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# Bicelles in structure–function studies of membrane-associated proteins

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**Abstract**

Bicelles are a novel form of long-chain/short-chain phospholipid aggregates, which are useful for biophysical and biochemical studies of membrane-associated biomolecules. In this work, we review the development of bicelles and their uses in structural characterization (primarily via NMR, circular dichroism, and fluorescence) of membrane-associated peptides. We also show that bicellar phospholipids are substrates for lipolytic enzymes. For this latter work, we employed a $^{31}$P NMR enzymatic assay system to examine the kinetic behavior of cobra venom phospholipase $A_2$ toward a variety of bicellar substrates. This enzyme hydrolyzed all bicelle lipids at rates comparable to those found for the enzyme action on traditional micellar substrates, which are the best substrates for this enzyme. In addition, we found that this $PLA_2$ showed no significant preference for long-chain or short-chain phospholipids when they were presented as mixtures in bicelles.


*Keywords:* Phospholipase $A_2$; Bicelle; Model membrane; NMR

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## 1. Introduction

### 1.1. Membrane mimetics

Although information on the structure and function of soluble proteins is expanding at an incredible rate, it has been more difficult to obtain data on membrane-associated biomolecules. This is due in part to the fact that membrane-bound proteins can be difficult to work with, both mechanistically and structurally. In addition, natural membranes are themselves intrinsically complex and difficult to manipulate. The large size of these complexes makes their use in standard structural studies, i.e., X-ray crystallography and NMR, very difficult. The presence in natural membranes of numerous other molecules besides the enzyme of interest adds to the difficulty in conducting studies of the interaction of enzymes with membranes. Therefore, it is important to develop model membrane systems that will allow one to examine the enzyme action on pure phospholipid bilayers and at the same time allow one to examine the structure and orientation of the enzyme when a membrane is associated.

Current model membrane systems that have been employed in functional studies of lipolytic enzymes include vesicles, micelles, mixed micelles, and short-chain/long-chain unilamellar vesicles (SLUVs) [1–6]. These systems have proven quite valuable for measuring the activity of various enzymes and allow one to vary the phospholipid composition. Cells carefully control their membrane composition to modulate the enzyme activity or its distribution. For example, it has been suggested that hydrophobic mismatch between the hydrophobic stretch of a transmembrane peptide and the bilayer thickness is a control mechanism for sorting proteins in the Golgi apparatus and endoplasmic reticulum (ER) [7,8]. Therefore, it is important to consider specific membrane properties that could affect the biological activity of an associated macromolecule. An appropriate membrane mimetic would allow the incorporation of different amounts of naturally occurring phospholipids and other membrane components to accurately represent the composition of a cell membrane. This can be easily done for each of the models listed above.

These models have been used extensively to study the action of phospholipase $A_2$ ($PLA_2$), a lipolytic enzyme that hydrolyzes the *sn*-2 fatty acid ester of phospholipids. These studies have shown that the physical state in which the phospholipids are presented can dramatically affect the enzyme activity. For example, although $PLA_2$s are active on a lipid bilayer ($<50\,\mu mol\ min^{-1}\ mg^{-1}$), the addition of a detergent such as Triton X-100 (TX-100) to form mixed micelles dramatically increases the enzyme activity ($1000–2000\,\mu mol\ min^{-1}\ mg^{-1}$). There are numerous possible reasons for this activation. They include changes in phospholipid packing or conformation or changes in the enzyme conformation or orientation at the interface.

Thus, these model systems are much simpler than native membranes and have proven useful in studying enzyme kinetics. They have, however, not solved the problems of studying the enzyme structure and orientation in the presence of a membrane. While phospholipid vesicles are much smaller than native membranes, it has still proven difficult to generate protein crystals for X-ray analysis when they are present. Vesicles are also not suitable for high-resolution NMR techniques due to low reorientation rates that lead to significant line broadening. On the other hand,

micelles, which are significantly smaller than vesicles, have been used successfully in obtaining NMR data for membrane-associated peptides [9–12]. However, the phospholipid packing in micelles is dramatically different from that of vesicles or bilayers. The curvature of the micelle surface is also much greater than that for a vesicle or a membrane. These differences could alter the way an enzyme interacts with these structures. For example, studies on some channel forming peptides have shown that the intrinsic curvature strain of a bilayer appeared to affect the peptide activity and/or mechanism of action [13,14].

Thus, while these model systems have allowed the analysis of various important biochemical functions, they are not amenable to correlating this to structural data. The bilayered micelle, "bicelle," is a model that has the potential to address this issue. The bicelle may allow one to study both function and structure in the same system.

### *1.2. Bicelles*

Sanders and Prestegard introduced the bicelle for solid-state NMR studies of membrane-associated biomolecules [15,16]. Bicelles are discoidal lipid aggregates composed of long-chain phospholipid and either detergent or short-chain phospholipid. As shown in Fig. 1, the distinguishing structural feature of a bicelle is a central planar bilayer formed by the long-chain phospholipid, surrounded by a rim of short-chain phospholipid or detergent that shields the long-chain lipid tails from water. Bicelle size is controlled both by the ratio of long-chain:short-chain phospholipid ($q$) and the total phospholipid concentration ($c_L$). Dimyristoyl phosphatidylcholine (DMPC) is often used as the long-chain phospholipid component and it can be doped with phospholipids that have identical chain lengths but different headgroups (e.g., dimyristoyl phosphatidylserine (DMPS) or dimyristoyl phosphatidylglycerol) to alter the charge characteristics of the interface and provide

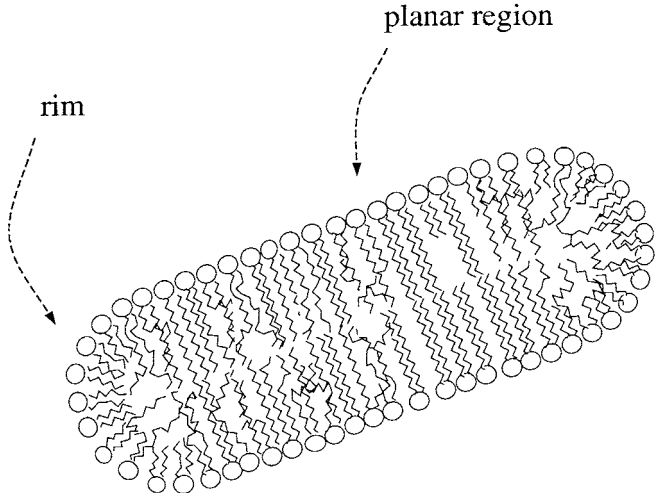


Fig. 1. Schematic representation of a bicelle showing the planar region, composed of long-chain phospholipid, surrounded by a rim of short-chain phospholipid.

versatility in phospholipid composition [17]. Bicelles can also be prepared with dipalmitoyl phosphatidylcholine (DPPC) or dilauryl phosphatidylcholine (DLPC) to vary the total bilayer thickness (manuscript in preparation). The rim, on the other hand, can be composed of either a bile-salt derivative such as 3-(cholamidopropyl)dimethylammonio-2-hydroxy-1-propanesulfonate (CHAPSO) or a short-chain phospholipid such as dihexanoyl phosphatidylcholine (DHexPC) [18–20]. Since the long-chain phospholipid in bicelles is sequestered into the planar core region, devoid of short-chain phospholipid or detergent, the core region of the bicelles mimics a section of natural membrane much better than standard micelles. Micelles have very little planar surfaces and the detergent is usually uniformly dispersed through out the phospholipid. Thus, the bicelles may provide a better membrane model in which to study membrane-associated biomolecules. For example, studies of diacylglycerokinase activity in bicelles were found to be maintained over time while enzyme activity in a micellar system was unstable [21].

The size and properties of these lipid aggregates can be varied as a function of lipid ratio and concentration to provide a variety of similar bicelle phases that are amenable to different types of biological studies. At high ratios of long- to short-chain phospholipids ($q > 3$) and high-total phospholipid concentration ($c_L \sim 15$–$25\%$ (w/w)), bicelles form discoidal lipid aggregates of approximately 4080 kDa and 500 Å diameter [22]. These phospholipid mixtures spontaneously align in magnetic fields of >2 T (Fig. 2A) at temperatures above the transition temperature ($T_m$) of the long-chain phospholipid. This property has made it possible to use solid-state NMR techniques to examine both the orientation of a peptide associated with the bicelle with respect to the bicelle surface and the effects of the peptide on lipid packing within the bicelle [16,20,21,23–27]. In this phase (high $q$), DMPC bicelles have been shown to form stacks of perforated lamellae, where the bicelles have formed edge-to-edge contacts, whereas, below the $T_m$, these phospholipid mixtures have been shown to contain the standard bicelle morphology [28,29].

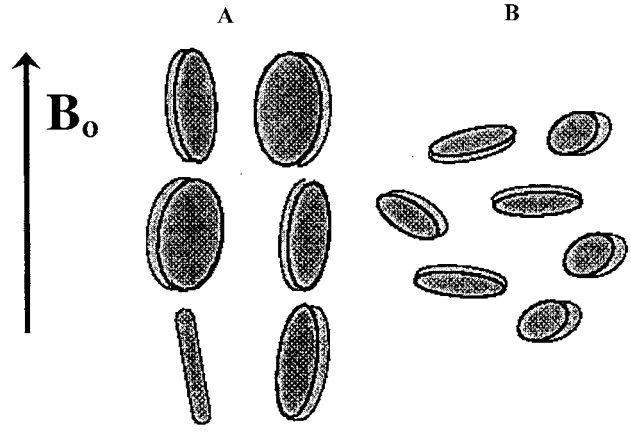


Fig. 2. Schematic representation of bicelle alignment in the magnetic field. (A) Large bicelles ($q > 3$ and $c_L \sim 15$–$25\%$ (w/w)) align with their normals oriented perpendicular to the field while small bicelles ($q < 1$ and $c_L \sim 10$–$15\%$ (w/w)) are unaligned.

As the amount of short-chain phospholipid is increased ($q < 1$ and $c_L \sim 5$–15% (w/w)), bicelles form an unaligned phase (Fig. 2B) that is suitable for high-resolution NMR studies [26,27,30,31]. Several complementary techniques have been used to show that these bicelles, which are much smaller than their alignable counterparts (252 kDa and 80 Å diameter), remain discoidal with segregated lipid pools [22,32,33]. This is of the same morphology as that observed for bicelles in the aligned phase. These *isotropic* bicelles exist over a wide range of sample conditions and have a viscosity amenable to solution-state studies of peptides and proteins [30]. Their *isotropic* nature makes them suitable for high-resolution NMR studies of protein structure. These *isotropic* bicelles were found to be stable over a wide range of phospholipid ratios ($q = 0.05$–0.5) and temperatures (15 and 37 °C) and maintained a free (not bicelle associated) short-chain phospholipid concentration of 7 mM for DHexPC. If the total phospholipid concentration is between 1% and 5% (w/w), the short-chain phospholipid dissociates from the bicelle to maintain the free short-chain phospholipid concentration at 7 mM. This increases $q$, which, in turn, leads to a doubling of the bicelle size over this concentration range. At total phospholipid concentrations less than 1% (w/w), the bicelles undergo a drastic change in morphology, resulting in all of the short-chain phospholipids leaving the bicelle, causing the long-chain phospholipids to form a large soluble lipid aggregate, presumably vesicular in structure [33].

Thus, bicelles offer a unique system in which different techniques can be employed to examine the interaction of proteins and model membranes. The aligned phase bicelles, $q > 3$, allow the use of solid-state NMR techniques to determine the orientation of the protein and to determine the orientation of the phospholipids. The *isotropic bicelles* can be used to conduct NMR structural studies of the enzyme. In both cases, the protein sees the identical structure and environment so that the results should be directly comparable.

The last requirement for a membrane model is that enzymes that act on native membranes are able to act on the bicelles in the same fashion. Previous studies of diacylglycerol kinase (DAGK) activity in various large bicelle systems ($q \geqslant 2.5$) have shown that the enzyme activity was comparable to those found in mixed micelles and vesicles [34]. We present data below that indicate that small isotropic bicelles ($q = 0.5$) also provide an excellent substrate for phospholipase $A_2$, a lipolytic enzyme. Thus, the isotoropic bicelles appear to be a good membrane model for at least this enzyme. The aligned phase can be used to determine the orientation of the enzyme at the surface. The *isotropic* phase can be used to analyze the structure of the enzyme and to measure the enzyme activity.

## 2. Methods

### 2.1. Protein expression and purification

The gene for $PLA_2$ (from the Indian cobra *Naja naja naja*) was previously subcloned into a yeast expression vector containing an AOX1 promoter and transfected into the protease-deficient strain of *Pichia pastoris*, SMD1168 [35]. Large-scale fermentation and subsequent purification to homogeneity of $PLA_2$ were performed as described previously [35,36].

### 2.2. Modified Dole assay for enzyme activity

Bicelle samples for the modified Dole assay [37] were prepared by first dissolving the long-chain phospholipid (Avanti Polar Lipids, Alabaster, AL) in chloroform with a sufficient amount of radiolabeled DMPC (1-myristoyl-2-[$^{14}$C]myristoyl phosphatidylcholine) (gift from New England Nuclear, Boston, MA) or DPPC (1-palmitoyl-2[$^{14}$C]palmitoyl phosphatidylcholine) (New England Nuclear, Boston, MA), to provide approximately 100,000 CPM per 500 μL assay. The chloroform was removed using $N_2$ and by placing the samples under vacuum for at least 1 h. Stock bicelle solutions (25% (w/w) for samples containing DHexPC or CHAPSO and 20% (w/w) for samples containing DHepPC) were then prepared by first vortexing the dried long-chain phospholipid in an appropriate amount of $H_2O$. DHexPC, DHepPC, or CHAPSO from a stock solution was then added to the long-chain phospholipid dispersion to achieve a $q = 0.5$. The samples were vortexed and heated at 50 °C until clear. Assay samples were prepared by addition of the appropriate amounts of stock bicelle solution, 1.0 M $CaCl_2$, and 1.0 M *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] (Hepes), pH 6.9. Samples were diluted to a final volume of 500 μL, yielding final concentrations of 50 mM total phospholipid, 10 mM $CaCl_2$, and 200 mM Hepes.

Samples were placed in a water bath at temperatures above the $T_m$ of the long-chain phospholipid (40 °C for DMPC samples and 45 °C for DPPC samples). The assay was initiated by the addition of 25 ng $PLA_2$. After the hydrolysis had been allowed to proceed for the desired amount of time, the reaction was quenched by the addition of 2.5 mL Dole reagent (2-propanol:heptane:$H_2SO_4$ 20:5:1 (v/v/v)), followed by about 0.1 g silica gel to sequester any remaining phospholipid. Heptane and milliQ water, 1.5 mL each, were then added. After vortexing and phase separation, 500 μL heptane, containing the free fatty acids, was removed and passed through a column containing approximately 0.1 g silica gel. The column was then washed with 1 mL ether and 5 mL scintillation cocktail was added to the flowthrough. Samples were counted on a Packard TR1600 Liquid Scintillation Analyzer. All experiments were performed in either duplicate or triplicate.

### 2.3. $^{31}$P NMR assay for enzyme activity

Stock bicelle solutions were prepared as described for the Dole assay without the radiolabeled phospholipid. NMR samples were also prepared in the same manner as for the Dole assay, with the addition of an appropriate amount of $D_2O$ (Cambridge Isotope Labs, Andover, MA) to yield a final concentration of 10% (v/v).

One-dimensional $^{31}$P spectra were recorded on a Bruker DRX600 spectrometer equipped with a 5 mm BBI probe using a proton-decoupled single-pulse experiment with 128 scans. All spectra were recorded with 8192 complex points and a sweep width of 100 ppm. The carrier frequency was set to 0 Hz. DMPC samples were equilibrated at 40 °C and DPPC samples were equilibrated at 45 °C before the addition of enzyme. Twenty-five ng $PLA_2$ (from a 10 ng/μL stock solution) was then added directly to the NMR tube, which was then vortexed and immediately placed back in the magnet. Spectra at various time points were acquired over a period of

2 h. All experiments were processed with the Bruker XWINNMR software package. Each experiment was repeated in triplicate.

## 3. Results and discussion

### 3.1. $PLA_2$ activity on bicelles

To determine if lipolytic enzymes can act on bicelles, we have studied the hydrolysis of bicelles by cobra venom $PLA_2$. $PLA_2$s are water-soluble lipolytic enzymes that catalyze the hydrolysis of the *sn*-2 ester bond of phospholipids.

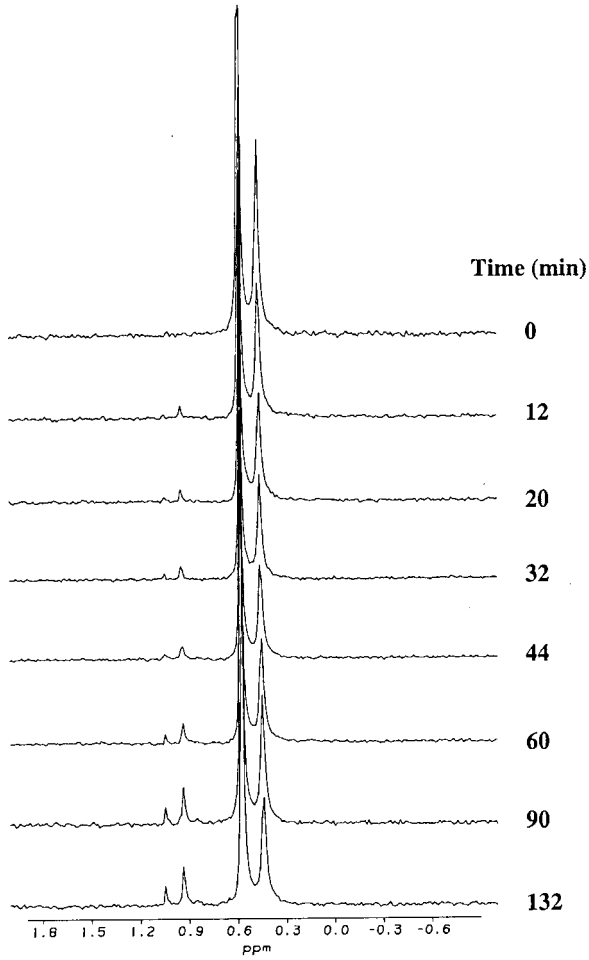


Fig. 3. 1D $^{31}P$ NMR spectra of DMPC/DhexPC bicelles during hydrolysis by $PLA_2$. The sample contained $q = 0.5$, $c_L = 50$ mM, 10 mM $CaCl_2$, 200 mM Hepes, pH 6.9, and 25 ng enzyme added at $t = 0$ min. Spectra were acquired at 40 °C.

Since bicelles contain two potential substrates for $PLA_2$ hydrolysis, we employed a $^{31}P$ NMR assay that allowed us to separately and concurrently detect the hydrolysis of each bicelle component. Fig. 3 shows an example of 1D $^{31}P$ NMR spectra acquired during the hydrolysis of DMPC/DHexPC bicelles by $PLA_2$. The long- and short-chain phospholipid peaks were assigned as described previously [33]. The long-chain lysophospholipid peak was assigned by examining the $^{31}P$ NMR spectra of DMPC/CHAPSO bicelles that had been subjected to long-term hydrolysis. The short-chain lysophospholipid peak was assigned to the remaining peak. The peaks are from upfield to downfield: DMPC, DHexPC, 1-myristoyl-2-OH-phosphatidylcholine (Lyso-DMPC), and 1-caproyl-2-OH-phosphatidylcholine (Lyso-DHexPC). Both the phospholipid and lysophospholipid peaks were integrated and the percentage hydrolysis and subsequent specific activity were calculated for each phospholipid component.

Since NMR is not a typical assay for $PLA_2$ activity, the Dole radioactive assay for $PLA_2$ hydrolysis of the long-chain phospholipid was also performed to confirm the results obtained in the NMR experiments. Fig. 4 compares the measured $PLA_2$ hydrolysis of DPPC in DPPC/DHepPC bicelles as measured by $^{31}P$ NMR and the radioactive Dole assay. (Since only radiolabeled long-chain phospholipid is available, we were only able to monitor the hydrolysis of the DPPC.) The values for long-chain lipid hydrolysis reported by both assays were within experimental error of each other. This indicates that the NMR assay accurately reports phospholipid hydrolysis and that the

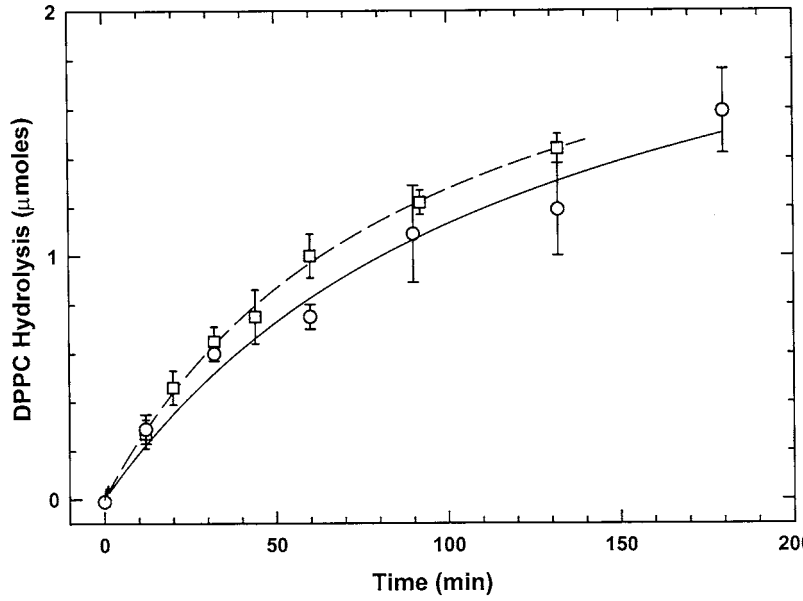


Fig. 4. Comparison of DPPC hydrolysis in DPPC/DHepPC bicelles as measured by $^{31}P$ NMR (□, dotted line) and radioactivity (○, solid line). Samples were prepared with $q = 0.5$, $c_L = 50$ mM, 10 mM $CaCl_2$, and 200 mM Hepes, pH 6.9, and hydrolysis was initiated with the addition of 25 ng $PLA_2$. Measurements were performed at 45 °C.

specific activities calculated from the NMR data can be directly compared to those obtained from other standard $PLA_2$ assays. Fig. 5 shows the total hydrolysis of both the long- and short-chain bicelle phospholipids as a function of time. In all cases, the hydrolysis was linear from 12 to 60 min. The following specific activities were obtained from these data: DPPC/DHepPC, $1320 \pm 80$; DMPC/DHepPC, $880 \pm 40$; DPPC/DhexPC, $560 \pm 80$; DMPC/DhexPC, $680 \pm 80$; DMPC/CHAPSO, $640 \pm 77$. The later three were within experimental error. The bicelles containing DMPC/DHepPC were slightly higher while the DPPC/DHepPC was twice the others. All of these rates are similar to these seen for the cobra $PLA_2$ on mixed micelles of DPPC with Triton X-100, which is one of the best substrates for this enzyme.

The surface packing of the long-chain phospholipid in the core and the short-chain phospholipid on the rim should differ dramatically. Since the activity of $PLA_2$s varies dramatically with changes in the lipid packing, we examined the individual rates of hydrolysis of the long- and short-chain phospholipids. Fig. 6 shows specific activities calculated between 12 and 44 min of hydrolysis. Within experimental error, the short- and long-chain phospholipids were hydrolyzed at similar rates. It would appear that the differences in the packing of phospholipids

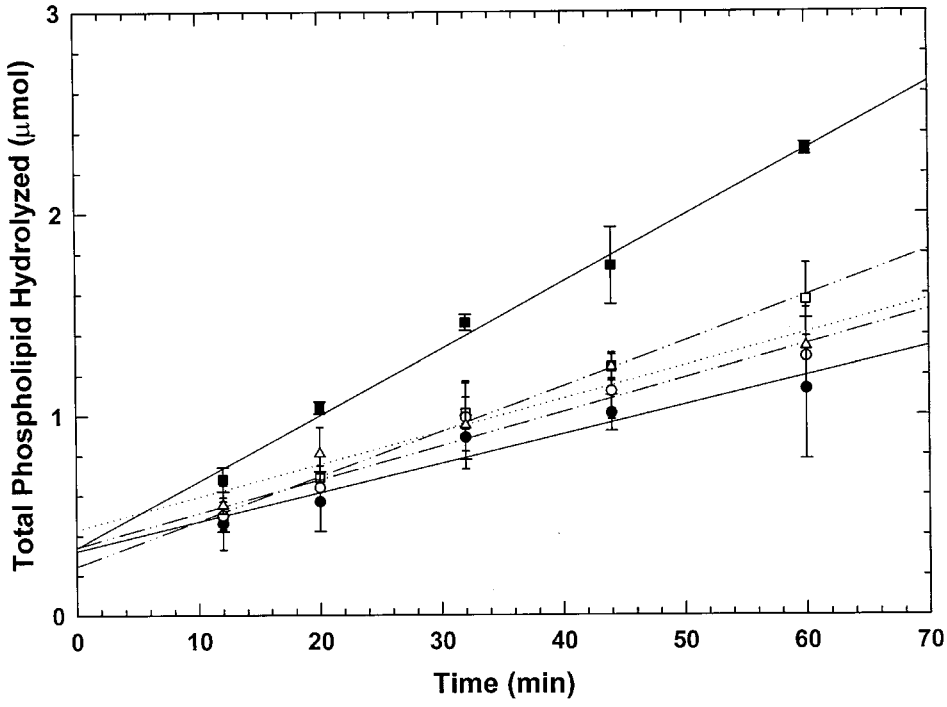


Fig. 5. Total bicelle hydrolysis by $PLA_2$ as measured by $^{31}P$ NMR. DPPC/DHepPC (■, solid line), DMPC/DHepPC (□, dashed line), DPPC/DhexPC (●, solid line), DMPC/DhexPC (○, dashed line), and DMPC/CHAPSO (Δ, dotted line). Samples were prepared with $q = 0.5$, $c_L = 50$ mM, 10 mM $CaCl_2$, 200 mM Hepes, pH 6.9, and 25 ng enzyme added at $t = 0$ min. DMPC spectra were acquired at 40 °C and DPPC spectra were acquired at 45 °C.

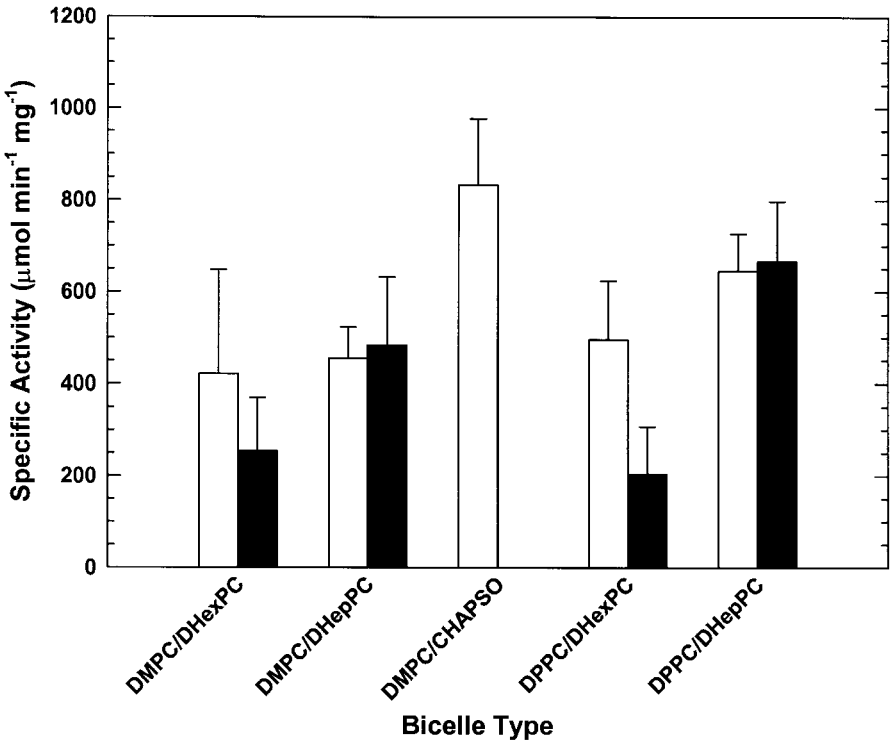


Fig. 6. Comparison of $PLA_2$-specific activities as determined by $^{31}P$ NMR on phospholipid components of different bicelle systems; long chain (open bar), short chain (closed bar). Specific activities determined for hydrolysis between 12 and 44 min. All samples contained $q = 0.5$, $c_L = 50$ mM, 10 mM $CaCl_2$, 200 mM Hepes, pH 6.9, and 25 ng $PLA_2$. DMPC experiments were performed at 40 °C and DPPC experiments were performed at 45 °C.

in the edge and the planar core region do not seem to affect the activity of the cobra venom $PLA_2$.

From these data, we have shown that $PLA_2$ is fully active on an isotropic bicellar substrate. Once substrate hydrolysis has begun, the cobra venom enzyme shows no strong phospholipid preference. Overall, both phospholipids were hydrolyzed at micellar rates. Since we have shown previously that these isotropic bicelles are discoidal aggregates composed of a long-chain lipid bilayer surrounded by a rim of short-chain lipid, the data presented here represent the hydrolysis of a long-chain phospholipid in a true planar bilayer. The observation of micelle like hydrolysis rates for both rim and bilayer phospholipids coupled with a lack of enzyme specificity for either the long- or short-chain phospholipid suggests that the enzyme may be binding to the bicelle at the border between the planar and rim regions. This area is likely to be discontinuous thereby producing defects that could increase the accessibility of the long-chain lipid to the enzyme.

This work also demonstrates the utility of small, unaligned bicelles for biochemical studies of peptides and proteins that require either a phospholipid substrate

or membrane-like environment for biological activity. In addition, by performing solid-state NMR studies in the anisotropic bicelle phase, the activity observed here could be related to the enzyme orientation at the membrane interface. This would further demonstrate the validity and versatility of bicelles as a model membrane system and their potential uses in deducing structure–function relationships for membrane-associated molecules.

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