

Enzymatic Activity of a Phospholipase A₂: An Electrochemical Approach

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A novel approach based on dc and ac cyclic voltammetric techniques to evaluate interfacial enzymatic activity is presented. As an illustrative example, the hydrolytic effect of phospholipase A₂ (PLA₂) on an L- α -dioleoylphosphatidylcholine (DOPC) monolayer adsorbed onto a mercury electrode surface has been studied and kinetic parameters have been evaluated. The enzymatic activity obtained in this study is of the order of 10⁻¹ min⁻¹. A dramatic effect of [Ca²⁺] on the hydrolysis of DOPC monolayers by PLA₂ has been observed where the hydrolytic rate constant exhibits a peak-shaped dependence on [Ca²⁺] with a maximum rate at a Ca²⁺ concentration of about 6 mM. Ca²⁺ concentrations above 6 mM appear to have an inhibitory effect on the hydrolysis. Control studies with the nonhydrolyzable substrate analog, N-oleoyl-D-sphingomyelin (SM), demonstrate that the PLA₂ does not simply displace the intact phospholipid molecules from the electrode surface. Thus, the observed electrochemical response arising from PLA₂ is due solely to those enzyme (PLA₂) molecules that are adsorbed onto the mercury electrode surface following the displacement of the hydrolytic products (fatty acids and lysophospholipids). It has also been found that Ca²⁺ has virtually no effect on the adsorption dynamics of PLA₂ onto a mercury electrode surface, with the adsorption rate constant being of the order of 10⁶ min⁻¹ over a Ca²⁺ concentration range of ca. 0–10 mM. A comparison of the chemical reaction (hydrolysis) rate constant (10⁻¹ min⁻¹) with the adsorption rate constant (10⁶ min⁻¹) suggests that the rate-limiting step of the enzymatic reaction is the chemical step itself rather than the binding step of the enzyme from the bulk solution to the interface, consistent with other reports in the literature.

Introduction

Phospholipases A₂ are a family of ubiquitous, small, and water-soluble lipolytic enzymes. These enzymes specifically catalyze the hydrolysis of the 2-acyl ester bond of the L- α -3-*sn*-glycerophospholipids, releasing the corresponding fatty acids and lysophospholipids (for reviews, see refs 1–3 and references therein). Although these enzymes can be found both within and without the cells, the majority of studies have been focused on the extracellular ones, partly because studies of intracellular enzymes have been complicated by the variation of the subcellular site of their location as well as by the interference of phospholipases A₁ that coexist in many cells. Extracellular phospholipases A₂ are abundant in pancreatic tissue and juice and in the venom of snakes and arthropods. Since their discovery late last century, there have been numerous studies geared at the understanding of the related biological functions and regulations, providing insight into the protein–membrane interactions involved. In particular, during the past 2–3 decades, significant progress has been achieved with X-ray crystallographic studies,⁴ in terms of the characterization

of the three-dimensional structure of the molecules as well as of the active center, and the so-called interfacial recognition site.^{1–4} From these, structure/function relationships as well as the activation and reaction mechanisms have been established.⁵ It has been found that although there is a wide variety of sources of the enzymes, they show a very high degree of homology of the amino acid sequence, especially that of the active site and of the disulfide groups. These enzymes show significantly higher affinity and activity to aggregated assemblies of phospholipid molecules (e.g., membranes, vesicles, micelles, bilayers, and monolayers), i.e., the so-called interfacial activation at the lipid/water interface. Thus, a great deal of effort has been devoted to the study of these model membrane systems.^{6,7} Among those, studies on phospholipid monolayers have been more widely carried out to illustrate the enzymatic mechanism,⁷ due to the

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advantages of the requirement of only small quantities of substrates as well as of the ready characterization and control of the monolayer structure. However, these studies have, so far, been confined to monolayers at air/water or oil/water interfaces, which are very difficult to probe with electrochemical techniques.

Since phospholipases A_2 , as well as phospholipids, are generally charged, their interactions with each other are strongly dependent on their charged states.^{5g} Thus, electrochemistry can be an effective way to provide new insights on their properties, due to the high mechanical stability and strong resistance of model biological membranes to electrode potential. Recently, new methodologies have been developed which allow ready fabrication of monolayer films of phospholipids onto electrode surfaces,⁸ and these have paved the way for the study of the enzymatic activity of PLA_2 onto solid-supported phospholipid substrates. In an effort to develop a novel approach for evaluating the PLA_2 enzymatic activity with electrochemical techniques, we have previously carried out studies on the adsorption dynamics and the surface reactions of porcine pancreatic phospholipase A_2 onto a mercury electrode surface.^{9,10} Such preliminary studies are essential for a more thorough understanding of the behavior of the phospholipases A_2 at the electrode/solution interface. It has been previously demonstrated that PLA_2 adsorbs strongly onto mercury electrode surfaces, and it was speculated that the adsorption site is one of the seven disulfide groups, likely the Cys61–Cys91, which is very close to the N-terminus of the enzyme molecule and the interfacial recognition site.^{1,2,10,11}

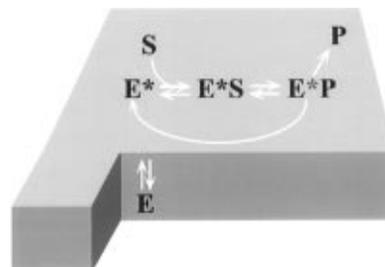
In this manuscript we present a novel approach based on electrochemical techniques to study the enzymatic activity of phospholipases, taking advantage of the recent developments in the preparation of phospholipid monolayers on mercury electrode surfaces. Employing such a solid-supported phospholipid monolayer system, we have probed the interactions involved between a phospholipase A_2 and the phospholipid molecules present in the monolayer. By combining them with our earlier studies of the interfacial behavior of phospholipase A_2 , as well as of DOPC adsorbed on mercury electrodes, we have been able to evaluate the interfacial enzymatic activity of phospholipase A_2 on an adsorbed monolayer of DOPC.

It is well established that Ca^{2+} is an essential cofactor in the activation of PLA_2 , and it has been determined that the primary Ca^{2+} binding site is the active center, where it is involved directly in the catalytic hydrolysis process of phospholipids. There have also been studies that suggest the presence of a second possible binding site found elsewhere on the molecular surface.^{1–3} Taking these observations as a point of departure, we have also studied the effects of $[Ca^{2+}]$ on the hydrolysis by PLA_2 of L- α -dioleoylphosphatidylcholine (DOPC) monolayers adsorbed on a mercury electrode surface.

Experimental Section

1. Materials. Porcine pancreatic phospholipase A_2 (PLA_2 , from Sigma, originally suspended in 3.2 M $(NH_4)_2SO_4$, pH 5.4, at a concentration of 6.3 mg/mL) was used as received. L- α -

Scheme 1. Schematic Illustration of the Interfacial Reaction Mechanism of Phospholipase A_2 (Scooting Mode): E, Enzyme (PLA_2) Molecule; E^* , (Interfacial) Activated Enzyme; S, Phospholipid Assembly; P, Hydrolytic Products



dioleoylphosphatidylcholine (DOPC, from Fluka) and *N*-oleoyl-D-sphingomyelin (SM, from Sigma) were also used as received. Their solutions (0.20 mg/mL) were prepared with chloroform (Fisher, Analytical Reagent) and stored below 0 °C. Electrolyte solutions (0.20 M) were prepared with ultrapure salts (all from Aldrich, of at least 99.99% purity). These were dissolved in water that was purified with a Millipore Milli-Q system and buffered with tris(hydroxymethyl)aminomethane (Tris) (Sigma, 99.0–99.5%) and hydrochloric acid (Aldrich, 99.999%), depending on the desired pH.

2. Procedure. The experimental setup has been described previously.^{8c,8d,9,10} The preparation, transfer, and characterization of phospholipid monolayers onto mercury electrode surfaces have been reported in the work of Lecompte,^{8a} and Nelson,^{8b} and in our earlier publications.^{8c,d} Prior to the introduction of phospholipase A_2 into the working solution, both dc and ac cyclic voltammetric techniques were employed to characterize and ensure the quality of the deposited phospholipid monolayer. The observation of sharp and well-defined voltammetric signatures in both dc and ac voltammetric scans was taken as clear evidence of a well-ordered and compact monolayer of DOPC adsorbed onto the mercury electrode surface. These electrochemical responses were taken as those at time zero. Following injection of the phospholipase A_2 solution (1 μ L) with a microliter syringe, the solution was magnetically stirred for 1 min. To follow the electrochemical responses, dc and ac signals were captured at various time intervals (shorter at the beginning and longer at the end). In the kinetic analysis, the ac, rather than the dc responses, were employed since the magnitude of the response could be more easily related to coverage.

Results and Discussions

Extensive studies of the hydrolysis activity of PLA_2 on phospholipid substrates have demonstrated that the interfacial reactions favor the so-called "scooting mode" (depicted in Scheme 1),^{1–4} which consists of two main steps: the binding of PLA_2 to the lipid/water interface and the subsequent interfacial enzymatic reactions where these enzyme molecules remain on the interface between cycles of hydrolytic reactions of phospholipid molecules. Here, the binding of PLA_2 to the lipid/water interface is a key step, in that it regulates the magnitude of the interfacial activation of the enzyme.^{1,2} Therefore, an appreciation of the magnitude of the binding rate constant is essential for understanding the interfacial catalysis and activation, especially for the determination of the rate-limiting step.

Since the interfacial enzymatic reaction is Ca^{2+} dependent, it is important to probe the effects of Ca^{2+} on the adsorption kinetics of PLA_2 onto the electrode surface (i.e., the binding step). By employing the previously described experimental procedures,^{9,10} we have studied the effect of the Ca^{2+} concentration on the adsorption dynamics of PLA_2 onto a mercury electrode surface and found that there is only a relatively small variation (data not shown) over a Ca^{2+} concentration range of ca. 0–10 mM. The adsorption rate constants are all of the same order of magnitude,

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about 10^6 min^{-1} , in the presence and absence of Ca^{2+} , which is about 7 orders of magnitude greater than the enzymatic reaction rate constant (10^{-1} min^{-1}) obtained below (vide infra), consistent with literature results that the rate-limiting step of the enzymatic reaction is the chemical (hydrolysis) step.¹²

A brief consideration of the scooting mode (Scheme 1) within the present framework is appropriate. It is generally assumed that this mechanism requires a high anionic surface charge of the substrate. In our experiments, the measurements were carried out under potential control where the potential was typically held at -0.30 V . From our previous studies on the adsorption dynamics of PLA₂, we have shown that, at -0.30 V , PLA₂ is strongly adsorbed onto Hg surfaces forming a compact monolayer with anions interacting with the adsorbed monolayer. In addition, we have also shown that the rate of adsorption of PLA₂ under our experimental conditions is much faster than the enzymatic rate of hydrolysis. Thus, although we cannot conclusively establish it, it is quite likely that, under the experimental conditions employed, the reaction does follow the scooting mode. However, it should also be mentioned that our interpretation of the enzymatic reactions is not necessarily dependent on whether the scooting mechanism is followed. Rather, it provides a framework for the discussion and interpretation of the kinetic results.

Figure 1a shows the time dependence of the dc cyclic voltammetric response for a DOPC monolayer adsorbed onto a mercury electrode surface, where it is apparent that, upon the introduction of PLA₂ into the solution, the twin peaks (present over the potential range from -0.80 to -1.0 V and labeled 1 and 2, respectively), which are characteristic of a compact and well-ordered DOPC monolayer, begin to decay. Concomitant to that, one can also observe the development of a pair of well-defined voltammetric peaks (labeled 3 in the figure) centered at about -0.70 V which, on the basis of on previous results,^{9,10} we ascribe to the reduction/oxidation of a disulfide bond within the adsorbed layer of the enzyme. Because the voltammetric response of the adsorbed DOPC is not Faradaic in nature (i.e., there are no redox active centers in DOPC over this potential range) but rather arises as a result of a reorganization of the interfacial charge (i.e., double-layer in nature), the data in this figure are intended to provide only a qualitative sense of the reaction. Figure 1b shows the corresponding out-of-phase (90°) ac voltammetric responses, where it can be seen that the twin peaks also decayed with time and, again, there was the appearance of a pseudocapacitance peak at about -0.70 V associated with the adsorbed PLA₂ molecules. Here, one should also notice that between -0.30 and -0.70 V , the ac signal increased after the introduction of PLA₂ to the system. Since this potential regime is the so-called minimum capacitance region, where the interfacial structure of DOPC monolayers is unaffected by the electrode potential (structurally intact), the ac current, and therefore the interfacial capacitance (more details below), can be related to the surface coverage of PLA₂. By measuring the time evolution of the ac response, one could follow the kinetics of PLA₂ adsorption onto the electrode surface in the presence of an adsorbed monolayer of DOPC. From these data, the hydrolytic activity of PLA₂ could, in turn, be evaluated.

However, appropriate blank experiments needed to be carried out since the observation of a response from

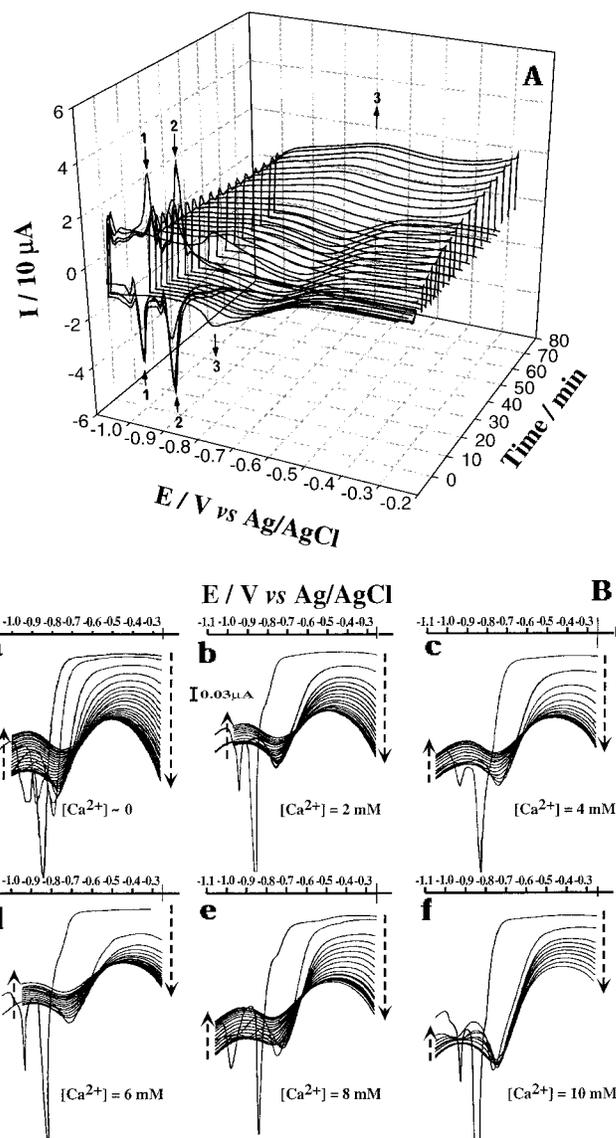
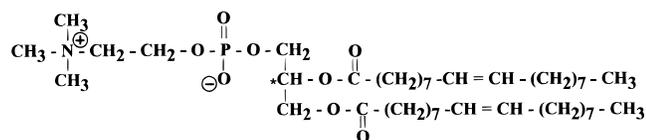
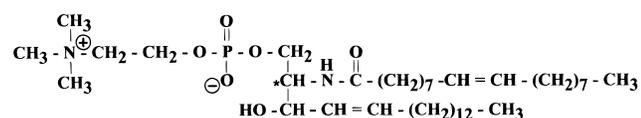


Figure 1. Cyclic voltammograms of a DOPC monolayer on a mercury electrode surface upon the introduction of PLA₂. PLA₂ concentration was $0.09 \mu\text{M}$. Supporting electrolyte solution was 0.20 M KCl buffered with 0.010 M Tris/HCl ($\text{pH} = 8.6$). HMDE area 1.82 mm^2 . (a) The dc responses. $[\text{Ca}^{2+}] = 2 \text{ mM}$. Sweep rate was 50 V/s . Total elapsed time period 70 min . (b) Corresponding out-of-phase (90°) ac responses at various Ca^{2+} concentrations. The ac frequency was 73 Hz and the dc potential ramp was 10 mV/s . Elapsed time periods for **a**, no added Ca^{2+} , 120 min ; **b**, $[\text{Ca}^{2+}] = 2 \text{ mM}$, 70 min ; **c**, $[\text{Ca}^{2+}] = 4 \text{ mM}$, 50 min ; **d**, $[\text{Ca}^{2+}] = 6 \text{ mM}$, 50 min ; **e**, $[\text{Ca}^{2+}] = 8 \text{ mM}$, 70 min ; **f**, $[\text{Ca}^{2+}] = 10 \text{ mM}$, 95 min .

adsorbed PLA₂ could arise as a result of two possibilities: either the PLA₂ molecules displaced intact phospholipid molecules from the surface or the PLA₂ hydrolyzed the adsorbed DOPC, displaced the hydrolytic products (namely, the fatty acid and the lysophospholipid molecules), and subsequently adsorbed onto the electrode surface. Clearly, both processes would give rise to the same electrochemical response.

In order to distinguish between these two processes, we carried out a control experiment with sphingomyelin (SM). SM and DOPC have the same polar head group (choline) with their main differences being the two hydrocarbon chains and their linkages to the glycerol backbone (Figure 2). Since in SM the *sn*-2 linkage is an amide and not an ester bond (as in DOPC), SM is not an active substrate for and cannot be hydrolyzed by PLA₂. Thus, if PLA₂ displaces SM from the electrode surface (as would be

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L- α -Dioleoylphosphatidylcholine (DOPC)

N-oleoyl-D-sphingomyelin (SM)

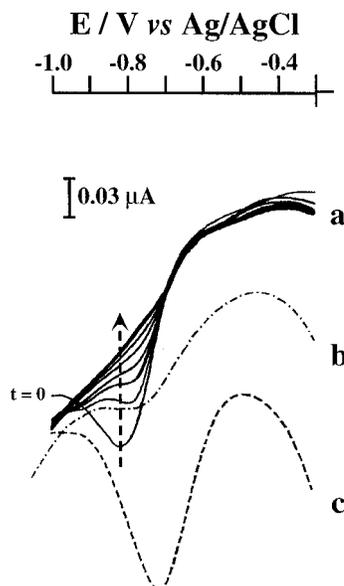
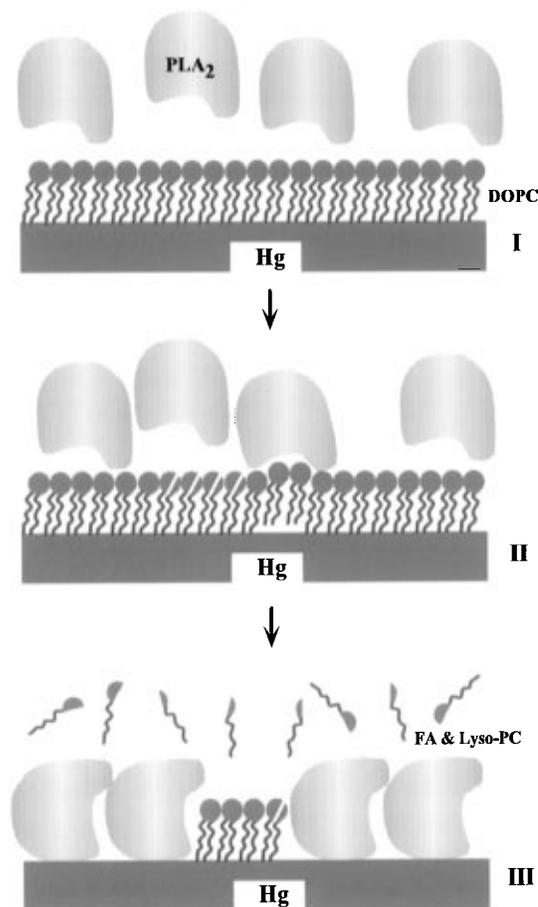
Figure 2. Molecular structures of DOPC and SM.

Figure 3. (a) The ac responses of a SM monolayer adsorbed on a mercury electrode surface upon the injection of PLA₂ (to a concentration of 0.09 μM). Experimental conditions were the same as in Figure 1. Total elapsed time 40 min. (b) The ac response after the negative potential limit was expanded to -2.0 V and then returned to the original value. (c) Steady-state ac current of a bare mercury electrode (same area) in the same electrolyte solution containing PLA₂ without adsorbed DOPC.

evidenced by the appearance of a voltammetric wave at -0.70 V due to adsorbed PLA₂), we would conclude that it would also displace adsorbed DOPC. On the other hand, if no such voltammetric wave is observed, then reasoning by analogy, one could safely assume that DOPC is also not physically displaced by PLA₂ so that any voltammetric response at -0.70 V would necessarily have to arise via hydrolysis of DOPC by PLA₂ as described above. Figure 3 shows the electrochemical response of an SM monolayer adsorbed onto a mercury electrode surface which exhibits a voltammetric response which, analogous to DOPC monolayers, we ascribe to an electrode potential induced reorientation (first scan in Figure 3a). Upon the introduction of PLA₂ into the solution, the structural transition peak, at about -0.80 V, of SM decreases in amplitude, suggesting that PLA₂ molecules form an overlayer on top of the SM monolayer. However the region between -0.30 and -0.70 V remains virtually unchanged, indicating that the SM monolayer structure was still intact, which suggests that PLA₂ molecules do not displace the SM monolayer nor adsorb onto the electrode surface. Figure 3b shows the ac response after a few cycles at a fast sweep rate (50 V/s) during which the negative potential limit

Scheme 2. Schematic Drawing of the Hydrolysis Reaction Mechanism of a Solid-Supported Phospholipid Monolayer^a



^a Note: the drawing is not to scale.

was extended to -2.0 V and, afterward, returned to the original value. It can be seen that even after sweeping the potential to such a negative value, which might desorb some of the SM molecules from the electrode surface, the amount of adsorbed PLA₂ molecules, indicated by the pseudocapacitance peak at -0.75 V, is still quite small, especially when compared to the response for a bare mercury electrode (Figure 3c) exposed to the same solution. (That is, Figure 3c is the response for PLA₂ directly adsorbed onto a Hg electrode surface in the absence of DOPC.) This suggests a strong binding of the SM monolayer to the mercury electrode surface and a very low displacement efficiency of SM molecules by PLA₂. Given the similarity between SM and DOPC molecules, we assume that the same holds for monolayers of DOPC on mercury. Moreover, the responses described in Figure 3 were independent of changes in the Ca²⁺ concentration, as would be expected. On the other hand, the hydrolysis reaction of DOPC by PLA₂ was dependent on the Ca²⁺ concentration (vide infra), again, consistent with the proposed reaction.

Therefore, we conclude that the displacement from the electrode surface of intact DOPC phospholipid molecules by PLA₂ is very slow, so that in the case of DOPC the voltammetric response centered at -0.75 V arises from PLA₂ molecules that hydrolyzed adsorbed DOPC molecules, displaced the hydrolysis product molecules and subsequently adsorbed onto the Hg electrode surface. This sequence of events is depicted in Scheme 2. Hence, the time evolution of this adsorption process (of PLA₂) reflects the actual hydrolysis step, and its analysis can be employed for an evaluation of the interfacial enzymatic activity.

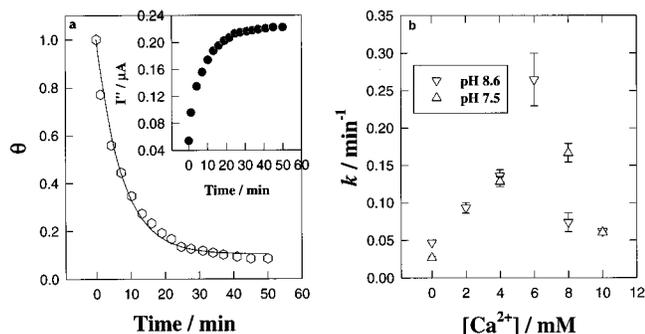


Figure 4. Hydrolysis kinetics of DOPC monolayers by PLA₂. (a) Reaction progress in terms of the surface coverage of DOPC molecules. Experimental conditions are the same as in Figure 1a. Inset shows the development of ac current at -0.51 V vs time. (b) $[Ca^{2+}]$ effect on the hydrolysis rate of DOPC monolayers at different solution pH (from Figure 1b).

Implicit in this statement is the assumption that the rate of adsorption of PLA₂ to the mercury electrode surface is much faster than the rate of hydrolysis of DOPC by PLA₂ as was indeed found to be the case (vide infra).

In the absence of a Faradaic reaction, the ac current response from an adsorbed layer is directly proportional to its capacitance (C_1), and since the capacitance is also proportional to the surface coverage of adsorbates in the case of a mixed adsorbed layer, one should be able to monitor the decrease of the phospholipid (DOPC) coverage (or equivalently the increase in the PLA₂ coverage) by measuring the appropriate ac current.¹⁰ At time t , where the surface coverage of phospholipid molecules is $\theta(t)$, the corresponding ac current can be expressed as

$$I' = \theta I'_{PC} + (1 - \theta)I'_E \quad (1)$$

where I' is the measured ac current during the hydrolysis reactions; I'_{PC} is the ac current for a pure DOPC monolayer; I'_E is the ac current of PLA₂ at saturation coverage. The current is measured at a potential (-0.51 V) where no Faradaic redox reaction of the adsorbed PLA₂ molecules is present. (Also recall that at this potential DOPC has no redox response.)

Equation 1 can be recast in terms of the fractional surface coverage (θ) as

$$\theta = \frac{I'_E - I'}{I'_E - I'_{PC}} \quad (2)$$

Figure 4a shows the decrease in the DOPC surface coverage with time (the inset shows the time dependence of the ac current due to PLA₂ adsorption). The fractional surface coverage (θ) is obtained from the ac current using eq 2. Symbols are the experimental data of the surface coverage of DOPC, and they are fit very well by first-order kinetics (solid line), which suggests that the hydrolysis process follows such kinetics. This is consistent with monolayer studies with the so-called first-order trough¹³ where the surface pressure was maintained constant during the action of PLA₂ onto the monolayer film and the observed reaction followed first-order kinetics. In the present study on solid-supported monolayers, it is most likely that the equimolar hydrolytic products (1-acyllysophosphatidylcholine and the long-chain fatty acid) initially remain on the surface, which reportedly form

very ordered, stable structures¹⁴ and consequently help maintain the surface pressure. On the other hand, when these molecules are displaced by PLA₂, the compactness of the strongly adsorbed PLA₂ layer would not be expected to alter the surface pressure significantly. From the fitting, the reaction rate constant can be evaluated.

As mentioned earlier, the activity of PLA₂ is known to be Ca²⁺ dependent. Figure 4b shows the effect of Ca²⁺ concentration on the hydrolysis of DOPC monolayers where a peak-shaped dependence is apparent. The rate constant is of the order of 10^{-1} min^{-1} under the two pH conditions studied (pH 7.5 and 8.6) with a maximum value obtained at a Ca²⁺ concentration of about 6 mM. Moreover, comparison of the reaction rate constant with the adsorption rate constant (recall that its value was of the order of 10^6 min^{-1}) strongly suggests that the rate-limiting step indeed is the chemical (hydrolysis) step, which is consistent with literature findings.¹² It is also apparent from the figure that even in the case where there was no intentionally added Ca²⁺, there was still some hydrolytic activity. We ascribe this to residual Ca²⁺ present in the enzyme preparation as stated by the manufacturer.

Since we do not know what fraction of the adsorbed PLA₂ molecules that give rise to the electrochemical response are actually involved in the enzymatic reaction, it is difficult to obtain the exact specific interfacial activity of PLA₂. Nevertheless, we can estimate a lower level for the activity of PLA₂ on this solid-supported substrate by assuming that all the PLA₂ molecules are involved in the reaction. Using this value yields a specific activity of the order of $1 \text{ } \mu\text{mol}/\text{min}/\text{mg}$ of protein.

Ongoing studies are geared at a better understanding of these processes as well as the study of other enzymatic reactions using the procedures described here. Results from these investigations will be reported elsewhere.

Conclusions

A novel approach, based on dc and ac voltammetric techniques, for evaluating the interfacial enzymatic activity of porcine pancreatic phospholipase A₂ toward a monolayer of DOPC adsorbed on a mercury electrode surface has been presented. It has been found that the adsorption kinetics of PLA₂ from the bulk solution onto the electrode surface, with an adsorption rate constant of the order of 10^6 min^{-1} , is much faster than the interfacial hydrolysis steps (with a rate constant of the order of 10^{-1} min^{-1}), in good agreement with literature results that the rate-limiting step is the chemical (hydrolysis) reaction.¹² There was virtually no effect of Ca²⁺ on the dynamics of enzyme adsorption. However, the concentration of Ca²⁺ had a significant effect on the rate of hydrolysis by PLA₂ of adsorbed DOPC monolayers exhibiting a maximum value at a Ca²⁺ concentration of 6 mM. Control experiments with sphingomyelin (a nonhydrolyzable analog) suggest that the displacement rate of the intact DOPC phospholipid molecules by PLA₂ is very slow, thus allowing for the assertion that the electrochemical responses arising from the interaction between PLA₂ and adsorbed DOPC monolayers are due to those PLA₂ molecules that catalyze the hydrolysis of the phospholipid molecules, subsequently displace the hydrolytic products, and then adsorb onto the electrode surface.

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