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METASTABLE PHASE BEHAVIOR OF A SPHINGOLIPID ANALOGUE

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The phase behavior of the sphingolipid analogue 1-palmitoyl-2-tridecanylcarbamyloxy-*sn*-glycero-3-phosphocholine (CM-PC) has been studied by differential scanning calorimetry. When CM-PC is cooled at rates >5 K/min, subsequent heating runs exhibit metastable behavior: a low enthalpy exotherm is observed at about 9°C ($\Delta H = -(1\text{–}2)$kcal/mol), followed by a high enthalpy endotherm at 38°C ($\Delta H = 13$ kcal/mol). Systematic variation of cooling/heating protocols indicates that CM-PC exhibits two low temperature states, one metastable and the other stable. Cooling from the liquid crystalline state results in formation of the metastable low-temperature polymorph I, which must transform into the stable low-temperature polymorph II before the liquid crystalline state can be reached again. This metastable thermal behavior is virtually identical to that recently reported for synthetic palmitoyl cerebroside (Ruocco, M.J., Atkinson, D., Small, D.M., Skarjune, R.P., Oldfield, E. and Shipley, G.G. (1981) Biochemistry 20, 5957–5966) and for bovine brain *n*-acylcerebrosides (Curatolo, W. (1982) Biochemistry, 21, 1761–1764). The observation of the metastable phase behavior of CM-PC indicates that the sphingosine backbone is not a prerequisite for such metastable behavior. Furthermore, the carbamyl group in CM-PC is reversed in orientation compared with the amide of sphingolipids (-NH-CO- vs. -CO-NH-), suggesting that the intermolecular hydrogen bonding potential, rather than some highly specific steric or conformational constraint, is responsible for the observed metastability of sphingolipids.

Introduction

A wide variety of synthetic and natural phospholipids have been shown to undergo readily reversible acyl chain order-disorder transitions at characteristic temperatures. Some polar lipids, however, exhibit phase behavior which is more complicated. Palmitoylcerebroside, for instance, exhibits two low-temperature states, one metastable and the other stable [1]. Similar behavior has also been observed for glucocerebroside from Gaucher's spleen [2], and for *n*-acyl-cerebrosides from bovine brain [3,4]. Such metastability is not confined to glycolipids, since stearoylsphingomyelin exhibits similar behavior [5]. In general, the calorimetric behavior of these systems includes the following features: (a) an exotherm on heating in the gel state, characteristic of a kinetically limited transition from a metastable to a stable state, and (b) an unusually high enthalphy (ΔH) for the acyl chain order-disorder transition. X-ray diffraction studies have demonstrated that the stable gel forms of stearoylsphingomyelin and palmitoylcerebroside are characterized by acyl chain packing modes which are more ordered than that of the gel state

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Abbreviations used: CM-PC, 1-palmitoyl-2-tridecanylcarbamyloxy-*sn*-glycero-3-phosphocholine; DSC, differential scanning calorimetry; DPPC, dipalmitoylphosphatidylcholine.

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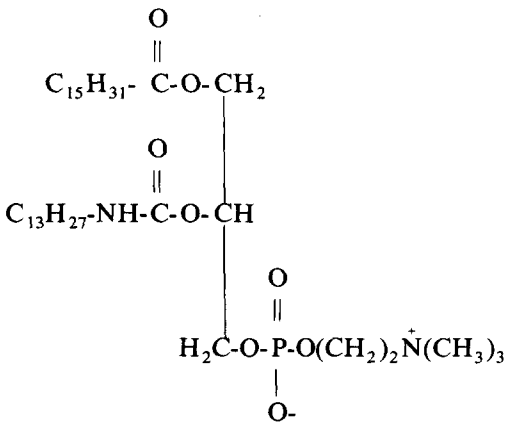
of diacyl phospholipids [1,5]. The crystal structures of ceramide, psychosine, and cerebroside indicate that intermolecular hydrogen bonding occurs in the crystalline state of these compounds [6–8]. It has been suggested that intermolecular hydrogen bonding may also be involved in formation of the stable gel state of cerebrosides [1,4].

We now present a calorimetric study of the sphingolipid analogue 1-palmitoyl-2-tridecanylcarbamyloxy-*sn*-glycero-3-phosphocholine (CM-PC), whose structure is shown:



This phospholipid possesses intermolecular hydrogen bonding potential via its carbamyl group, which is reversed in orientation compared to the amide of sphingolipids (-NH-CO- vs. -CO-NH-). This unusual analogue exhibits thermal behavior which is almost identical to that of *n*-acylcerebrosides, and provides strong evidence for the role of interlipid hydrogen bonding in the formation and stabilization of the stable gel state of sphingolipids.

Methods

1-Palmitoyl-2-tridecanylcarbamyloxy-*sn*-glycero-3-phosphocholine (CM-PC) was synthesized as previously described [9], and was purified by Sephadex LH-20 column chromatography followed by preparative thin-layer chromatography on silica gel plates.

CM-PC samples in $CHCl_3/CH_3OH$ (2:1, v/v) were dried under N_2 in Perkin-Elmer 50 μl DSC pans, desiccated under vacuum overnight, hydrated with 30 μl distilled deionized H_2O, and sealed. The lipid concentration was approx. 17 weight %. Scanning calorimetry was carried out using a Perkin-Elmer DSC-2 scanning calorimeter.

Results

In Fig. 1a is presented a differential scanning calorimetry (DSC) trace (heating run, 5 K/min) of a multilamellar dispersion of CM-PC which was previously cooled at 40 K/min. Two thermal transitions are observed: (A) an exotherm at about 9°C with $\Delta H = -1.7$ kcal/mol, and (B) a large endotherm at 38°C with $\Delta H = 13.1$ kcal/mol. The small exotherm A is indicative of the presence of a metastable low temperature state which undergoes an exothermic transition to a more stable state on heating. This metastable behavior was investigated by heating through the exotherm A, stopping at a temperature (24°C) just before the major endo-

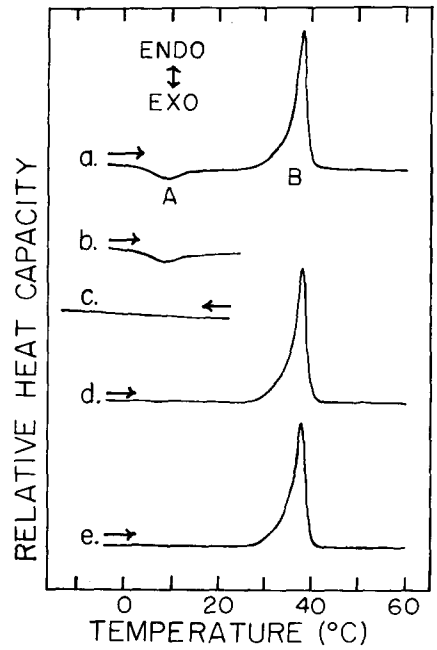


Fig. 1. Differential scanning calorimetry traces of multilamellar dispersions of CM-PC. (a) Heating run at 5 K/min which followed a 40 K/min cooling run. (b, c, d) CM-PC dispersion was (b) heated from −10°C to 24°C at 5 K/min, then (c) immediately cooled from 24°C to −10°C at 5 K/min, then (d) immediately heated from −10°C to 60°C at 5 K/min. (e) Heating run at 5 K/min which followed a 0.6 K/min cooling run.

TABLE I

ENTHALPY DATA FOR CM-PC AS COOLING RATE IS VARIED

The cooling rate was varied; all heating runs were at 5 K/min. n.m., not measured.

Cooling rate (K/min)	ΔH(kcal/mol)				
	Cooling run			Subsequent heating run	
	Txn C	Txn D	Σ	Txn A	Txn B
40	n.m.	n.m.	n.m.	−1.7	13.1
20	−6.6	−0.3	−6.9	−1.5	12.9
10	−6.7	−1.2	−7.9	−0.9	12.7
5	−6.5	−2.9	−9.4	0	12.8
2.5	−6.0	−4.0	−10.0	0	12.6
1.25	−6.0	−4.8	−10.8	0	12.7
0.6	−6.0	−4.8	−10.8	0	12.5

therm B, and then cooling (Figs. 1b and 1c). No transitions were observed during the cooling run, and a subsequent heating run (Fig. 1d) reveals only the major endotherm B. Thus transition A is irreversible.

The metastable behavior of CM-PC was further investigated by varying the cooling rate, and observing the effect upon subsequent heating runs. A heating run at 5 K/min is presented in Fig. 1e which followed a cooling run at 0.6 K/min. The exotherm A is not observed in this case. Thus slow cooling results in formation of the stable low temperature state exclusively. Examination of cooling runs at various rates is especially instructive. In Fig. 2 are shown cooling runs at 10 K/min, 2.5 K/min, and 0.6 K/min. At 10 K/min, two cooling transitions are observed: a relatively sharp transition C at approx. 27°C, followed by a small broad transition D at approx. 8°C. As the cooling rate is decreased, the following effects are noted: (i) both exotherms increase in temperature with a larger increase for the small exotherm D, (ii) both exotherms become sharper, and (iii) the relative enthalpy of the low temperature exotherm D increases *. In Table I are presented enthalpy data for cooling runs at various rates, and for subsequent heating runs at 5 K/min. As the cooling rate is decreased, the enthalpy of the sharp cooling transition C remains essentially invariant, while the enthalpy of the broader transition D increases significantly. As the cooling rate is decreased, the

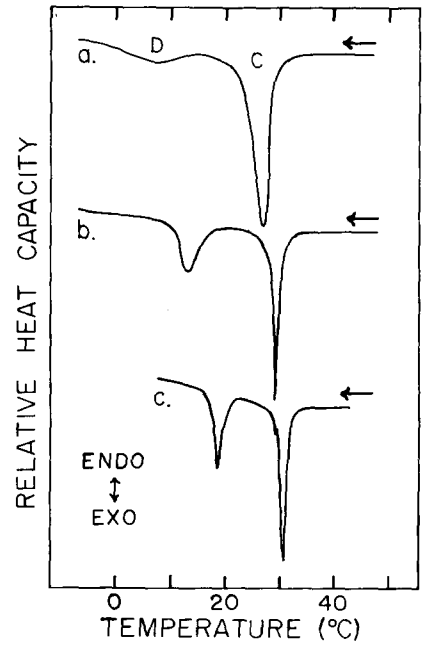


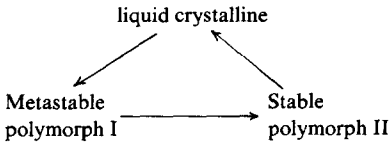
Fig. 2. Cooling behavior of CM-PC, at various cooling rates: (a) 10 K/min, (b) 2.5 K/min, (c) 0.6 K/min. The relative areas of the thermal profiles as shown do not reflect the relative enthalpies; see Table I for enthalpy as a function of cooling rate.

* In the slow cooling run (0.6 K/min) of Fig. 2c, an additional small peak is resolved at approx. 29°C. We believe this transition to be the order-disorder transition of a small quantity of the reversed-chain isomer, i.e. 1-tridecanyl-carbamyloxy-2-palmitoyl-*sn*-glycero-3-phosphocholine. A small percentage of this reversed-chain isomer is expected due to acyl chain migration during CM-PC synthesis [14].

enthalpy of the heating exotherm A in the subsequent heating run decreases. Following cooling runs at 5 K/min or less, the exotherm A is not observed in subsequent heating runs. The enthalpy of the major heating transition B exhibits no significant dependence on the previous cooling rate.

Discussion

The calorimetric data presented above clearly demonstrate that the sphingolipid analogue CM-PC undergoes metastable thermal behavior. This behavior can be schematically summarized:



The evidence for this scheme is as follows. As shown in Fig. 1a, a heating run which follows a fast cooling run exhibits an exotherm at approx. 9°C. The evolution of heat by the sample at this transition indicates that a portion of the sample is transformed from a metastable state (polymorph I) into a more stable state (polymorph II). Figs. 1b and 1c demonstrate that this polymorph I → polymorph II transition is irreversible. Subsequent heating through the major endotherm B results in transformation of polymorph II into the liquid crystalline (fluid acyl chain) state. The transition temperature of the major endotherm B (38°C) is close to that of the order-disorder transition of dipalmitoylphosphatidylcholine (41°C), as expected since CM-PC has a palmitoyl chain in the *sn*-1 position, and an *sn*-2 chain which is approximately one C-C bond length shorter than a palmitoyl chain.

Fig. 1e demonstrates that the heating exotherm A is absent after a slow cooling run, indicating that slow cooling results in the formation of polymorph II exclusively. This behavior is further clarified by examination of the cooling behavior as the cooling rate is varied. On cooling, two exothermic transitions are observed. The sharp exotherm C exhibits a ΔH which is invariant with cooling rate, while the smaller exotherm D increases in enthalpy as the cooling rate is decreased (Fig. 2; Table I). The most reasonable interpretation of these observations is that the large cooling exotherm C reflects the liquid crystalline → metastable polymorph I transition. The lower temperature cooling exotherm D reflects the metastable polymorph I → stable polymorph II transition. Since the temperature dependence of the polymorph I → polymorph II transition varies with cooling rate, it is kinetically limited; i.e. it occurs at a rate which is slower than the experimental cooling rate. As the cooling rate is decreased, the total cooling enthalpy (−10.8 kcal/mol at 0.6 K/min; Table I) approaches the enthalpy of the major heating endotherm B (12.5 kcal/mol) of the subsequent heating run. However, for all heating/cooling protocols studied, the total exothermic ΔH (cooling ΔH, or cooling ΔH plus ΔH of subsequent heating exotherm A) is less than the endothermic ΔH for heating transition B. We conclude that a portion of the polymorph I material converts into stable polymorph II noncooperatively under the conditions studied. To summarize, cooling CM-PC from the liquid crystalline state results first in formation of metastable polymorph I, which is subsequently transformed into the stable polymorph II, either on slow cooling or on subsequent heating. The major endotherm B at 38°C is the stable polymorph II → liquid crystalline phase transition.

The enthalpy of the major heating endotherm B is significantly larger than that observed for the gel ↔ liquid crystalline transition of synthetic diacyl phospholipids [10,11]. The disruption of intermolecular hydrogen bonds may account for part of this enthalpy, but the extent of this contribution is difficult to estimate since residual hydrogen bonding (between lipids or with water) may persist in the liquid crystalline state. Since the change in entropy ΔS is equal to $\Delta H/T$, the difference in entropy between the gel and liquid crystalline states is significantly larger for CM-PC than for phosphatidylcholines in general. Assuming reasonably that the liquid crystalline states are similar, we conclude that the low temperature polymorph II of CM-PC is a more ordered structure than the gel state of diacyl phospholipids.

The metastable thermal behavior of the sphingolipid analogue CM-PC is similar to that previously observed for stearoylsphingomyelin,

palmitoylcerebroside, and *n*-acylcerebrosides from bovine brain [1,3–5]. For stearoylsphingomyelin and palmitoylcerebroside, it has been directly shown by X-ray diffraction that the stable low temperature polymorph possesses a highly ordered acyl chain packing mode. Sphingomyelin and cerebroside possess an amide group as a common feature, with the attendant capability of forming intermolecular hydrogen bonds. Cerebroside, of course, possesses additional intermolecular hydrogen bonding potential via its glycosyl headgroup. The present work with CM-PC demonstrates that the sphingosine backbone is not a necessary prerequisite for the formation of unusually stable acyl chain packing modes. Furthermore, the carbamyl group in CM-PC is reversed in orientation compared with the amide of sphingolipids (-NH-CO- vs. -CO-NH-), suggesting that the intermolecular hydrogen bonding potential, rather than some highly specific steric or conformational constraint, is responsible for the observed metastability.

Recent work by Chen et al. [12] has demonstrated that dipalmitoylphosphatidylcholine (DPPC) can exhibit metastable thermal behavior, and is capable of forming a second low temperature gel state which is more ordered than the commonly observed gel state. The metastable thermal behavior reported for DPPC differs from that of CM-PC and *n*-acylcerebrosides in two major respects. First, the kinetic barrier to the formation of the more ordered gel state of DPPC is considerable: incubation at 0°C for 3 days is required. In the case of CM-PC (or *n*-acylcerebrosides) the stable low temperature polymorph II is attained relatively quickly, within minutes at slow cooling rates. Second, on heating, the more ordered gel state of DPPC undergoes a transition at approx. 18°C (referred to as the 'subtransition' by Chen et al. [12]) to the normally observed gel state ($\Delta H \sim 3$ kcal/mol); continued heating results in the well-described thermal pretransition (35°C) and order-disorder transition (41°C). The stable low temperature polymorphs of CM-PC and *n*-acylcerebrosides, on the other hand, undergo no transitions on heating until the order-disorder transition to the liquid crystalline state occurs. We propose that intermolecular hydrogen bonding provides a mechanism for overcoming the kinetic barrier to the formation of highly ordered acyl chain packing modes in polar lipids, perhaps by ordering the lipids laterally into an anisotropic two dimensional lattice. Furthermore, the presence of intermolecular hydrogen bonding results in stabilization of the ordered acyl chain packing; thus the stable polymorph II undergoes no cooperative phase changes as the temperature is increased, until the ordered → liquid crystalline transition is reached.

The capability of forming interlipid hydrogen bonds may have in vivo significance in the lateral stabilization of liquid crystalline membranes, as suggested by Pascher [6]. In this regard, it is interesting to note that vesicles composed of carbamylphosphatidylcholine are stable to phospholipase A_2 attack and to serum-induced leakage [9,13]. The in vivo significance, if any, of the metastable behavior of sphingolipids is at present unknown. We have suggested that, in the case of the cerebroside-rich myelin membrane, such metastability is undesirable and is prevented by the presence of amide-linked hydroxy fatty acids in brain cerebrosides [4].

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