 mM Tris, 1 mM NaCl (pH 8.0 at 25 °C).

No.	Lipid-protein complex	Mixed with	k_d (s^{-1})
19	TTGPMe·phospholipase A_2	none	< 0.0002
20	TTGPMe·phospholipase A_2	EGTA	< 0.0022
21	DMGPMe·phospholipase A_2	EGTA	0.037
22	Dioctyl-GPC·phospholipase A_2	EGTA	70
23	Dioctyl-GPMe·phospholipase A_2	EGTA	4
24	TTGPMe·prophospholipase A_2	EGTA	2.9

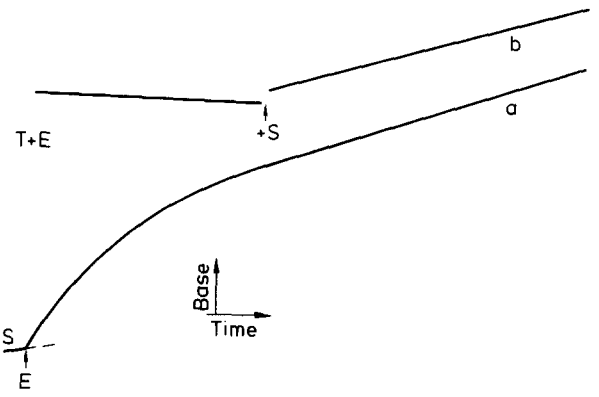


Fig. 6. The reaction progress curve for the hydrolysis of DMGPMe (S) vesicles with (top) and without TTGPMe (T) vesicles (bottom) by prophospholipase A_2. These pH-stat titrations were done in 0.5 mM $CaCl_2$ and 1 mM NaCl at 25 °C. Details are given in ref. 2 and the concentrations in Fig. 7. The sequence of addition is given in the figure.

Kinetics of hydrolysis of DMGPMe vesicles by prophospholipase A_2

Although prophospholipase A_2 binds to anionic interfaces [3,14,15], it does not exhibit catalytic activation at the interface [1]. Therefore, it is of interest to examine the rate of hydrolysis of vesicles of anionic phospholipids by prophospholipase A_2. As shown in Fig. 6, the reaction progress curve for the hydrolysis of DMGPMe vesicles by most preparations of prophospholipase A_2 is complex, i.e., a rapid first-order component superimposed on a slow steady-state rate of hydrolysis. Data not shown here demonstrate that the extent of hydrolysis does not depend upon the enzyme concentrations, and that the steady-state pseudo-zero-order rate of hydrolysis shows a hyperbolic dependence upon the concentration of DMGPMe. This kinetic behavior is different from the behavior of phospholipase A_2 on DMGPMe vesicles [2]. The difference suggests that during the course of its action on vesicles prophospholipase A_2 can rapidly undergo intervesicle exchange, which is not the case with phospholipase A_2. Such a kinetic difference between these two enzymes is further emphasized by the observations described below.

The relative contribution of the initial first order component in the reaction progress curve of prophospholipase A_2 (curve a, Fig. 6) is quite different in different preparations of prophospholipase A_2. This is due to the presence of phospholipase A_2 as an impurity, whose faster rate of interfacial catalytic turnover would mask the slower zero-order rate of hydrolysis by prophospholipase. In order to study the kinetics of hydrolysis by prophospholipase without any complications from phospholipase A_2 we devised a protocol to eliminate the contribution due to hydrolysis by phospholipase A_2. As shown in Fig. 6 (curve b), the rate of hydrolysis fo DMGPMe vesicles by prophospholipase A_2 can be measured without any complicating effect of the trace amounts of phospholipase A_2. This interpretation is based on the observation that the binding of phospholipase A_2 to TTGPMe vesicles is essentially irreversible [2–5] and that the binding of prophospholipase A_2 to TTGPMe vesicles is reversible, as shown earlier in this paper. Thus, TTGPMe vesicles added initially to the reaction mixture irreversibly remove phospholipase A_2, whereas prophospholipase A_2 remains available for the catalytic action on the substrate vesicles which are subsequently added to initiate the hydrolysis. The steady-state zero-order rate of hydrolysis under these conditions depends upon the mole fraction of the substrate and TTGPMe vesicles. This behavior would be expected if prophospholipase A_2 readily undergoes intervesicle exchange, i.e., it can hop to other vesicles. Phospholipase A_2 is not able to do so on vesicles of anionic phospholipids [2–5].

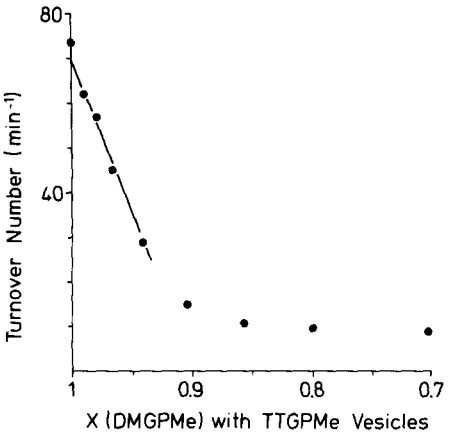


Fig. 7. The turnover number for the hydrolysis of DMGPMe vesicles by prophospholipase A_2 as a function of the mole fraction of TTGPMe vesicles. The reaction was initiated by adding DMGPMe vesicles to a mixture of prophospholipase A_2 and vesicles of TTGPMe. Other conditions were as given in legend to Fig. 6.

As shown in Fig. 7, the steady-state rate of hydrolysis by prophospholipase A_2 decreases with the decreasing mole fraction of the substrate vesicles mixed with TTGPMe vesicles. The initial part of this curve can be extrapolated to obtain the pseudo-zero-order initial rate of hydrolysis of DMGPMe vesicles alone. Thus, the rate of catalytic turnover by prophospholipase A_2 is found to be 1.1 per s. From the difference between the extrapolated value and the actual value of the steady-state rate of hydrolysis one can compute the amount of phospholipase A_2 present as an impurity in a prophospholipase A_2 preparation. The catalytic turnover rate of phospholipase A_2 on DMGPMe vesicles under the first-order kinetic conditions of the scooting mode of hydrolysis is more than 60 per s [2]; therefore, in the prophospholipase A_2 preparation used in this study (Fig. 7), about 0.18% phospholipase A_2 is present as an impurity. At present there is no other method by which this result can be substantiated. This level of impurity is not expected to influence the results of stopped-flow kinetics for binding because the corresponding signal will not be detectable.

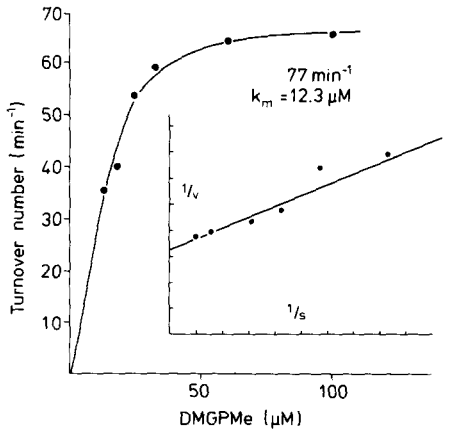


Fig. 8. Dependence of the catalytic turnover number for prophospholipase A_2 for the hydrolysis of DMGPMe vesicles under the steady-state pseudo-zero-order conditions described in Fig. 6 (top). The ratio of DMGPMe vesicles to TTGPMe vesicles in the reaction mixture was maintained at 20 and the inhibitory effect for TTGPMe vesicles was corrected by extrapolation to zero mole fraction of TTGPMe as shown in Fig. 7.

As shown in Figs. 6 (curve b) and 7 the steady-state rate of hydrolysis by prophospholipase A_2 can be obtained under a variety of conditions. This rate of hydrolysis is linear as a function of the enzyme concentration (data not shown) and it shows a hyperbolic dependence on the substrate concentration. From the data shown in Fig. 8 the maximum rate of catalytic turnover by prophospholipase A_2 is calculated as 1.2 per s. From the kinetic data shown in Fig. 8 the apparent affinity of prophospholipase A_2 for DMGPMe vesicles is calculated as 12.3 μM, whereas the apparent dissociation constant calculated from the binding experiments (Ref. 4, or for example Fig. 5, top) is about 18 μM. These results show that the catalysis by prophospholipase A_2 occurs with considerable intervesicle exchange, i.e., in the hopping mode.

Discussion

The time-course of binding of phospholipase A_2 to vesicles of TTGPMe is described by two rate constants (k_a and k_b). On the other hand, the binding of prophospholipase A_2 is described only by a single second-order rate constant (k_a) that appears to be the diffusion-limited encounter of the protein with target vesicles. Interaction of phospholipase A_2 with vesicles and micelles of substrate phospholipids or analogs is dominated by a slower first-order rate constant (k_b) of about 4 per s. The rate constant for desorption of bound

prophospholipase A_2 (k_b) is 2.9 per s in the presence of EGTA. On the other hand, k_d for bound phospholipase A_2 is less than 0.002 per s in the presence of EGTA, and the rate of intervesicle exchange of the bound enzyme is less than 0.0002 per s in the absence of EGTA. The significance of these rate constants can be elaborated to account for the kinetics of interfacial catalysis by phospholipase A_2 in the scooting mode and by prophospholipase A_2 in the hopping mode.

According to Scheme I there is no correlation between the rate constants for binding of the enzyme to the interface and the rate of interfacial catalytic turnover in the scooting mode, which involves only the steps shown in the box. Since the binding of phospholipase A_2 to the interface occurs in the pre-steady state phase, the value of k_b suggests that the lag period would be less than 1 s, as observed for the hydrolysis of vesicles of anionic phospholipids, as well as for micelles of dioctanoylphosphatidylmethanol and dioctanoylphosphatidylcholine. While it is possible that the rapid binding of phospholipase A_2 to micelles is facilitated by the unique dynamics of their interface, it appears unlikely that the latency period of several minutes observed during the hydrolysis of the monolayers of dioctanoylphosphatidylcholine and its homologs is due to an intrinsically slow rate constant for penetration of the enzyme from the aqueous phase to the monolayer (E to E* step). In contrast to the original suggestion by Verger et al. [6], but in analogy with the behavior of vesicles of zwitterionic phosphatidylcholines [8,9], we propose an alternative explanation. As shown elsewhere [8,9], the equilibrium binding of phospholipase to vesicles of pure dimyristoylphosphatidylcholine is very poor (the apparent dissociation constant is greater than 10 mM), and it is not noticeably perturbed by the gel-fluid phase transition properties. However, the dissociation constant decreases to about 0.01 mM in the presence of a critical mole fraction of the products of hydrolysis [8,9]. In these ternary codispersions k_b is about 4 per s for binding and there is no detectable lag period in the reaction progress curve. Moreover, the equilibrium binding of phospholipase A_2 to these ternary codispersions is appreciably modulated by the gel-fluid phase transition [18]. This is because the critical anionic charge density at the interface, which is required for the binding of phospholipase A_2, is optimal only near the phase transition temperature above a critical mole fraction of the products in the bilayer.

On the basis of the following evidence, the above explanation for the origin of the latency period can also be extended to account for the origin of the latency phase during the hydrolysis of monolayers of zwitterionic phospholipids: phospholipase A_2 does not penetrate monolayers of nonhydrolyzable *sn*-1-PC [16]; catalytically inactive phospholipase A_2 modified at His-48 does not bind to monolayers of PC [16]; the rate of binding of phospholipase A_2 is rapid in monolayers of anionic phospholipids [7]; only 5% of the total enzyme is bound to monolayers of zwitterionic phospholipids even under steady-state conditions for hydrolysis [16] which suggests that the E to E* equilibrium is not in favor of E* as required for interfacial catalysis in the scooting mode but without hopping. Based on these observations on monolayers and in analogy to the behavior of phospholipase A_2 in vesicles we postulate that the binding of phospholipase A_2 to monolayers of zwitterionic phosphatidylcholines is determined by the critical mole fraction of fatty acid or both the products of hydrolysis that remain in the monolayer during the course of hydrolysis. Obviously, the equilibrium partition coefficient for short chain fatty acids is in favor of their distribution in the aqueous phase rather than the monolayer; however, a significant steady-state level of fatty acid could remain in monolayers during their hydrolysis. Indeed, Zographi et al. [17] have shown that the rate of desorption of the products of hydrolysis from their monolayers in the absence of the substrate occurs with a rate constant of about 0.005 per s. This implies that in monolayers that are being actively hydrolyzed a substantial amount of the products remains in the monolayer for a significant period of time. The steady-state concentration and the rate of desorption of the products are expected to depend upon the chain length and the mole fraction of the substrate as well as the surface pressure of the monolayer. Although this conclusion is qualitatively consistent with the observed effects of such variables on the lag period and steady-state kinetics of hydrolysis, unfortunately quantitative data

regarding the effects of these variables on the concentration and distribution of the products in monolayers are not available. It is probably pertinent to note that the effect of many of these variables can be normalized in terms of the packing density of phospholipids in the monolayer [17]; this behavior is consistent with the suggestion that the latency phase in monolayers has its origin in the steady-state concentration of the products of hydrolysis.

The magnitudes of k_b and k_b also provide an insight into a possible mechanism of interfacial activation that has not been explicitly considered before. According to the Scheme I the binding (k_b) and desorption (k_d) steps are part of the catalytic turnover in the hopping mode but not in the scooting mode. Thus, a change from hopping to scooting mode would significantly increase the catalytic turnover number. Elsewhere [2–5] we have elucidated that the catalysis in the scooting mode constitutes the basis for efficient interfacial catalysis because the binding and desorption of the enzyme from the interface is not a part of the turnover cycle. Introduction of such slower interfacial steps in the hopping mode of catalysis could appreciably reduce the rate of the overall catalytic turnover. Indeed, a process with a rate constant of about 4 per s would give a considerably lower catalytic turnover rate compared to the value of over 60 per s observed for hydrolysis of DMGPMe vesicles by phospholipase A_2 [2]. Since bound prophospholipase A_2 undergoes intervesicle exchange much more readily than does phospholipase A_2, binding to the interface and desorption from the interface could be a part of its catalytic turnover cycle. The experimentally measured maximum rate of catalytic turnover by prophospholipase A_2 on DMGPMe vesicles is about 1.2 per s. This is quite consistent with the observed value of $k_d = 2.9$ per s for the desorption of prophospholipase A_2 from vesicles of TTGPMe in the presence of EGTA. It is possible that a yet another step of this magnitude is also involved but not detectable by fluorescence methods.

The origin of the molecular changes underlying k_b and k_d is not known. Such a slow process could arise from a minor conformational change in the enzyme, or from the desolvation of the microinterface between the enzyme and the bilayer of micelles. A third possibility invoking aggregation of phospholipase A_2 in the interface is not considered here because it is inconsistent with the kinetic observations reported elsewhere [2]. Low activation energy (about 4 kcal/mol from Fig. 3) and the dependence of k_b on the nature of the interface tend to favor the second possibility. The first two possible explanations for the origin of k_b and k_d are not necessarily mutually exclusive because it is possible that the binding of the enzyme to the interface and a consequent desolvation of the microinterface could promote a conformational change accompanied by a rearrangement of the hydrogen-bonding or hydration network in the bound phopholipase A_2. Prophospholipase A_2 binds without desolvation [11] and k_b is not seen in the time course of its binding. The binding and catalytic action of phospholipase A_2 do not exhibit an anomalous effect as a function of temperature or the phase transition of bilayer. This is because binding of phospholipase A_2 is dominated by ionic interactions with the anionic interface. Most probably there is no direct interaction of phospholipase A_2 with the acyl chain region of the bilayer [2,11] although desolvation of the microinterface would appreciably perturb the acyl chains and give rise to hydrophobic interactions. Such ionic interactions would not be expected with zwitterionic interfaces unless a critical anionic charge density, required for binding of phospholipase A_2, is introduced by the products of hydrolysis. Since phospholipase A_2 does not recruit the anionic amphiphiles in the interface, the local anionic charge density due to phase separation of the anionic amphiphiles in the zwitterionic interface would be susceptible to thermotropic or solute-induced phase transition or to surface pressure.

To recapitulate, the values of k_b and k_d provide a kinetic basis for understanding the catalytic turnover in the scooting and the hopping modes of interfacial catalysis. The value of k_b and k_d reported in this paper provide a kinetic basis for the interfacial activation. The 50-fold difference between the catalytic turnover rate in the scooting and the hopping modes on DMGPMe vesicles is based on several assumptions, and as such, it should be considered only a lower limit. The turnover rate for catalysis by phospholipase A_2

(60 per s) is a very conservative estimate based on the turnover rate under the first-order kinetics that is characteristic for catalysis in the scooting mode. On the other hand, the turnover rate for prophospholipase A_2 is an upper limit based on the extrapolated V_m values for the hydrolysis in an anionic interface which binds prophospholipase A_2 with a relatively high affinity. It is also possible that the bound prophospholipase A_2 could undergo two or more catalytic turnover cycles before leaving the interface, and thus the underlying effective interfacial on- and off-rates could be appreciably slower. For zwitterionic interfaces the contribution of the interfacial rates can only decrease the overall catalytic turnover number because the binding affinity of phospholipase as well as that of prophospholipase A_2 is considerably lower. Thus, a difference of well over 1000-fold in the rates of hydrolysis in the scooting versus the hopping modes is conceivable under the conditions where the affinity of phospholipase A_2 is much higher than that of prophospholipase.

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