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PHOSPHOLIPIDS CHIRAL AT PHOSPHORUS. STEREOCHEMICAL EFFECTS ON THE THERMOTROPIC PROPERTIES OF THIOPHOSPHATIDYLCHOLINES AND THIOSPHINGOMYELINS

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SUMMARY

The effect of the configuration at the phosphorus atom in diastereomers of phosphorothioyl analogues of lecithin and sphingomyelin on their thermotropic phase behaviour has been studied. It was found that the effect of varying the configuration of the phosphate function concerns primarily the properties of the subphase of lecithins. (Sp)-1,2-dipalmitoyl-sn-glycero-3-thiophosphocholine [(Sp)-DPPsC] and (Rp+Sp)-DPPsC are characterized by the thermal behaviour reminiscent of natural diacylphosphatidylcholines. (Rp)-DPPsC exists in the metastable gel phase exhibiting typical transitions of natural compound. This phase readily rearranges to a subphase showing only one broad high enthalpy endotherm. Contrary to the behaviour of natural stearoylsphingomyelin its phosphorothioate analogues do not display metastability. The difference in the properties of (Rp)- and (Sp)-2-N-stearoylsphingosyl-1-thiophosphocholine [(Sp)-SPsM] is larger than this between diastereomers of DPPsC. Our results suggest that the configuration of the phosphate in diastereomers of thiophospholipids is most important in defining properties of the subtransition of phospholipid bilayer.

INTRODUCTION

The effect of chirality on the molecular interactions of membranes has attracted increasing attention in recent years (ref.1). The main problem is whether chiral discrimination or recognition factors exist in the bilayer matrix. The general approach to enantiomer discrimination has been to compare the properties of enantiomerically pure phospholipid with those of the racemic mixtures. So far the only clear evidence that such discrimination operates indeed comes from the results of Arnett et al. (ref 1,2) on the pressure area

any disparity between the monolayers of racemic and enantiomeric DMPE. The apparent differences found in DSC curves of DPPC bilayers may originate from the different degree of purity of samples used for comparison. Thus, the lack of enantiomers discrimination reported by Arnett and Gold (ref.5) using extremely pure phospholipid preparations seems more convincing.

Chiral discrimination in membranes may also be induced by diastereomer interactions of components bearing multiple chiral centers. This kind of chiral interactions is present in true biological membranes constituting of sphingolipids, sterols and proteins in addition to glycerophospholipids. Recently, we have utilized P-chiral analogues of glycerophospholipids for the studies aimed at exploring the stereochemical aspects of membrane biochemistry. Our approach is thus more consistent with the situation of interaction of diastereomeric compounds occuring in biological membranes than with the "diastereomer discrimination" defined by Arnett (ref.1) as any measurable difference between diastereomeric pairs.

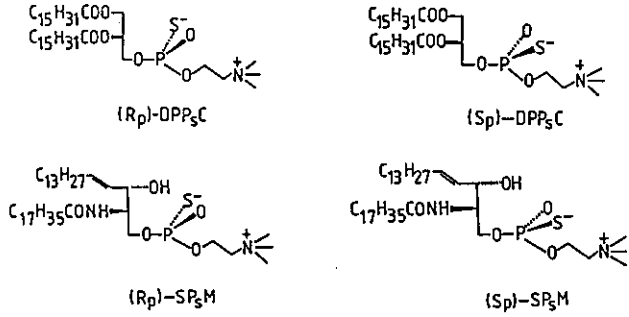

Figure 1. Structures of phosphorothioate analogues of dipalmitoylphosphatidylcholine and stearoylsphingomyelin (charges are ommitted for simplicity).

In the case of phosphorothioyl analogues of DPPC we are comparing the properties of two diastereomeric phospholipids. Since the configuration at carbon-2 of the glycerol backbone is fixed a large difference in the properties of (Rp)- and (Sp)-DPPsC may suggest involvement of the phospholipid head group in stereospecific interactions or conformational change at the polar part of molecule inflicted by the reversal of the configuration at phosphorus.
At this moment it is worthwhile mentioning that change of the thiophosphate configuration has very little effect on the structure of monomeric phospholipid as illustrated by very small differences detected in nmr spectra of DPPsC in solution (refs.6-8)

Whether the phosphate function is involved in chiral interactions is best

illustrated by the stereospecificity of phospholipase A_2 (refs.7-9) and lecithin-cholesterol acyltransferase (LCAT,ref.10). In the latter system the K_m and V_{max} values are indistinguishable for DPPC, (Rp)-DPPsC and (Sp)-DPPsC. On the other hand PLA_2 showed high stereospecificity toward (Rp)-DPPsC (ref.9). These results suggest that in PLA_2 hydrolysis stereospecific iteractions of the phosphate with the enzyme play important role, and that they are not essential in acyltransferase reaction. Concerning biophysical properties of diastereomers of DPPsC we have been able to demonstrate that (Rp), (Sp) and (Rp+Sp)-DPPsC bilayers display different physical characteristics in the liquid-crystalline state as revealed by ^{14}N nmr, ^{31}P nmr (ref.11) and FT-IR (ref.12). These differences detected for aggregates in contrast to monomers have prompted us to investigate thermal phase behaviour of multilamellar vesicles of DPPsC and SPsM in detail.

MATERIALS and METHODS

DPPC was purchased from Avanti and was used without further purification. (Rp+Sp)-DPPsC was synthesized chemically and resolved into (Rp)- and (Sp)-isomers based on the stereospecific hydrolysis of (Rp)-DPPsC by bee venom phospholipase A_2 as described previously (ref.7). d-Erythro-N-stearoyl-sphingomyelin (SPM) and its phoshorothioyl analogue (Rp+Sp)-SPsM were obtained according to the recently published procedure (ref.13). The mixture of diastereomers was separated into individual compounds by chromatography. Diastereomeric purity was assessed by ^{31}P nmr in $CDCl_3$ or CD_3OD using Bruker WM-300 and Bruker AM-500 nmr spectrometers. Diastereomeric purity of phosphorothioyl analogues of DPPC and SPM were considered greater than 99 %. Chemical purity of lipid samples was monitored by ^1H nmr on Bruker WP-200 and Bruker AM-500 and by tlc. Final purification of phospholipids was accomplished by 6-7 precipitations from acetone : ethanol or acetone : chloroform. Typically, purification was continued until constant T_m and $t_{1/2}$ were obtained. DSC curves were obtained with Microcal scanning calorimeter, model MC-1 for PC's and model MC-2 for SPM's. Dispersions for DSC measurements were prepared by hydration of phospholipid at 60°C in 20mM PIPES buffer, pH 7.4 (DPPC) or 2.5 mM MOPS buffer, pH 7.0 (SPM) with shaking. Phase transition enthalpies were calculated with ±10% accuracy. Scanning rates varied from 27 deg/min for subtransitions of DPPC to 70 deg/min for SPM's. Our standard conditions were 5% (wt/wt) phosphatidylcholine and 0.4% sphingomyelin in corresponding buffers.

RESULTS

DSC profiles of phosphatidylcholines

The DSC heating curves were obtained first in an effort to reproduce the

also given in Table 1. The mixture of diastereomers of DPPsC and (Sp)-DPPsC showed very similar phase transition behaviour with pretransitions at 43.8°C and 43.7°C and main transition at 44.8°C and 45.0°C, respectively. In addition, the associated pretransition enthalpies of DPPsC are greater than that of DPPC by ~50 % and the half widths ($\Delta t_{1/2}$) of the pretransitions of DPPsC are also substantially reduced.

Table 1. Summary of Subtransition, Pretransition and Main Transition Parameters of DPPC and DPPsC and Main transition of SPM and SPsM

Compound	Subtransition			Pretransition			Main Transition		
	T_s °C	$\Delta t_{1/2}$ (K)	ΔH Kcal/mole	T_p °C	$\Delta t_{1/2}$ (K)	ΔH Kcal/mole	T_m °C	$\Delta t_{1/2}$ (K)	ΔH Kcal/mole
DPPC (ref.14)	18.4	3.0	3.2	35.1	1.8	1.1	41.1	0.18	6.9
DPPC (this work)	18.3	2.1	2.8	35.1	1.5	1.1	41.5	0.27	7.2
(Rp + Sp)-DPPsC	21.7	2.6	2.6	43.8	0.75	1.7	44.8	0.26	6.8
(Sp)-DPPsC	22.0	2.8	2.9	43.7	0.75	1.6	45.0	0.27	7.1
(Rp)-DPPsC									
equilibrated							45.9	1.5	13.4
nonequlibrated				42.7	1.0	1.4	44.9	0.36	7.4
SPM							44.7	0.6	6.8
(Rp)-SPsM							46.1	0.4	5.6*
(Sp)-SPsM							49.5	3.0	7.7*
(Rp + Sp)-SPsM							45.9	2.0	6.1*

* tentative values

(Rp)-DPPsC showed anomalous behaviour in that only a single broad transition at 45.9°C was seen (ΔH = 13.4 Kcal/mole). The shape of the profile remained unchanged after the sample had been subjected to further purification steps or when a second batch of synthetic material is used. The character of the profile returned to "normal" with pretransition at 42.8°C and main transition at 44.7°C when this sample was "contaminated" with 15 % of (Sp)-DPPsC.
High enthalpy of the main transition of pure (Rp)-DPPsC, asymmetry of the peak and its broadness suggest that it may be a composite of several two-state transitions.

Metastability of gel phases of DPPsC

After the dispersion of (Rp)-DPPsC had been heated at 70°C, cooled quickly to 25°C and scanned immediately, a normal pattern of DSC profile appeared (Figure 3a). The transition parameters (Table 1) are similar to those of Sp- and (Rp+Sp)-DPPsC. As is shown in Figure 3b-d the gel phase of (Rp)-DPPsC

relaxes rapidly to a lower energy phase displaying one broad endotherm. The rate of the rearrangement of the gel phase of (Rp)-DPPsC is much higher relative to the rate of appearance of the subtransition in the case of DPPC (ref.14) and (Sp)-DPPsC. In accordance with this the admixture of the (Sp)-isomer (3%) reduces the rate of the gel-subgel phase relaxation of (Rp)-DPPsC.

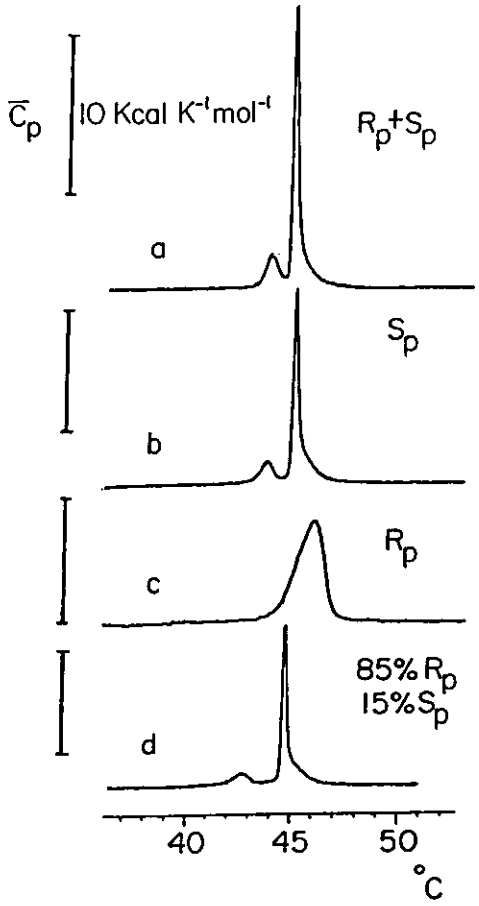

Figure 2. DSC traces of (Rp+Sp)-DPPsC (a), (Sp)-DPPsC (b), (Rp)-DPPsC (c), (85% Rp + 15% Sp)-DPPsC (d).

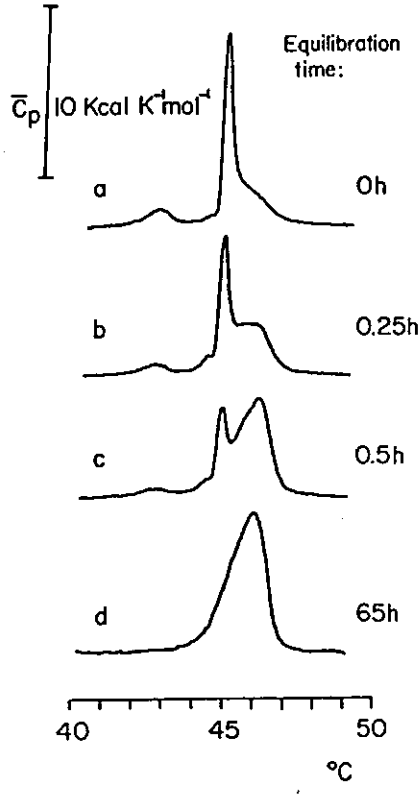

Figure 3. DSC traces of (Rp)-DPPsC of >99% diastereomeric purity. Sample was equilibrated at 25 C for: a) 0 h, (b) 0.25 h, (c) 0.5 h, (d) 65 h

On the other hand (Sp)- and (Rp+Sp)-DPPsC are kinetically more stable in the gel phase relative to DPPC. The dependence of the subtransition on the incubation time at 0°C for (Sp)- and (Rp+Sp)-DPPsC is shown in Figure 4.

days for b). It therefore may be concluded that the relaxation rate of the gel phase to the subgel phase fall in order: (Rp)-DPPsC > DPPC > (Sp)-DPPsC = (Rp+Sp)-DPPsC. The phase behaviour of the isomeric DPPsC described above have been confirmed by X-ray diffraction studies (ref.15)

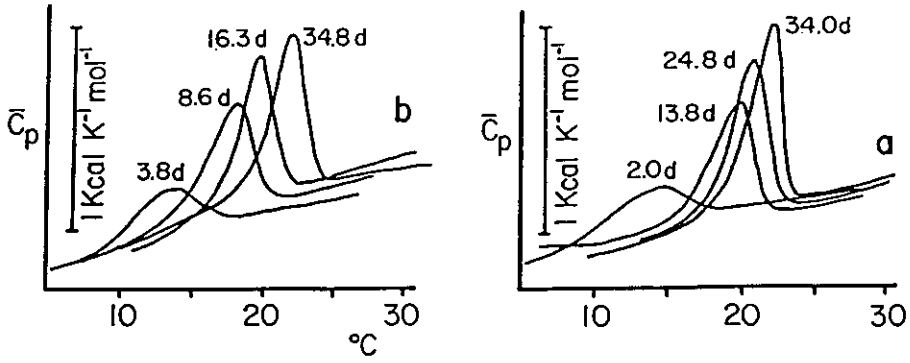

Figure 4. Dependence of subtransition on the incubation time at 0°C for (Rp + Sp)-DPPsC (a) and (Sp)-DPPsC (b). The duration of incubation at 0°C in days are shown above the traces.

DSC of d-erythro-N-stearoylsphingomyelin and its phosphorothioyl analogues.

Melting profiles of d-erythro-N-stearoyl-SPM are shown in Figure 5. The sample of phospholipid dispersion heated directly after phospholipid hydration at 60°C exhibits a single sharp endotherm (peak I) at 44.7°C ($\Delta t_{1/2}$=0.5 deg) with an enthalpy of the transition 6.8±0.2 Kcal/mole (trace a). The sharpness of the transition is greatly affected by the presence of impurities and broader peaks are seen at intermediate stages of sphingomyelin purification (see Materials and Methods). When the dispersion previously heated above T_m is held at the temperature below 25°C for several hours and rescanned another reversible broad endotherm (peak II) appears at 36°C.

The enthalpy of this new transition is time and temperature dependent (compare traces b and c) and the rate of its appearance increases as the temperature of incubation decreases. At 24°C the half-life of the metastable gel phase is ~5 h. The dispersion which had been heated above 36°C, and cooled quickly down to 20°C displayed only the phase transition at 44.7°C upon next heating scan (traces d and e). When the sample heated above 36°C was again incubated at 20°C melting profile with two endotherms (I and II) was observed again. The enthalpy and the temperature of transiton I are not influenced by the appearance of the transition II. The enthalpy of the main phase transition is however affected by incubation of the sample at lower temperatures. Storing of the dispersion at

5°C for 5 days causes asymmetric broadening of the main transition with concomittant increase of its enthalpy, disappearance of the transition II, and formation of the new broad transition (III) at 33.4°C (2.8 Kcal/mole, trace h). After shorter times of incubation at 7°C the two domains coexist: one characterized by the transitions I and II and the second displaying transitions III and IV is apparent in traaces g and f. The broadness and high enthalpy of transition IV in such sample is reminiscent of the behaviour of d,l-erythro-stearoylsphingomyelin reported by Estep et al. (ref.16).

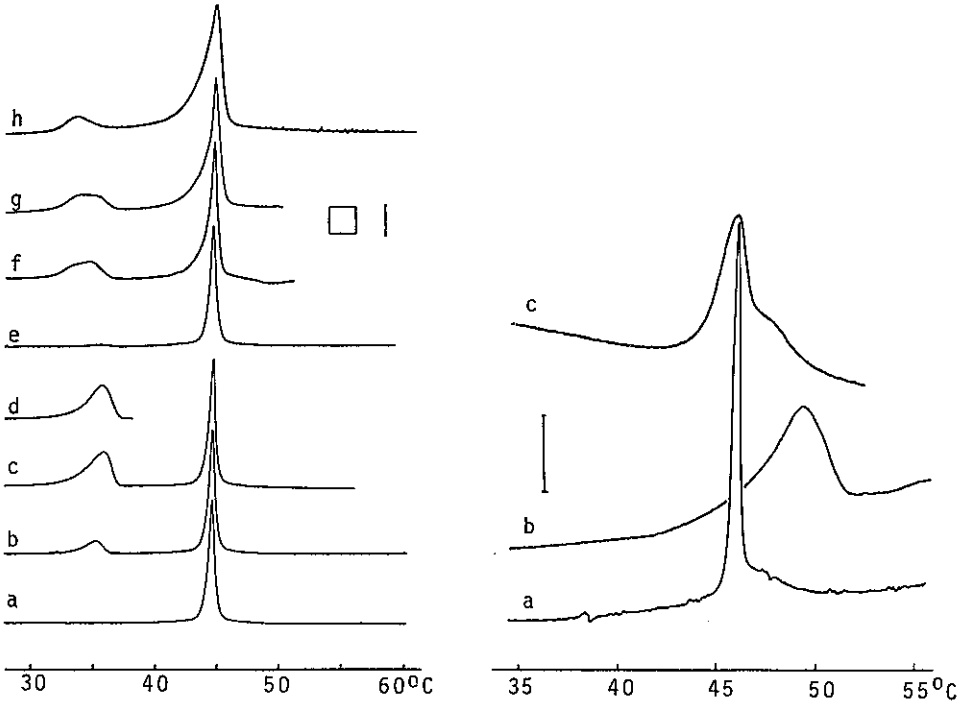

Figure 5. DSC curves of d-erythro-stearoylsphingomyelin: obtained immediately after hydration (a), after 2.6 h at 24°C (b), 15 h at 20°C (c), 7h at 20°C (d), reheated immediately after scan d (e), 12 h at 7°C (f), 4 days at 7°C (g), 5 days at 5°C (h). Bar - 2 Kcal/mole, square - 10 mcal, sample size 2 mg.

Figure 6. DSC curves of SPsM isomers. (Rp)-SPsM (a), (Sp)-SPsM (b), (Rp + Sp)-SPsM (c). Bar - 1 Kcal/mole, sample size - 2 mg.

The DSC curves of (Rp)- and (Sp)-SPsM are shown in Figure 3. While (Rp) SPsM is characterized by the sharp transition at 46.1°C ($\Delta t_{1/2}$ = 0.4 deg) associated with relatively low enthalpy (ΔH = 4.0 Kcal/mole), (Sp)-SPsM displays a broad unsymmetrical endotherm centered around 49.5°C ($\Delta t_{1/2}$ = ~3 deg, ΔH = 6.0 Kcal/mole). The behaviour of (Rp+Sp)-SPsM is characterized by the transition at 45.9°C (ΔH = 6.0 Kcal/mole) of intermediate width and a poorly resolved shoulder at high temperature side (~48°C). Contrary to the behaviour of the natural compound the analogues do not exhibit metastable behaviour as the incubation of all SPsM samples at lower temperatures did not induced any measurable changes in their DSC curves.

DISCUSSION

Based on the large differences in physical properties of (Rp)- and (Sp)-DPPsC bilayers in their liquid-crystalline state we have expected strong effects of the phosphate configuration on the thermal behaviour of these compounds. In interpreting physical properties of thiophospholipids, the properties which are different from natural compounds but are insensitive to the configuration at phosphorus can be attributed to the effect of O→S substitution. The differences between stereoisomers should be attributed to the effect of the configuration of the phosphate group. It is clear from the results presented in Table 1 that O→S substitution raises the temperature of all phase transitions for both DPPsC and SPsM. In case of DPPsC the magnitude of this increase falls in the order T_p (ca.9°C)> T_m (ca.3.5°C) = ~T_s (ca.3.0°C). Furthermore, there is substantial effect of sulfur substitution on the ΔH and the width of the pretransition. Sulfur substitution also has some effect on the kinetics of gel-subgel phase relaxation. The effect of the configuration of the phosphate is most significant on the subtransition and on the stability of the subphase. This conclusion is especially justified since the subphase has been shown to possess highly ordered molecular and chain packing modes in the bilayer (ref.17). Molecular events occurring at the subtransition are a subject of the dispute. The results of FT-IR (ref.18) and X-ray (ref.19) studies seem to suggest that the hydration change which takes place at this transition is the main driving force for the gel-subgel phase rearrangement. This conclusion is not supported by recent data on the deuterium isotope effect of D_2O medium on the phase transitions of phosphatidylcholines which show that subtransition is least affected by D_2O (ref.20). The large configurational effect of the phosphate function suggests possible involvement of this function in the overall conformation change occuring at subtransition. It is also likely that the ionic interaction of phosphate with the ammonium cation of the neighbouring phospholipid molecule are important to the

stability of the subphase. X-Ray crystal data for DMPC clearly show the inequivalence of two terminal oxygens of phosphate group (ref.21). If the packing of polar groups in the subphase involves similar interactions between adjacent phosphocholine groups as those in the crystal form, the configurational change of the phosphate would result in the observed differences in the subtransitions of DPPsC isomers.

As expected introduction of the sulfur into the polar function of sphingomyelin has a greater effect on the thermal behaviour of SPM bilayers than it has on the properties of DPPC. Diastereomers of SPsM do not exhibit metastable behaviour characteristic of natural SPM. The broad endotherm in the DSC curve of (Sp)-SPsM is a composite of several phase transitions. The time required for the equilibration of the calorimeter prior to starting the scan could be sufficient for complete relaxation of the metastable gel phase. Under our standard conditions metastable states would not have been detected if their half life-time was 10-20 min. On the other hand the sharp endotherm of (Rp)-SPsM suggests that it is likely to be a single transition and that Rp-SPsM is kinetically stable at the gel phase. The properties of diastereomers of SPsM are so different that they exhibit incomplete miscibility manifested by the asymmetry of the main endotherm of (Rp+Sp)-SPsM.

The differences in the properties of natural phospholipids and their phosphorothioyl analogues and between the stereoisomers of these phosphorothioates with opposite configurations at phosphorus are more pronounced in phases with tighter molecular packing and less motional freedom such as gel phase and subgel phase. It seems reasonable that these differences are more marked in case of sphingomyelin bilayers since sphingomyelin head group is known to occupy smaller area (ref.22) and molecular packing in SPM bilayers may be tighter due to existing network of hydrogen bonds at the interface region. In case of sphingomyelin bilayers we have found recently that thermal behaviour of SPM dispersions is very sensitive to configuration at carbon C_3 of the sphingosine (ref.23). Thermal behaviour of purified d-erythro-C18-SPM also differs from the reported one for d,l-erythro-C18-SPM.

Limited amount of data do not allow for more sophisticated evaluation of the observed effects. Further studies dedicated to explain the observed differences between stereoisomers of phosphorothioyl analogues of phospholipids in aggregated forms are in progress.

ACKNOWLEDGEMENTS

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