Phospholipids Chiral at Phosphorous: Use of Chiral Thiophospholipids to Study the Mechanism of Phospholipase A_2

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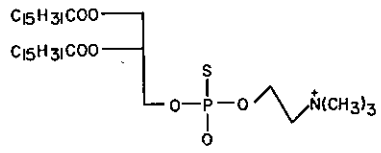
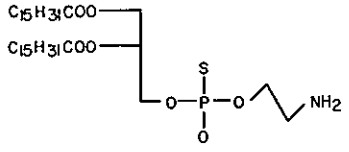
INTRODUCTION

Nucleoside phosphorothioates are useful probes in studying the stereochemistry and mechanism of enzymatic reactions involving nucleotides and nucleic acids, as described in several papers of this Symposium. This article deals with the phosphorothioate analogues of phospholipids, DPPsC and DPPsE[3], and the use of these compounds to study the mechanism of phospholipase A_2 (PL A_2) from bee venom.



DPP_sC



DPP_sE

PL A_2 catalyzes hydrolysis of the fatty acid ester at the sn-2 position of phospholipids. The reaction is characterized by four types of specificity: (a) substrate specificity, which requires a phosphate ester adjacent to a fatty acid ester (2) (phosphatidylcholines are the best

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Abbreviations used: DOPsC, 1,2-dioleoyl-sn-glycero-3-thiophosphocholine; DPPC, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine; DPPsC, 1,2-dipalmitoyl-sn-glycero-3-thiophosphocholine; DPPsE, 1,2-dipalmitoyl-sn-glycero-3-thiophosphoethanolamine; DSC, differential scanning calorimetry; GPsC, sn-glycero-3-thiophosphocholine; Mops, 3-(N- morpholino)propanesulfonic acid; MPPsC, 1-palmitoyl-sn-glycero-3-thiophosphocholine; and PL, phospholipase.

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substrates for PL A_2 from many different sources, but the choline side chain is not an absolute requirement); (b) surface specificity, which requires aggregated substrates (in the form of micelles or vesicles, but not monomers) with a suitable length of acyl chains for optimal activity (3,4); (c) stereospecificity, which requires L configuration at the C_2 of phospholipids (i.e. the phosphate group should be at the sn-3 position of glycerol) (2); and (d) metal ion specificity, which is highly specific to Ca^{2+} (5,6).

We report on the synthesis and configurational analysis of chiral thiophospholipids, kinetic study of PL A_2 using DPPsC as substrates, as well as biophysical properties of DPPsC isomers. The results are discussed in terms of the substrate specificity of PL A_2 (i.e. the effect of sulfur substitution at phosphorus), the surface specificity (due to different surface properties of DPPC and isomers of DPPsC), the stereospecificity (due to the created chiral center at phosphorus), and the metal ion specificity (ca^{2+} vs. Cd^{2+}).

MATERIALS AND METHODS

Bee venom PL A_2 was purified from lyophilized whole venom of Apis mellifera (Sigma, grade IV) according to the procedure of Shipolini et al. (5). Other enzymes were obtained from Sigma or Boehringer. Chloride or nitrate salts of Ca^{2+} and Cd^{2+} were ultrapure grades from Alfa.

Spectrophotometric assays were performed on a Uvikon 820 UV-visible spectrophotometer. ^{31}P NMR experiments were carried out on Bruker WM-300 or WP-200 NMR spectrometer. Differential scanning calorimetry was performed on a Microcal MC-1 scanning microcalorimeter.

RESULTS AND DISCUSSION

Synthesis and Configurational Determination

DPPsC and DPPsE were synthesized by condensation of choline and ethanolamine, respectively, with $PSCl_3$ and 1,2-dipalmitoyl-sn-glycerol (7-9). The synthesized DPPsC and DPPsE consist of two diastereomers due to the second chiral center at phosphorus. The two diastereomers gave distinguishable ^{31}P chemical shifts in $CDCl_3$, and the isomer resonating at the lower field was designated as isomer A and the other as isomer B, as summarized in Table I. The separation of diastereomers and the elucidation of absolute configuration at phosphorus are outlined in Figure 1. First, it was found that PL A_2 and PL C catalyze stereospecific

TABLE I. ^{31}P Chemical Shifts of Thiophospholipids

Compound	Condition	R_p (isomer B)	S_p (isomer A)	Ref.
DPPsC	$CDCl_3$	56.07	56.12	10
	D_2O/5% Triton X, pH 8	57.205	57.133	
MPPsC	CH_3OD	59.336	59.275	10
	D_2O/5% Triton X, pH 8	56.904	56.961	
GPsC	D_2O	55.799	56.026	
DPPsE	$CDCl_3$	59.82	59.95	11
	$CDCl_3 + Et_3N$	59.13	59.29	
	CH_3OD	60.099	60.081	
DOPsC	$CDCl_3$	56.584	56.611	

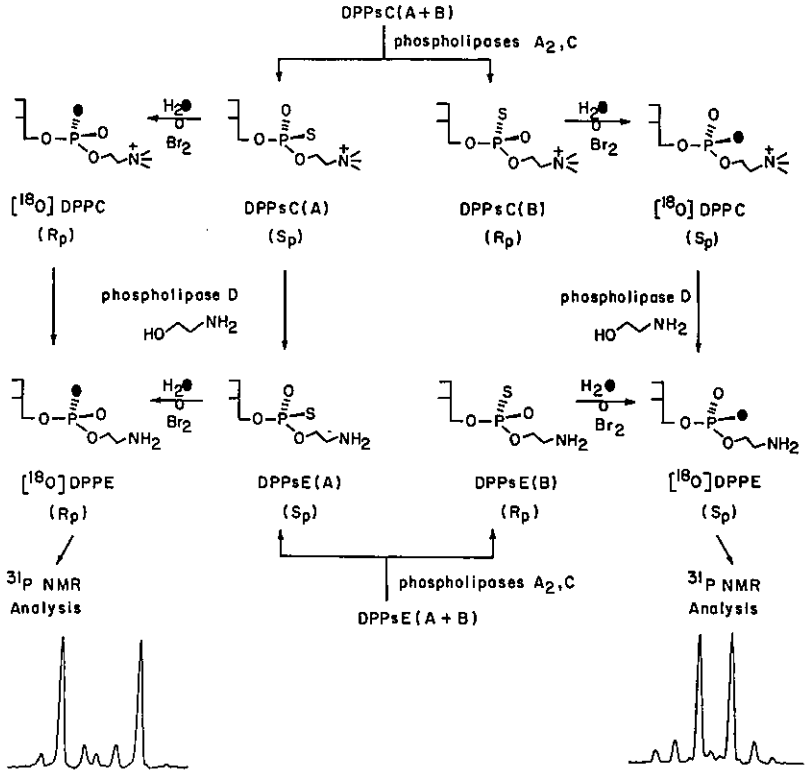

Figure 1. Outline of procedures for the configurational analysis of chiral thiophospholipids.

hydrolysis of isomer B and isomer A, respectively, of both DPPsC and DPPsE (8-11). Figures 2 and 3 show the stereospecific hydrolysis of DPPsC, catalyzed by PL A_2 and PL C, respectively, by ^{31}P NMR. Thus, although chromatographic separation of diastereomers of DPPsC and DPPsE is still unsuccessful, separation can be achieved by the stereospecific hydrolysis of PL A_2 and PL C. In the case of PL A_2, the product of hydrolysis can be reacylated to give pure isomer B of DPPsC or DPPsE. It was also found that PL D catalyzes stereospecific transphosphatidylation of DPPsC(A) to DPPsE(A) (11).

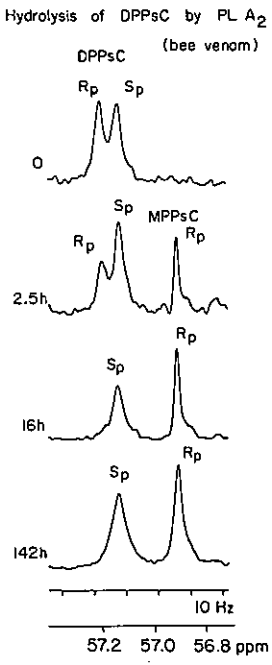

Figure 2. Stereospecific hydrolysis of (R_p)-DPPsC by bee venom PL A_2 followed by ^{31}P NMR at 81.0 MHz, 37°C. Sample conditions: 20 μmol of DPPsC in 2 mL of 50 mM Mops buffer, pH 7.2 containing 35% D_2O, 5% Triton X-100, 0.25 mM EDTA, 2.5 mM $Ca(NO_3)_2$, and 3.8 μg of PL A_2. (Reproduced from ref. 10 with permission.)

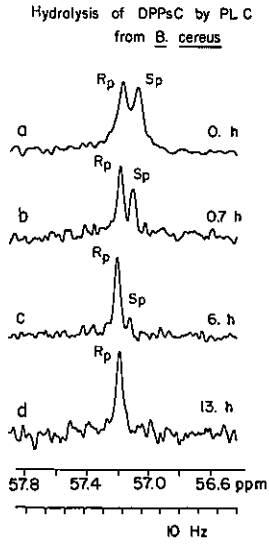

Figure 3. Stereospecific hydrolysis of (S_p)-DPPsC by PL C from *B. cereus* followed by ^{31}P NMR at 81.0 MHz, 37°C. Sample conditions: 50 μmol of DPPsC in 2 mL of D_2O containing 80 mg of sodium deoxycholate and 5 μg of PL C. (Reproduced from ref. 10 with permission.)

The configurations of DPPsC and DPPsE were then correlated with those of [^{18}O]DPPC and [^{18}O]DPPE, respectively, via stereospecific desulfurization mediated by Br_2 or CNBr, a reaction known to proceed with inversion of configuration (12-17). As described elsewhere, [^{18}O]DPPC can be converted to [^{18}O]DPPE by PL D (retention of configuration) (18), and the configuration of [^{18}O]DPPE can be analyzed by ^{31}P NMR (19).

Different biochemical problems may require thiophospholipids of different lengths of acyl chains. These can, in principle, be synthesized from sn-1,2-diacylglycerols as described above. However, many 1,2-diacylglycerols (other than dipalmitin) are very unstable. A more feasible and simpler procedure, as shown in Figure 4, is to hydrolyze (R_p)-lyso-DPPsC and (S_p)-DPPsC with tetrabutylammonium hydroxide. The resulting (R_p)- and (S_p)-GPsC, respectively, can then be reacylated by appropriate acid chloride or anhydride to form desired chiral thiophospholipids. The ^{31}P chemical shifts of GPsC and other thiophospholipids are also listed in Table I. Recently, the phosphorothioate analogues of a sphingomyelin have also been synthesized (K. Bruzik, private communication).

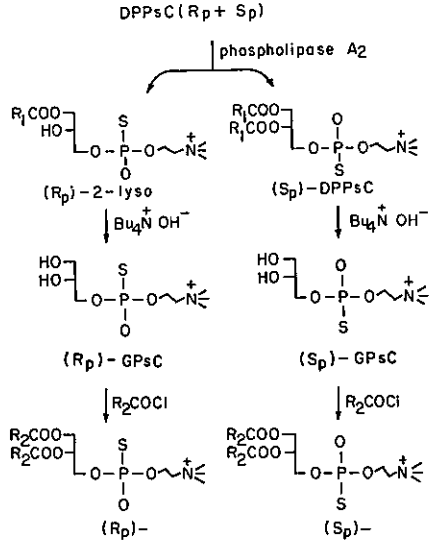

Figure 4. Outline of synthetic procedures for chiral thiophosphatidylcholines of different acyl groups.

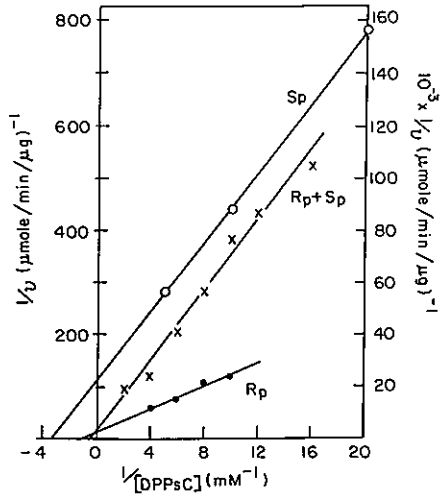

Figure 5. Lineweaver-Burk plots of (R_p)- and (R_p + S_p)-DPPsC (left vertical scale) and (S_p)-DPPsC (right vertical scale). The substrate was sonicated in a 0.2 mM Mops buffer, pH 7.23, containing 0.1 mM p-nitrophenol, 5 mM $CaCl_2$, 0.1 mM EDTA, and 0.1% (w/w) Triton X-100 (1.5 mM).

Kinetic Properties of Bee Venom PL A_2

The catalysis of lipophilic enzymes may involve "surface interaction" (step 1), "substrate binding" (step 2), and the chemical reaction (step 3) (20):

$$E{\cdot}Ca^{2+} + S \overset{\text{step 1}}{\rightleftharpoons} E{\cdot}Ca^{2+}\cdots S \overset{\text{step 2}}{\rightleftharpoons} E{\cdot}Ca^{2+}{\cdot}S \overset{\text{step 3}}{\longrightarrow} E{\cdot}Ca^{2+}{\cdot}P$$

Various assay methods and kinetic models for PL A_2 have been proposed (6, 21-23). For the purpose of comparing DPPC and isomers of DPPsC, we used an isotropic solution of mixed micelles by sonicating the substrate with Triton X-100. The release of fatty acid was monitored spectrophotometrically using p-nitrophenol as a pH indicator (24). Under this condition linear Lineweaver-Burk plots were obtained (Figure 5), and the apparent K_m and V_{max} are summarized in Table II.

TABLE II. Summary of Kinetic Data of Bee Venom PL A_2 (1)

Metal	Substrate	K_m (mM)	V_{max} (μmole/min/mg)
Ca^{2+}	DPPC	1.67	1850
Ca^{2+}	($\underline{R}_p$)-DPPsC	0.85	76
Ca^{2+}	($\underline{S}_p$)-DPPsC	0.30	0.044
Ca^{2+}	($\underline{R}_p$+$\underline{S}_p$)-DPPsC	2.1 (1.05)[a]	64
Cd^{2+}	DPPC	6.4	17.6
Cd^{2+}	($\underline{R}_p$)-DPPsC	0.24	0.069
Cd^{2+}	($\underline{S}_p$)-DPPsC		0.0044

[a]Number in parenthesis is the K_m of ($\underline{R}_p$)-DPPsC only.

The V_{max} of ($\underline{R}_p$)-DPPsC is 1/24 of that of DPPC, but 1727 times of that of ($\underline{S}_p$)-DPPsC. Such differences are not unusual in enzymatic reactions involving nucleotides (25,26), but it is intriguing for PL A_2, since the bond cleaved is far away from the phosphate group, and the enzyme is not specific to the choline side chain.

The apparent K_m values represent combined properties of steps 1 and 2. The relative invariance of the K_m values suggests that the differences in the V_{max} between DPPC and ($\underline{R}_p$)-DPPsC, and between ($\underline{R}_p$)- and ($\underline{S}_p$)-DPPsC, are not due to surface specificity. Rather, they are due to substrate specificity and stereospecificity, respectively. The results can be best interpreted by stereospecific binding between Ca^{2+} and the P—O^- in ($\underline{R}_p$)-DPPsC.

Effect of Cd^{2+} Substitution on Stereospecificity

Recently, Jaffe and Cohn (27) have shown that a "reversal of

stereospecificity" upon substitution of Mg^{2+} or Ca^{2+} (which prefers oxygen over sulfur) by Cd^{2+} (which prefers sulfur over oxygen) can be viewed as a direct evidence for the coordination between the divalent metal ion and the sulfur-substituted phosphate group. We therefore investigated the effect of Cd^{2+} on the stereospecificity of PL A_2 toward isomers of DPPsC.

We have shown that Cd^{2+} is a competitive inhibitor (against Ca^{2+}) of PL A_2, with K_i = 0.25 mM. Before we can compare the stereospecificity between Ca^{2+}/PL A_2 and Cd^{2+}/PL A_2, it is necessary to show that Cd^{2+} can actually activate apo-PL A_2, other than function as a competitive inhibitor of Ca^{2+}/PL A_2. A problem encountered was that the apo-PL A_2 still retained 1-2% of residual activity, which might be higher than the true activity of Cd^{2+}/apo-PL A_2.

Use of the least active isomer, ($\underline{S}_p$)-DPPsC, allowed unequivocal demonstration of activation of apo-PL A_2 by Cd^{2+}, as well as determination of the relative activity of Ca^{2+}/PL A_2 and Cd^{2+}/PL A_2 for the hydrolysis of ($\underline{S}_p$)-DPPsC, by use of ^{31}P NMR. Figure 6 shows the time course of hydrolysis of ($\underline{S}_p$)-DPPsC (4.1 mM) by Ca^{2+}/PL A_2 (a) and Cd^{2+}/PL A_2 (b), as well as control experiments (c) in the absence of PL A_2 or metal ions.

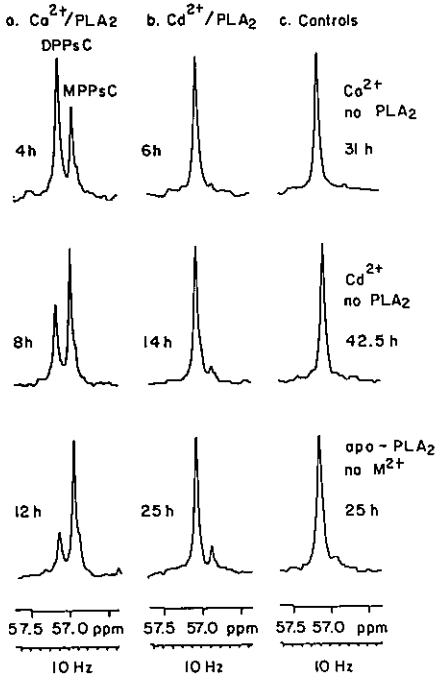

Figure 6. ^{31}P NMR spectra (at 81.0 MHz) showing hydrolysis of ($\underline{S}_p$)-DPPsC by Ca^{2+}/PL A_2 (a) and by Cd^{2+}/PL A_2 (b), as well as three control experiments with Ca^{2+} only, Cd^{2+} only, and apo-PL A_2 only (c). Sample conditions: 4.1 mM DPPsC, 4.2% Triton X-100, 4.6 mM M^{2+}, 1.34 mg of apo-PL A_2, and 26.4 mM Mops, pH 7.2. (Reproduced from ref. 1 with permission.)

In the absence of PL A_2, neither Ca^{2+} nor Cd^{2+} catalyzed hydrolysis of ($\underline{S}_p$)-DPPsC substantially. At 25 h, the hydrolysis was ca. 18% for Cd^{2+}/PL A_2, but only barely detectable (<5%) for apo-PL A_2, which clearly showed the activation of apo-PL A_2 by Cd^{2+}. The hydrolysis by Ca^{2+}/PL A_2 was 30% at 4 h, approximately 10 times faster than that of Cd^{2+}/PL A_2. The V_{max} of ($\underline{S}_p$)-DPPsC for Cd^{2+}/PL A_2 can thus be determined, while the K_m and V_{max} of DPPC and ($\underline{R}_p$)-DPPsC for Cd^{2+}/PL A_2 were determined spectrophotometrically and listed in Table II.

According to the data in Table II, the ratio V_R/V_S is 1727 for Ca^{2+}/PL A_2 and 15.7 for Cd^{2+}/PL A_2. Thus, substitution of Ca^{2+} by Cd^{2+} causes a decrease in the V_R/V_S ratio by a factor of 110, which is a large change though not large enough for a complete reversal of stereospecificity ($V_R/V_S < 1$). The question is whether these data support direct binding between M^{2+} and the phosphate group. A review of literature (1) indicates that the ratio $(V_R/V_S)\ Mg^{2+}/(V_R/V_S)Cd^{2+}$, defined as the "Mg/Cd ratio", is $\geq$ 50 or $\leq$ 1/150 in most cases where a reversal of stereospecificity occurred. In the case of hexokinase/ATPβS, Mg/Cd = 15 (Ca/Cd) (27). Thus, the Mg/Cd ratio of PL A_2, if obtainable, could be as high as 1650. Such a large change in stereospecificity strongly supports direct binding between Ca^{2+} and the pro-S oxygen of DPPC in the active site of PL A_2.

Biophysical Properties of DPPsC

In view of the large difference between isomers of DPPsC in the reaction catalyzed by PL A_2, it would be interesting to know the behavior of these compounds in the absence of proteins. Table III summarizes the biophysical properties of DPPC and DPPsC isomers in the forms of micelles, small unilamellar vesicles (SUV) (28), and multilamellar vesicles. In the monomers/micelles, the difference between DPPC and isomers of DPPsC are very small, and the critical micelle concentration is the same within experimental error. Like the natural DPPC, DPPsC form SUV upon injection of an ethanolic solution of thiophospholipids (20 mM) into a 0.15 M NaCl solution. However, the SUV from DPPC and isomers of DPPsC have different sizes, as indicated by electron micrographs and by ^{31}P NMR line widths (28). The differences between DPPC and DPPsC isomers seem to be manifested in the multilamellar phase. As also summarized in Table III, DPPC and isomers of DPPsC show different chemical shift anisotropy ($\Delta\sigma$) in ^{31}P NMR, and different quadrupolar splitting ($\Delta\nu_q$) in ^{14}N NMR, both of which are sensitive to the structural and motional properties of the phosphate

head group of phospholipids (29). It is noteworthy that the ^{31}P NMR and ^{14}N NMR properties of ($\underline{R}_p$+$\underline{S}_p$)-DPPsC are not an additive of the properties of the separate isomers.

TABLE III. Biophysical Properties of DPPC and DPPsC

Phase/Property	DPPC	$\underline{R}_p$-DPPsC	$\underline{S}_p$-DPPsC	$\underline{R}_p$+$\underline{S}_p$-DPPsC
Monomers/Micelles:				
a. Critical Micelle Conc. (in 9/1 MeOH/H_2O) ($MX10^5$)	8.3±0.7	6.9±1.4	8.1±0.1	7.0±0.8
Small Unilamellar Vesicles:				
a. Outer Radius (A)	115±30	210±70	240±75	
b. ^{31}P NMR line width (Hz)	24	35	50	48
c. Transition temp (^oC)	37.9	43.8	43.8	43.8
Multilamellar Phase:				
a. Chemical shift anisotropy in ^{31}P NMR (ppm)	46.7±1.2	37.2±1.4	34.3±1.4	30.9±1.2
b. Quadrupolar splitting in ^{14}N NMR (KHz)	10.26±0.1	7.9±0.1	7.5±0.1	7.5±0.1 7.7±0.1
c. DSC:				
Pretransition, $T_{m1}(^oC)$	35.1	none	43.7	43.8
ΔH(kcal/mole)	1.1		1.6	1.7
Main Transition, $T_{m2}(^oC)$	41.1	45.6	45.0	44.8
ΔH(kcal/mole)	6.9	14.7	7.1	6.8

Another observation, which is as surprising as the high stereospecificity of PL A_2, though the two may not necessarily be related, is that the configuration at phosphorus seems to have a large effect on the packing, and thus the phase transition properties of membranes. The DSC heating trace of natural DPPC is characterized by a pretransition (T_{m1}) at 35.3°C and a main transition (T_{m2}) at 41.4°C (30). As shown in Fig. 7, a and b, and the data summarized in Table III, ($\underline{R}_p$+$\underline{S}_p$)-DPPsC and ($\underline{S}_p$)-DPPsC show similar DSC traces, with T_{m1} and T_{m2} substantially higher than those of DPPC. The ($\underline{R}_p$)-isomer of DPPsC, however, exhibits a broad transition (Fig. 7c) with a significantly higher ΔH. As shown in Fig. 7d, addition of 15% ($\underline{S}_p$) isomer drastically converts the DSC curve of the ($\underline{R}_p$) isomer to the "normal shape", i.e., it shows a pretransition at 42.8°C and a main transition at 44.7°C, with ΔH values comparable to those of ($\underline{S}_p$)- and ($\underline{R}_p$ + $\underline{S}_p$)-DPPsC. The results clearly demonstrate the effect of the configuration at phosphorus on the phase transition properties of phospholipids in the multilamellar state.

The larger ΔH of ($\underline{R}_p$)-DPPsC suggests that the sample may undergo other transitions in addition to the "pretransition" and the "main transition". Figure 8a shows that when ($\underline{R}_p$)-DPPsC was heated at 70°C for

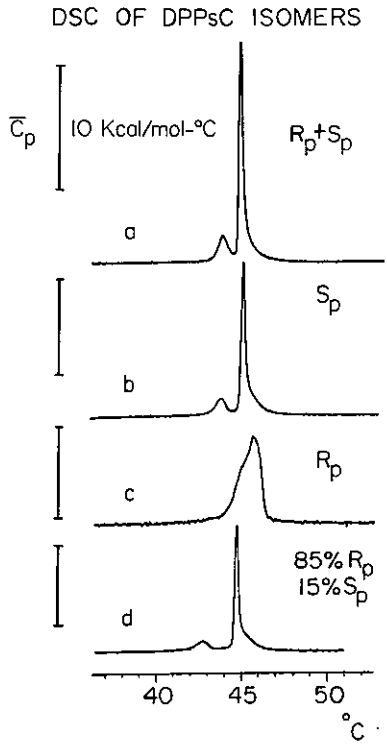

Figure 7. DSC traces of ($\underline{R}_p+\underline{S}_p$)-DPPsC (a), ($\underline{S}_p$)-DPPsC (b), ($\underline{R}_p$)-DPPsC (c), and (85% $\underline{R}_p$ + 15% $\underline{S}_p$)-DPPsC (d). The diastereomeric purity was >97% and >99% for ($\underline{R}_p$)-and ($\underline{S}_p$)-DPPsC, respectively. Phospholipid samples (2-6 mg) were transferred to the calorimeter cell with chloroform, and dried in vacuo at 50°C overnight. The sample was then suspended in 20 mM Pipes buffer,pH 7.4 (5% lipid by weight), by incubating at 50-60°C for 10 min. with occasional shaking, and kept refrigerated before scanning.

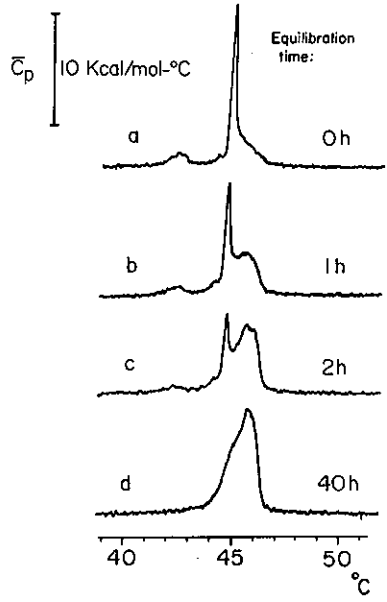

Figure 8. DSC traces of ($\underline{R}_p$)-DPPsC showing the existence of a "metastable gel phase". The sample (2.14 mg) was prepared as described in Figure 7, followed by heating at 70°C for 5 min. and equilibrating at 25°C before each scanning for 0 h (a), 1 h (b), 2 h (c), and 40 h (d).

5 min., cooled down to 25°C and scanned immediately, a "normal shape" was obtained. If the sample was equilibrated at 25°C (after heating at 70°C), it relaxed back to the "stable gel phase" with a time constant of 4 h, as shown in Fig. 8, b-d. Separate experiments indicated that heating at 50°C instead of 70°C gave essentially the same result. Thus, after heating at temperatures above the main transition, the ($\underline{R}_p$) isomer can exist in a "metastable gel phase" which shows similar thermotropic properties to ($\underline{S}_p$)- and ($\underline{R}_p$ + $\underline{S}_p$)-DPPsC.

CONCLUSION

We present the synthesis and configurational analysis of chiral thiophospholipids, and the use of these compounds to study the stereochemical mechanism of PL A_2. The difference between DPPC and ($\underline{R}_p$)-DPPsC is due to substrate specificity, whereas the difference between ($\underline{R}_p$)- and ($\underline{S}_p$)-DPPsC is due to stereospecificity. The biophysical properties of ($\underline{R}_p$)- and ($\underline{S}_p$)-DPPsC are different in every respect, but the surface specificity does not seem to play an important role in the kinetic properties of DPPsC isomers. Substitution of Ca^{2+} by Cd^{2+} causes the ratio of the maximal velocity, V_R/V_S, to decrease by a factor of 110. The results support direct binding of Ca^{2+} with the pro-S oxygen of the phosphate group of DPPC in the active site of PL A_2.

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