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Use of Short-chain Cyclopentano-phosphatidylcholines to Probe the Mode of Activation of Phospholipase A_2 from Bovine Pancreas and Bee Venom*

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A great mystery in the mechanism of phospholipase A_2 (PLA2) and many other lipolytic enzymes is the "interfacial activation" induced by micellar but not monomeric substrates. Equally mysterious is the lack of interfacial activation in bee venom PLA2, as opposed to PLA2s from pancreas and other sources. We have probed these problems using the conformationally restricted short-chain cyclopentano-analogues of diacylphosphatidylcholine (Cp-DC$_n$PC, all-*trans* isomer). In the reaction catalyzed by bovine pancreatic PLA2, Cp-DC$_8$PC behaved differently from DC$_8$PC in that its monomers and micelles showed comparable activities (but lower than the activity of DC$_8$PC). This result suggests that the activity of PLA2 can be regulated by substrate conformation and supports the "substrate conformation model" (Wells, M. A. (1974) *Biochemistry* 13, 2248–2257), but raises a question as to whether Cp-DC$_8$PC mimics monomers or micelles of DC$_8$PC. Conformational analysis by ^{1}H NMR revealed that monomeric Cp-DC$_8$PC was conformationally restricted near the carbonyl region, a property characteristic of micelles. Thus, monomeric CP-DC$_8$PC can be considered as a conformational analogue of micelles, but the important structural feature lies in the CH_2COO region instead of the glycerol backbone. CP-DC$_8$PC was then used to test a previous proposal that the bee venom PLA2 hydrolyzes monomers but not micelles (which would predict little or no activity for Cp-DC$_8$PC since its conformation is micelle-like whether below or above its critical micelle concentration). The results showed that Cp-DC$_8$PC is a relatively good substrate for the bee venom PLA2 in comparison with the pancreatic PLA2. This and other evidence together suggest that the bee venom PLA2 is not sensitive to the conformation of monomeric and micellar substrates and hydrolyzes both monomers and micelles. The results in both PLA2s demonstrate the usefulness of cyclopentano-phospholipids in probing the mechanism of phospholipases and the roles of substrate conformation in the catalysis of PLA2.

The mode of activation of phospholipase A_2 (PLA2)[1] is an important and controversial problem. Except for the bee venom PLA2 (Shipolini *et al.*, 1971) and the proenzyme of pancreatic PLA2, it has been well established that the activity of a substrate is greatly enhanced in the reaction catalyzed by PLA2 when the substrate concentration passes the critical micelle concentration (CMC). Many different (and opposing) models have been proposed to explain this phenomenon (for recent reviews, see Verheij *et al.*, 1981; Volwerk and de Haas, 1982; Dennis, 1983). These include, among others, the "dual phospholipid model" proposed by Roberts *et al.* (1977) to explain the kinetic properties of PLA2 from cobra venom, the "interfacial recognition site model" (IRS model) advocated by de Haas and his collaborators (Verger *et al.*, 1973; Pieterson *et al.*, 1974; Verheij *et al.*, 1981; Volwerk and de Haas, 1982; Volwerk *et al.*, 1986; Mao *et al.*, 1986) based on extensive studies of porcine pancreatic PLA2 and the "substrate conformation model" developed by Wells (1974, 1978) based on detailed kinetic studies on the hydrolysis of short-chain lecithins by PLA2 from *Crotalus adamanteus* venom. According to the substrate conformation model (the one supported by the results of this paper), PLA2 interacts with aggregated or monomeric substrate in essentially the same manner. An interface-induced conformational change is not functionally significant, and the so called "interfacial activation" is due to a conformational change of the substrate in an interface which allows for a higher fraction of productive interactions with the enzyme.

In contrast to the properties of pancreatic and snake venom PLA2s, Shipolini *et al.* (1971) reported that the activity of DC$_6$PC as a substrate of bee venom PLA2 increases linearly below CMC but levels off at CMC (11 mM). They suggested that the activity of micelles for this enzyme is negligible compared to that of monomers. Since the bee venom PLA2 has been an unpopular enzyme, this unusual behavior has been ignored in most discussions concerning the interfacial activation of PLA2, except the report that the bee venom PLA2 shows a tendency to denature at the air-water interface (Cohen *et al.*, 1976) and that it is activated by fatty acid or by acylation (Lawrence and Moores, 1975; Drainas *et al.*, 1978; Drainas and Lawrence, 1978).

This paper reports the use of the all-*trans* isomer of cyclopentanoid analogues of phosphatidylcholines (Cp-DC$_n$PC, see

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[1] The abbreviations used are: PLA2, phospholipase A_2; Cp-DC$_n$PC, cyclopentano-analogues of DC$_n$PC (DL, all-*trans* isomer, unless otherwise specified); DC$_n$PC, 1,2-diacyl-*sn*-glycero-3-phosphocholine (L-isomer, unless otherwise specified); CMC, critical micelle concentration; Et, ethyl; OMe, methoxy; Pr, propyl; MS, mass spectroscopy.

Fig. 1 for structure[2] and numbering systems) to probe the mode of activation of PLA2s from bovine pancreas and bee venom. The cyclopentanoid phospholipids have been developed by Sable, Hancock, and Lister (Hancock, 1981; Hancock *et al.*, 1977, 1982; Pajouhesh and Hancock, 1983, 1984; Lister, 1985; Jain *et al.*, 1984) as analogues of glycerophospholipids with restricted conformation at the glycerol backbone. Using the various geometric and positional isomers of long-chain Cp-$DC_{16}PC$, Lister (1985) demonstrated that the PLA2 from *C. adamanteus* shows a high specificity toward the all-*trans* (1,3/2-1P) isomer. This prompted us to prepare the short-chain analogue Cp-DC_8PC and Cp-DC_4PC to probe the mode of activation of PLA2.

EXPERIMENTAL PROCEDURES

Materials—The starting material DL-(1,3/2)-1-*O*-benzylcyclopentane-1,2,3-triol (1) was obtained from previous work (Hancock *et al.*, 1977) and made available for this work by H. Z. Sable. DC_8PC and DC_4PC were purchased from Avanti Polar Lipids, Inc. Bovine pancreatic PLA2 was purified according to the procedure of Dutilh *et al.* (1975). Bee venom PLA2 was purchased from Boehringer Mannheim. Both enzyme preparations migrate as single bands on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Other biochemicals were purchased from Sigma. All other chemicals were reagent grade.

Kinetic Studies—The assay was carried out with the aid of a Radiometer PHM 84 pH meter, an ABU 80 autoburette, and a TTT 80 titrator in the pH STAT mode. The amount of standardized sodium hydroxide necessary to maintain a constant pH was recorded. The quantity added is a direct measure of the amount of fatty acid released per unit time. Under no circumstances were the assay mixtures buffered.

Typically, a stock solution of L-DC_8PC or DL-Cp-DC_8PC was prepared in 41 °C water and then diluted to various concentrations in 3 ml of water containing 20 mM $CaCl_2$ and 40 mM NaCl. Assays run at low substrate concentrations were conducted in larger reaction volumes. The thermostatted reaction vessel containing the assay mixture was then flushed with argon with stirring to achieve a stable base-line pH. A measured quantity of standardized sodium hydroxide was used to set the pH at 8.0. The equilibration period and the kinetic runs were all carried out at 41 °C. At lower temperatures phase separation was observed when the substrate concentration was high, as discussed in Tausk *et al.* (1974b).

The stock solution of enzyme was added in a volume of 10 μl with a Hamilton syringe, and the quantity of 0.005 N NaOH (standardized daily) needed to maintain pH 8.0 was recorded. Initial velocities were measured within the first 3 min. The response was usually linear, except in the hydrolysis of Cp-DC_8PC by bee venom PLA2 where a curvature was observed even in the initial part, for unclear reasons. In such cases the initial velocity was measured by drawing a tangent line at time 0.

Between kinetic runs, the electrodes were soaked for 15 min in a solution of Terg-azyme detergent (Alconox, Inc.) and then rinsed exhaustively with distilled water. We found that a considerable quantity of enzyme would bind to the electrode assembly if this procedure was not followed.

Instrumental Methods—^1H NMR spectra were recorded on Bruker WP-200, AM-250 or AM-500 NMR spectrometers. ^{13}C and ^{31}P NMR spectra were obtained at 62.89 and 101.25 MHz, respectively, on a Bruker AM-250 NMR spectrometer, with broadband ^1H decoupling. Fast atom bombardment mass spectra were recorded on a VG-70-250S mass spectrometer.

^1H and ^{13}C chemical shifts were referenced to internal Me_4Si standard when organic solvents were used. For D_2O samples, sodium formate was added as internal reference (8.4633 ppm, calibrated against 3-(trimethylsilyl)-1-propanesulfonic acid, sodium salt). ^{31}P chemical shifts were referenced to external 85% H_3PO_4 at 25 °C.

Determination of CMC was performed on the AM-500 NMR spectrometer at 30 °C. The samples contained 11 mM each of NaCl and HCOONa and were exchanged with 99.996% D_2O two to three times. The chemical shifts were referenced to internal HCOONa.

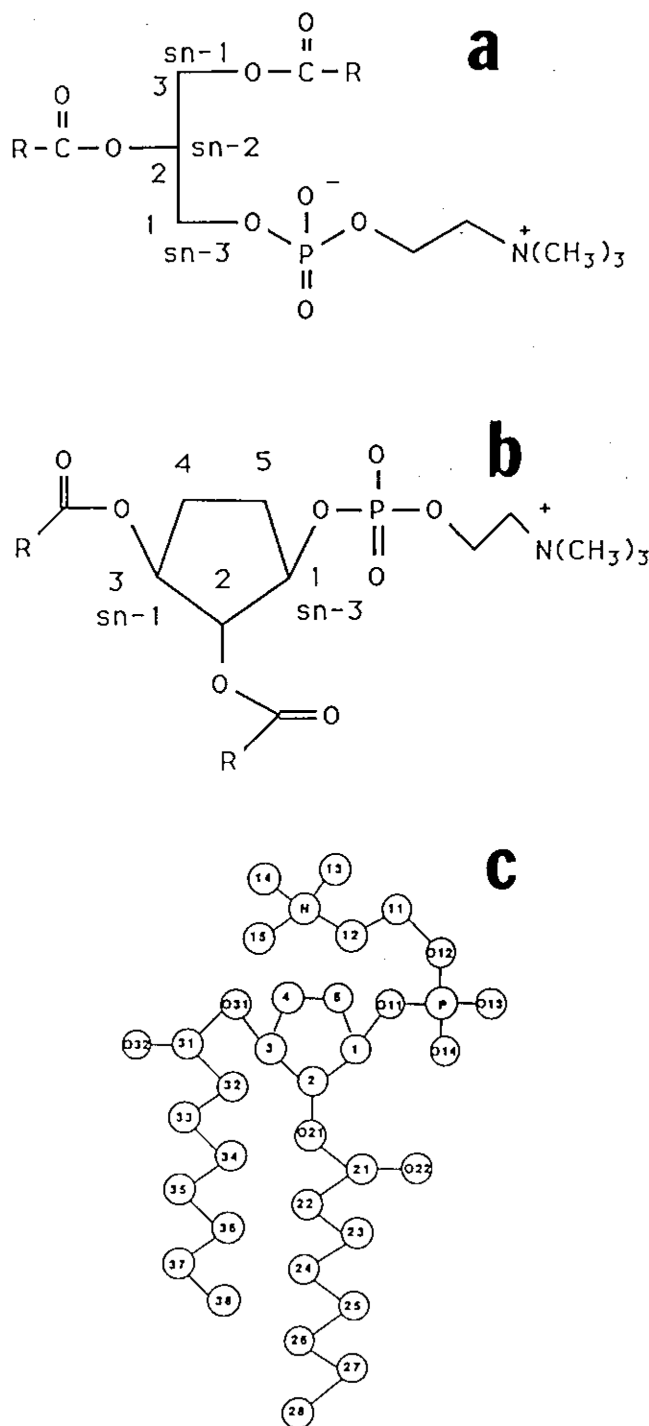

FIG. 1. *a*, structure of DC_nPC, with $R = C_{n-1}H_{2n-1}$. *b*, structure of Cp-DC_nPC, with $R = C_{n-1}H_{2n-1}$. *c*, the numbering system used in this paper for both DC_nPC and CP-DC_nPC. Notice that the numbering system for the Cp-compounds according to the Tentative Rules for Nomenclature of Cyclitols (IUPAC-IUB, 1968) will give number 1 to the phosphocholine group, in contrast to the *sn* system for glycerol phospholipids (IUPAC-IUB, 1977). We, therefore, adopt the Sundaralingam (1972) system which has been widely used in describing the crystal structure of phospholipids and agrees with the convention for Cp-analogues.

[2] Unless otherwise specified, the abbreviation DC_nPC represents the L-isomer of glycerol-PC, whereas the abbreviation Cp-DC_nPC represents the DL-(1,3/2-1P) isomer of cyclopentano-PC. Even though we used DL mixture for the kinetic studies of CP-DC_8PC, the D-isomer of phospholipids has been shown to be a pure competitive inhibitor with same binding constant as the L-isomer in the catalysis of PLA2 (Bonsen *et al.*, 1972; Verheij *et al.*, 1981). Thus it should not affect our conclusion regarding the activation of PLA2. As noted in the last paragraph, similar results have been obtained with the L-isomer of Cp-DC_6PC (Barlow *et al.*, 1988b).

Synthesis of Cp-DC$_8$PC—The procedure is outlined in Fig. 2. Unless otherwise specified, the ratio of solvents are volumetric ratios. **1** was converted to **2** by aclyation with octanoyl chloride, and **2** was converted to **3** by catalytic hydrogenation in the presence of 10% Pd-C. Both steps were performed according to the procedure for the synthesis of Cp-DC$_{16}$PC (Hancock *et al.*, 1977; Hancock, 1981). TLC data: $R_f = 0.7$ for **2** ($CHCl_3$/ethyl ether, 20/1); $R_f = 0.1$ for **3** (hexane/ethylacetate, 5/1). ^{1}H NMR data ($CDCl_3$, 200 MHz) for **2**: δ 7.3 (m, 5H, phenyl), 5.2 (t, J = 3.6 Hz, 1H, HC(2)), 5.0 (m, 1H, HC(3)), 4.65, 4.55 (AB, J = 11.1 Hz, 2H, CH_2 of benzyl), 3.8 (m, 1H, HC(1)), 2.3 (t, J = 7.4 Hz, 4H, $H_2C(22)$ and $H_2C(32)$), 2.2–1.6 (m, 4H, $H_2C(4)$ and $H_2C(5)$), 1.6 (m, 4H, $H_2C(23)$ and $H_2C(33)$), 1.3 (bs, 16H, $H_2C(24)$ to $H_2C(27)$ and $H_2C(34)$ to $H_2C(37)$), 0.9 (t, J = 7.4 Hz, 6H, $H_3C(28)$ and $H_3C(38)$). ^{1}H NMR data ($CDCl_3$, 200 MHz) for **3**: δ 5.1 (m, 1H, HC(2)), 4.8 (t, J = 4.5 Hz, 1H, HC(3)), 4.0 (m, 1H, HC(1)), 2.2 (m, 4H, $H_2C(22)$ and $H_2C(32)$), 2.2–1.6 (m, 4H, $H_2C(4)$ and $H_2C(5)$), 1.6 (t, J = 6.5 Hz, 4H, $H_2C(23)$ and $H_2C(33)$), 1.4 (bs, 16H, $H_2C(24)$ to $H_2C(27)$ and $H_2C(34)$ to $H_2C(37)$), 0.9 (t, J = 7 Hz, 6H, $H_3C(28)$ and $H_3C(38)$).

Introduction of the phosphocholine group to **3** was carried out by a procedure adapted from Bruzik *et al.* (1986). The reactions were carried out in a two-neck flask. Compound **3** (0.46 mmol) was dried by rotary evaporation with 3 × 5 ml of dry toluene and then added with 5 ml of chloroform (dried over P_2O_5) and 1 mmol of triethylamine (dried over NaH) through vacuum transfer. While the mixture was being stirred at room temperature, chloro(*N,N*-diisopropylamino)methoxyphosphine (0.55 mmol) (95%, Aldrich) was added to the flask through a dry syringe. After the reaction was judged to be complete (~ 15 min) on the basis of the disappearance of **3** on TLC, the solvent and excess triethylamine were removed under vacuum, and the reaction mixture was further dried *in vacuo* (0.2 mm Hg) for 4 h. The product **4** was not isolated and was subjected to the following steps directly.

Thoroughly dried tetrazole (1.84 mmol, Aldrich gold label) and choline tosylate (1.38 mmol, prepared by refluxing the mixture of choline chloride and *p*-toulene sulfonic acid monohydrate in toulene) were dissolved in tetrahydrofuran-acetonitrile (1:1, distilled over NaH) through vacuum transfer. After warming to 40 °C to ensure all salts were dissolved, the solution was added to **4** by a syringe under argon. After stirring for 17 h at room temperature, the reaction was judged complete on the basis of TLC. The solvents were removed by rotary evaporation and replaced with 10 ml of dry toluene (distilled over NaH). The heterogeneous mixture was cooled to −80 °C and added with 0.92 mmol of *t*-butyl hydroperoxide (3 M solution in 2,2,4-trimethylpentane). The suspension was stirred at room temperature for 22 h. The reaction mixture was then washed with 5 ml of 1.5 M trimethylammonium bicarbonate (pH 7) and the organic phase was evaporated to dryness repetitively with anhydrous toulene. To this anhydrous semisolid, toluene (over NaH, 3 ml) and anhydrous trimethylamine (over NaH, 0.5 ml) were added by vacuum transfer. The resulting solution was stirred at room temperature for 30 h. After the deprotection reaction was judged complete (TLC), trimethylamine and solvents were evaporated, and the crude product was extracted successively with chloroform/methanol/water (10 ml/20 ml/8 ml) and chloroform/water (10 ml/10 ml). The crude product from organic layers was concentrated and dissolved in 1 ml of chloroform/MeOH/water 66/33/4 and was purified by flash chromatography on silica gel (230–400 mesh) with chloroform/methanol/water (66/33/4) as the eluting solvent. The white solid obtained was then precipitated from acetone and dried *in vacuo* to give 212 mg of **5** (80% yield from **3**). TLC ($CHCl_3/CH_3OH/H_2O$, 66/33/4): $R_f = 0.2$. ^{1}H NMR (CD_3OD, 250 MHz): δ 5.15 (t, J = 3.7 Hz, 1H, HC(2)), 5.05 (m, 1H, HC(3)), 4.50 (m, 1H, HC(1)), 4.26 (m, 2H, $H_2C(11)$ (choline-$POCH_2$)), 3.61 (m, 2H, $H_2C(12)$ (choline-CH_2N)), 3.21 (s, 9H, choline-$N(CH_3)_3$), 2.34 (t, J = 7.2 Hz, 2H, $H_2C(22)$), 2.29 (t, J = 7.4 Hz, 2H, $H_2C(32)$), 2.26-1.80 (m, 4H, $H_2C(4)$ and $H_2C(5)$), 1.59 (m, 4H, $H_2C(23)$ and $H_2C(33)$), 1.3 (b, 16H, $H_2C(24)$ to $H_2C(27)$ and $H_2C(34)$ to $H_2C(37)$), 0.89 (t, J = 6.8 Hz, 6H, $H_3C(28)$ and $H_3C(38)$). ^{13}C NMR(CD_3OD, 62.896 MHz): δ 174.79 and 174.52 (C(21) and C(31)), 84.26 (d, $^3J_{CP}$ = 8.1 Hz, C(2)), 80.03 (d, $^2J_{CP}$ = 5.7 Hz, C(1)), 78.88 (C(3)), 67.54, (m, C(12)), 60.37 (d, $^2J_{CP}$ = 5.0 Hz, C(11)), 54.74 (t, $^1J_{CN}$ = 3.8 Hz, C(13), C(14), and C(15)), 35.03 (C(22) and C(32)), 32.84 (C(26) and C(36)), 31.02, 30.98, 30.09, 30.06, 29.15, 25.97 (C(23) and C(33)), 23.64 (C(27) and C(37)), 14.38 (C(28) and C(38)). ^{31}P NMR (CD_3OD, 101.256 MHz): δ 1.04. MS (fast atom bombardment), *m/e* 1072 (2M), 1071 (2M − 1), 537 (M + 1), 536 (M^+), high resolution MS calculated for $C_{26}H_{51}O_8NP$ 536.38, found 536.28.

Synthesis of Cp-DC$_4$PC—If the procedures of CP-DC$_8$PC were applied, the yield was only 8%. A better yield (58%) was obtained by a slight modification in the work-up procedure. The Et_3N-H_2CO_3 wash was omitted, and the column chromatography was performed on silica gel with 5 weight % of triethylamine and eluted with 100 ml of chloroform, 100 ml of chloroform/methanol 2:1, 100 ml of chloroform/methanol 1:1, and 200 ml of chloroform/methanol (1:2). Final elution with chloroform/methanol (1:2) gave the final product which was characterized as follows: TLC: R_f = 0.22 ($CHCl_3/MeOH/H_2O$, 66:25:4), R_f = 0.55 ($CHCl_3/MeOH/H_2O$, 5:4:1). ^{1}H NMR (CD_3OD, 500 MHz): δ 5.13 (t, 1H, J = 3.2 Hz, HC(2)), 4.99 (m, 1H, HC(3)), 4.49 (m, 1H, HC(1)), 4.27 (m, 2H, $H_2C(11)$), 3.64 (m, 2H, $H_2C(12)$), 3.22 (s, 9H, NMe_3), 2.33 (t, J = 7.3 Hz, 2H, $H_2C(22)$), 2.28 (t, J = 7.3 Hz, 2H, $H_2C(32)$), 2.2–1.8 (m, 4H, $H_2C(4)$ and $H_2C(5)$), 1.65 and 1.64 (t, J = 7.1 Hz, $H_2C(23)$ and $H_2C(33)$), 0.95 and 0.94 (t, J = 6.0 and 7.3 Hz, $H_2C(24)$ and $H_2C(34)$). ^{13}C NMR (CD_3OD, 62.896 MHz): δ 173.041 and 172.835 (C(21) and C(31)), 82.900 (C(2)), 78.35 and 77.00 (C(3) and C(1)), 66.40 (C(12)), 59.17 C(11)), 54.368 (C(13)), C(14), and C(15)), 36.147 and 36.093 (C(22) and C(32)), 30.035 and 28.185 (C(4) and C(5)), 18.348 (C(23) and C(33)), 13.574 (C(24) and C(34)). ^{31}P NMR (CD_3OD, 101.256 MHz): δ − 2.4. Mass spectroscopy (fast atom bombardment): *m/e* 848 (2M), 847(2M-1), 425(M + 1), 424(M^+), high resolution MS calculated for $C_{18}H_{35}O_8NP$ 424.253, found 424.20.

RESULTS AND DISCUSSION

Synthesis and CMC of Cp-DC$_8$PC and CP-DC$_4$PC—Cp-DC$_8$PC was synthesized from (1,3/2)-1-*O*-benzylcyclopentane-1,2,3-triol(**1**) according to Fig. 2. The use of ClP(OMe) $N(iPr)_2$ in the introduction of phosphocholine was adapted from Bruzik *et al.* (1986). Cp-DC$_4$PC was synthesized analogously. Details are described under "Experimental Procedures."

The CMC of DC$_8$PC and Cp-DC$_8$PC were determined by ^{1}H NMR in D_2O, on the basis of the changes in the resonances of the $H_2C(22)$ and $H_2C(32)$ groups, as described in Hershberg *et al.* (1976). The CMC values obtained at 27 °C were 0.25 ± 0.05 mM and 0.6 ± 0.1 mM for DC$_8$PC and Cp-DC$_8$PC, respectively. The value for DC$_8$PC is consistent with the published CMC values of 0.25 mM (Burns *et al.*, 1981), 0.19 mM (Pieterson, 1973), 0.17 mM (Wells, 1974), and 0.10–0.16 mM (Tausk *et al.*, 1974a; de Haas *et al.*, 1971; Roholt and Schlamowitz, 1961; Bonsen *et al.*, 1972). In order to mimic the condition of kinetic assays, we also determined the CMC of Cp-DC$_8$PC in the presence of bovine serum albumin (same

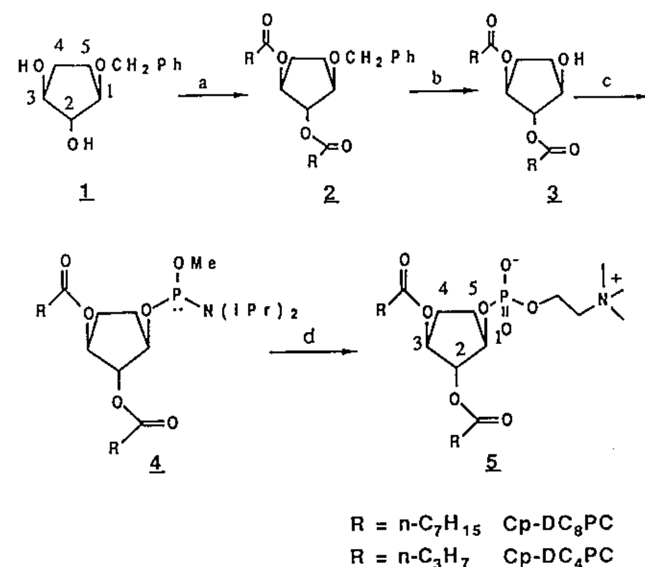

FIG. 2. **Procedures for the synthesis of Cp-DC$_8$PC and CP-DC$_4$PC.** Reagents: RCOCl, 4-pyrrolidinopyridine/pyridine (*a*); H_2, Pd-C/EtOAc (*b*); ClP(OMe)N(i-Pr)$_2$ (*c*); i) choline tosylate, tetrazole/THF-CH_3CN, ii) *t*-BuOOH/2,2,4-trimethyl-pentane-toluene, iii) Me_3N/toluene (*d*).

concentration as PLA2 in kinetic studies). The value was the same as that without bovine serum albumin within experimental errors. The ^{1}H NMR properties of Cp-DC$_8$PC are discussed in a later section.

Hydrolysis of DC$_8$PC and Cp-DC$_8$PC by Pancreatic PLA2—Fig. 3*a* shows the activity of DC$_8$PC at concentrations below and around CMC, and Fig. 3*b* shows the Lineweaver-Burk plot of the hydrolysis of DC$_8$PC above CMC. As expected, DC$_8$PC induces interfacial activation of the PLA2 from bovine pancreas. The K_m and V_{max} obtained from the plots are listed in Table I. The activity of monomers is only ~1/66 of that of micelles. Furthermore, the activity decreases substantially near CMC, which has also been observed by Wells (1974) for the hydrolysis of DC$_6$PC catalyzed by PLA2 from *C. adamanteus* venom.

In contrast to DC$_8$PC, Cp-DC$_8$PC behaved like a single species across the entire concentration range without showing an interfacial activation, except that the sharp decrease in activity near CMC was still present (Fig. 4*a*). Such a decrease near CMC assured that there was indeed a transition from monomers to micelles.

There are two ways to plot 1/v *versus* 1/[S] for the data in Fig. 4*a*. One is to plot it as a single component, as shown in Fig. 4*b*. Except for the two points near CMC, all other points in both monomeric and micellar ranges can be fitted with a straight line, which results in K_m = 0.44 mM and V_{max} = 0.81 units/mg. The second way to plot the data in Fig. 4*a* is to plot monomers and micelles separately. The plot for monomers will be the same as that shown in Fig. 4*b*. In the plot for micelles (Fig. 4*c*), we assume the activity is a summation of monomers and micelles and subtract the activity of monomers

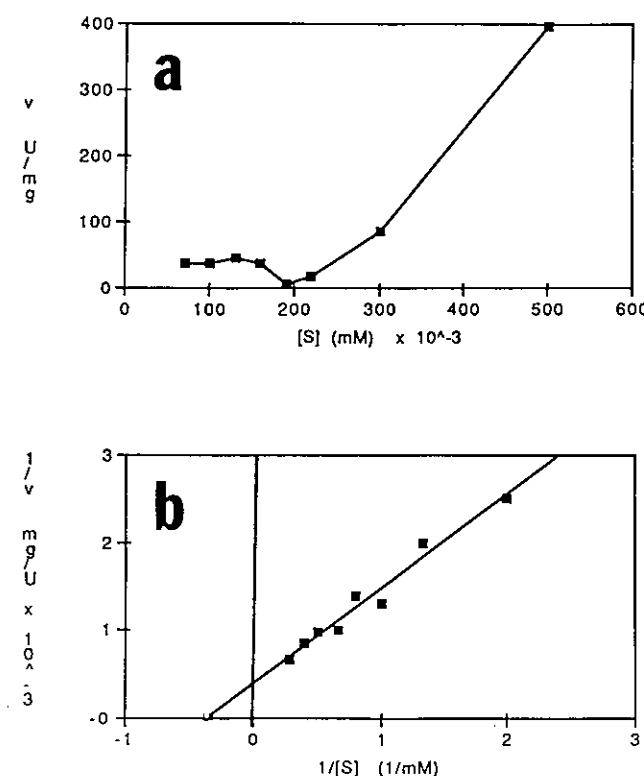

FIG. 3. *a*, bovine pancreatic PLA2-catalyzed hydrolysis of DC$_8$PC at concentrations *below and around* CMC. *b*, Lineweaver-Burk plot of the data *above* CMC.

TABLE I
Summary of kinetic data

PLA2 source	Substrate	State	K_m	V_{max}	Source
			mM[a]	*μmol min^{-1} mg^{-1}*	
Bovine pancreas	L-DC$_8$PC	Monomer		38	Fig. 3*a*
		Micelle	2.7	2510	Fig. 3*b*
	DL-Cp-DC$_8$PC	Monomer	0.44	0.81	Fig. 4*b*
		Micelle	1.8	0.48	Fig. 4*c*
Bee venom	L-DC$_8$PC	Monomer		~4000	Fig. 6*a*
		Micelle		~4000	Fig. 6*a*
	DL-Cp-DC$_8$PC	Monomer		~110	Fig. 6*b*
		Micelle		~110	Fig. 6*b*
C. adamanteus	DL-DC$_{16}$PC	Micelle	0.50	500	[b]
	DL-Cp-DC$_{16}$PC	Micelle	1.1	4.3	[b]

[a] In cases where the substrate is a DL mixture, the K_m value is expressed as the total phospholipid concentration.
[b] From Lister (1985).

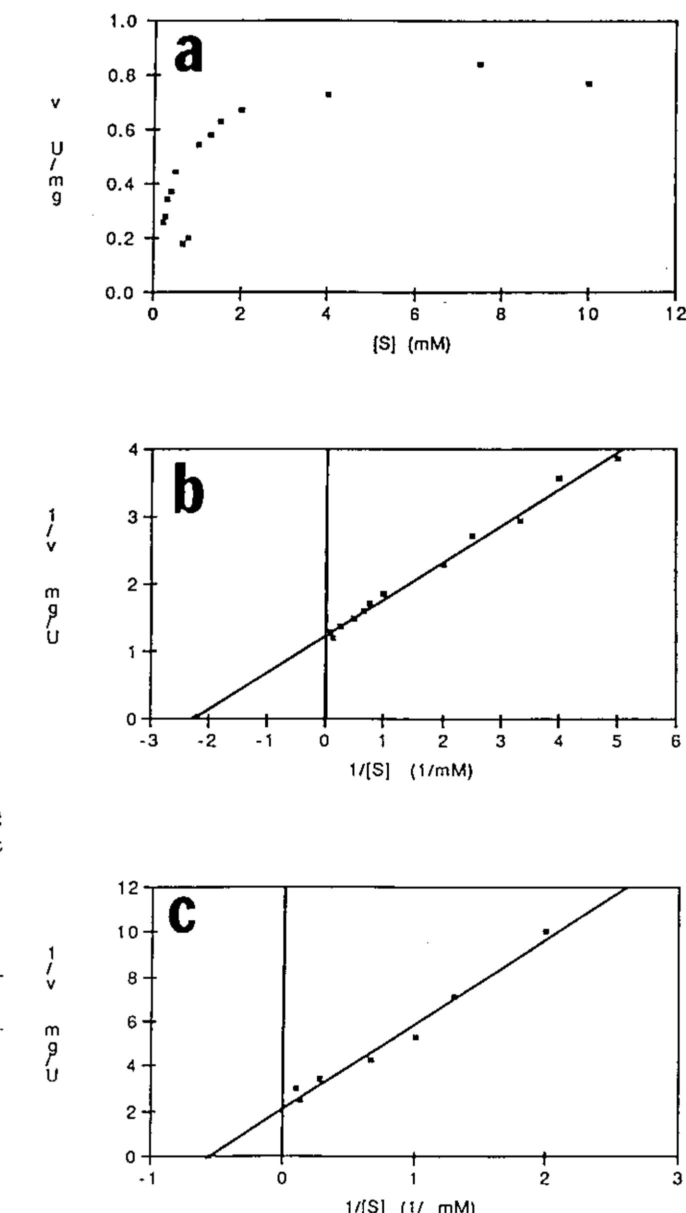

FIG. 4. *a*, hydrolysis of Cp-DC$_8$PC catalyzed by bovine pancreatic PLA2. As noted in Footnote 2, the substrate is a DL mixture. The concentration on the *x*-axis represents the total phospholipid, instead of the L-isomer. *b*, double-reciprocal plot of the data in *a*, except the two points near CMC. *c*, double-reciprocal plot of the micelle region in *a*, after correcting for the activity and concentration of monomers as described in the text.

(assuming it remains constant at 0.45 units/mg after CMC) from the observed activity. The concentration of micelles has also been corrected accordingly. The plot thus obtained for micelles gives K_m = 1.8 mM and V_{max} = 0.48 units/mg. Thus, Cp-DC_8PC micelles have similar activity, but higher K_m, relative to Cp-DC_8PC monomers. This seems to be the most reasonable interpretation. In any case, micellar Cp-DC_8PC does not induce an activation of bovine pancreatic PLA2.

Possible Explanation for the Kinetic Property of Cp-DC_8PC—A straightforward explanation of the lack of activation in Fig. 4*a* would be that no conformational change could occur between monomers and micelles of Cp-DC_8PC due to restricted conformation of the glycerol backbone. Such an explanation strongly supports the substrate conformation model and suggests that the so called interfacial activation is mediated by the conformation of the substrate, as has been advocated by Wells (1974) and Allgyer and Wells (1979).

Two questions, however, could be raised concerning the above interpretation. The first is that since the activity of Cp-DC_8PC is lower than that of either monomeric or micellar DC_8PC, we don't know whether the conformation of Cp-DC_8PC resembles that of monomeric DC_8PC (thus behaves like monomers even above CMC) or resembles that of micellar DC_8PC (thus behaves like micelles even below CMC). The second question is that ^1H NMR analysis and other spectroscopic studies suggested only minor changes in the glycerol backbone conformations of DC_6PC and DC_7PC between monomers and micelles (Roberts *et al.*, 1978; Hauser *et al.*, 1980; Burns *et al.*, 1982). Then, why should a restriction in the glycerol backbone conformation result in the observed change in the kinetic pattern? These two questions have been addressed by ^1H NMR analysis (at 500 MHz) of DC_8PC, DC_4PC, Cp-DC_8PC, and Cp-DC_4PC as described in the next section.

Conformational Analysis by ^1H NMR—According to several previous reports on the ^1H NMR studies of phosphatidylcholines (Hershberg *et al.*, 1976; Roberts *et al.*, 1978; Hauser *et al.*, 1980; Burns *et al.*, 1982; Lin *et al.*, 1987), the most notable and general changes between monomers and micelles are the following: (i) in monomers the $H_2C(22)$ and $H_2C(32)$ groups are only slightly different (two overlapping triplets in ^1H NMR). Upon micellization the chemical shift difference ($\Delta\delta$) increases from $\leq$0.02 ppm to 0.05–0.1 ppm. The $H_2C(32)$ group resides in a more hydrophobic environment and resonates at the higher field. (ii) The carbonyl region of the 2-acyl chain in micelles is so rigid that the two protons of the $H_2C(22)$ group become magnetically nonequivalent and give rise to two slightly separated triplets. Such a magnetic nonequivalence occurs in most micelle systems, but in none of the monomers studied. (iii) The chemical environment of the two acyl chains becomes so different in micelles that the two terminal methyl groups become distinguishable in the ^1H NMR spectra of the short-chain phospholipids DC_6PC and DC_7PC.

Fig. 5 shows the partial ^1H NMR spectra of DC_8PC monomers (Fig. 5*a*) and Cp-DC_8PC monomers (Fig. 5*b*). In Fig. 5*b* the $H_2C(22)$ and $H_2C(32)$ resonances are well separated, and the two protons of the $H_2C(22)$ group are magnetically nonequivalent. These satisfy criteria i and ii mentioned above for micelles of phosphatidylcholines. Thus, Cp-DC_8PC has restricted conformation in the carbonyl region of the acyl chains, and the conformation of its monomers happens to resemble that of micelles of glycerol phosphatidylcholines. Two structural factors could be responsible for such a conformation: (*a*) the two additional CH_2 groups, $H_2C(4)$ and $H_2C(5)$ may pose steric hindrance to the random motion of the two acyl chains, as revealed by model building. (*b*) The *trans* configuration of the two ester groups may help orient the two acyl chains in a micelle-like conformation. While it is unclear to us which of the two factors may predominate, we have further generalized this phenomenon by showing that the "$\Delta\delta$" values of monomeric Cp-compounds are always larger than those of the corresponding glycerol phospholipids under a variety of conditions, as summarized in Table II.

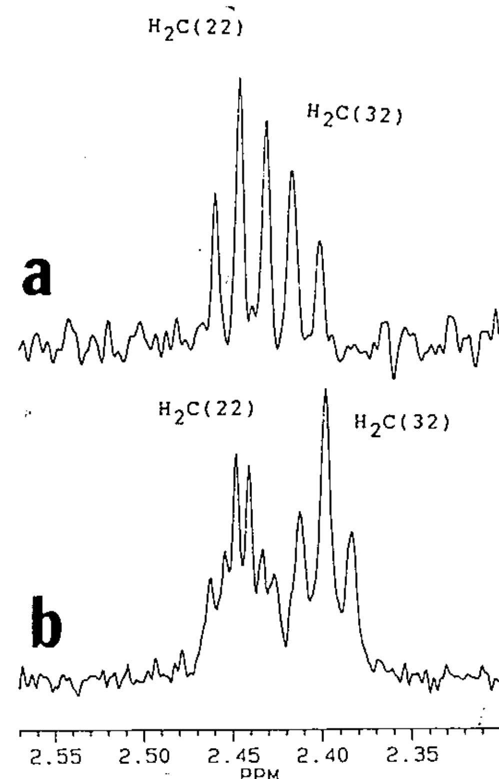

FIG. 5. **CH_2COO region of the ^1H NMR spectra (D_2O, 500 MHz) of monomeric DC_8PC (*a*) and monomeric Cp-DC_8PC (*b*).**

TABLE II

Comparison of $\Delta\delta$ values between monomers of Cp-compounds and the corresponding glycerol phospholipids

Compounds	Solvent	Pattern of $H_2C(32)$	Pattern of $H_2C(22)$	$\Delta\delta$(ppm)	Pattern of terminal CH_3
Cp-DC_8PC	D_2O	t[a]	dt[a]	0.040	t[a]
DC_8PC	D_2O	t	t	0.029	t
Cp-DC_8PC	CD_3OD	t	t	0.048	t
DC_8PC	CD_3OD	t	t	0.024	t
Cp-DC_4PC	D_2O	t	t	0.035	dt
DC_4PC	D_2O	t	t	0.028	dt
Cp-DC_4PC	CD_3OD	t	t	0.047	dt
DC_4PC	CD_3OD	t	t	0.022	dt

[a] t and dt stand for triplet and double triplet, respectively.

Properties of Cp-DC_8PC Micelles—An alternative interpretation for the lack of changes in activity at the CMC of Cp-DC_8PC is that Cp-DC_8PC forms poor micelles which cannot interact properly with the interfacial recognition site of PLA2. However, we have added Triton X-100 to Cp-DC_8PC attempting to improve the quality of the micelle surface, and found no significant increase in activity.

Even though Cp-DC_8PC possesses micelle-like conformation in the monomeric form, it does undergo a transition from monomers to micelles as evidenced by proton NMR experiments. The nonequivalence ($\Delta\delta$) between $H_2C(22)$ and $H_2C(32)$ increased from 0.045 to 0.058 ppm at CMC for Cp-DC_8PC and from 0.029 to 0.038 ppm for DC_8PC. Both continued to increase slowly above CMC. The chain-terminal methyl groups become nonequivalent immediately after CMC for Cp-DC_8PC and at higher concentration for DC_8PC. In addition, both compounds showed an additional set of broader peaks (possibly due to formation of larger micelles or small vesicles) when the concentration was well above CMC. Thus, the differences in the proton NMR properties of monomers and micelles are qualitatively similar between Cp-DC_8PC and DC_8PC. This suggests that the different kinetic behavior of the two compounds is not due to differences in their surface properties.

Hydrolysis of DC_8PC and Cp-DC_8PC Catalyzed by PLA2 from Bee Venom—Since monomeric Cp-DC_8PC mimics the conformation of micellar DC_8PC, it can be used to test the suggestion by Shipolini *et al.* (1971) that bee venom PLA2 hydrolyzes monomers but not micelles, which would predict little or no activity for Cp-DC_8PC relative to DC_8PC. Fig. 6 shows plots of the activity of DC_8PC (Fig. 6*a*) and Cp-DC_8PC (Fig. 6*b*) as a function of substrate concentration. Both plots resemble that of DC_6PC (Shipolini *et al.*, 1971) in showing a sharp, linear increase before CMC and leveling off after CMC, except that the "dip" near CMC was not detected in the case of DC_6PC. The plots in Fig. 6 and the data listed in Table I show that Cp-DC_8PC possesses ~2.7% of activity relative to DC_8PC below and above CMC. According to the data listed in Table I, this ratio is comparable to the ratio of Cp-$DC_{16}PC$ to $DC_{16}PC$ in the case of *C. adamanteus* PLA2 and is higher than that of Cp-DC_8PC to DC_8PC in the case of bovine pancreatic PLA2.

The above results suggest that DC_8PC and Cp-DC_8PC behave similarly as substrates of PLA2 in terms of the kinetic pattern of monomers and micelles. The difference in activity (by ~37 ×) is most likely a structural rather than a conformational effect on the basis of the above discussions. Thus, our interpretation of the unusual kinetic behavior of bee venom PLA2 (high activity toward monomers and no activation at CMC) is that his enzyme does not differentiate the conformation of substrates between monomers and micelles (either they can both activate the enzyme or the enzyme already exists in the activated form before substrate binding). According to this interpretation, micelles should also be good substrates. This is supported by the previous observation that mixed micelles of Triton X-100 and long-chain lecithins are good substrates for bee venom PLA2 (Upreti and Jain, 1978; Bruzik *et al.*, 1983; Tsai *et al.*, 1985), and that long-chain lecithin bilayers (sonicated and unsonicated) can also be hydrolyzed by the bee venom PLA2 (Upreti and Jain, 1978; Wilschut *et al.*, 1978), although with lower activity.

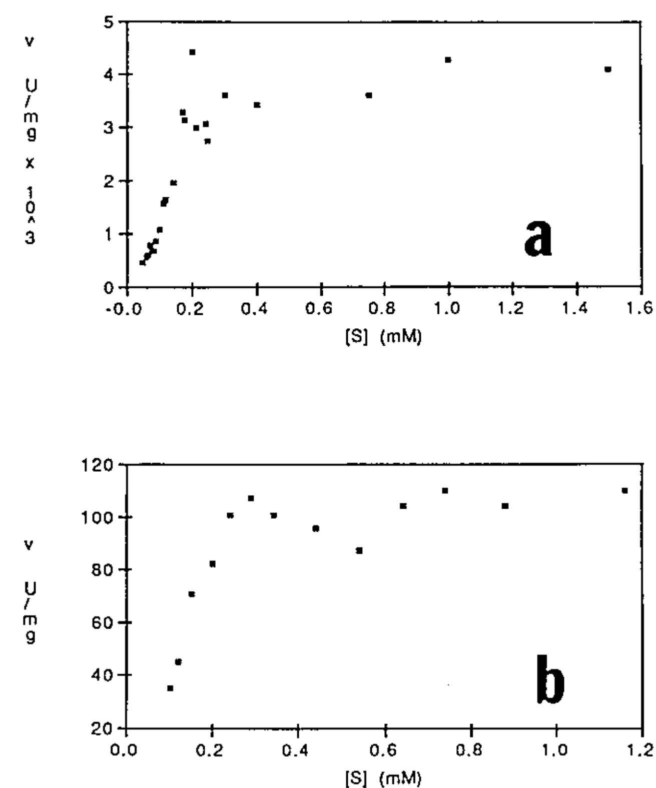

FIG. 6. **Bee venom PLA2-catalyzed hydrolysis of DC_8PC (*a*) and Cp-DC_8PC(*b*).**

Alternative Interpretations—Although the above results strongly favor the substrate conformation model as the explanation for the interfacial activation of PLA2 from bovine pancreas, they do not necessarily argue against other models. An alternative interpretation of our results on the pancreatic PLA2 is that the monomeric Cp-DC_8PC, possessing micelle-like conformation, can bind to the interfacial recognition site and activate the enzyme. A precedent of this behavior is that the PLA2 from *Naja melanoleuca* has been shown to be activated by forming lipid-protein aggregates at sub-micellar concentrations (van Eijk *et al.*, 1983, 1984). Such a model may also be applied to the bee venom PLA2. In other words, we can conclude that the bee venom PLA2 accepts micelles and monomers as opposed to the suggestion of Shipolini *et al.* (1971), but we cannot differentiate whether the enzyme preexists in the activated form (thus insensitive to the conformation of substrates) or it can bind to and be activated by monomers and micelles. It is also possible that more than a single model may function simultaneously and that different PLA2s may function by different models.

Conclusion— We have used conformationally restricted Cp-DC_8PC to probe the mode of activation in the catalysis of PLA2 from bovine pancreas and the lack of interfacial activation in the catalysis of PLA2 from bee venom. In the case of pancreatic PLA2, Cp-DC_8PC behaved differently from DC_8PC in that its monomers and micelles have comparable activities. The results support the substrate conformation model of Wells (1974), which attributes the interfacial activation to a conformational change of the substrate. Conformational analysis by ^1H NMR revealed that monomeric Cp-DC_8PC was conformationally restricted near the carbonyl region, a property characteristic of micelles. Thus, Cp-DC_8PC can be considered as a conformational analogue of micelles, but the important structural feature lies in the CH_2COO region instead of the glycerol backbone. The kinetic pattern of Cp-DC_8PC was similar to that of DC_8PC in the hydrolysis catalyzed by PLA2 from bee venom. This suggests that, unlike PLA2 from other sources, bee venom PLA2 is not sensitive to the conformation of monomeric and micellar substrates. It is likely to be activated by both monomers and micelles or to exist as an activated enzyme before substrate binding.

During the course of this work pure enantiomers of Cp-DC_6PC have been synthesized by a somewhat different procedure (Barlow *et al.*, 1988a) and used to probe the activation of PLA2 from other sources (Barlow *et al.*, 1988b). The chain lengths of the substrates and the sources of PLA2 are different between their work and ours, but the results are consistent and reenforce each other.

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