

## DSC-Studies of Phospholipids Containing Iso-Branched and $\omega$ -Cyclohexane Fatty Acids

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### Summary

Differential Scanning Calorimetry (DSC) and Differential Scanning Densitometry (DSD) was employed to study the phase behavior of phospholipids with iso-branched and  $\omega$ -cyclohexyl fatty acids. Phosphatidylcholines (PC) and phosphatidylethanolamines (PE) with iso-branched fatty acids show complicated phase behavior with several gel-gel transitions with odd-even effects. These odd-even effects are even more pronounced for phosphatidylcholines with  $\omega$ -cyclohexyl fatty acids (cyPCs). Odd-numbered cyPCs show a large main transition with strong hysteresis while the even-numbered cyPCs have several transitions but show no hysteresis. These differences in thermotropic behavior are also reflected in their molecular volumes. The odd-numbered cyPCs display much tighter packing in the gel phase than the even-numbered analogues. Binary mixtures of cyPCs with either straight chain PCs or iso-branched PCs show eutectic behavior. This is also observed when cyPCs of different chain length are mixed.

### Introduction

Bacteria living in extreme environments with high temperatures and low pH-values contain a number of unusual lipids which are not found in other cellular membranes. In the membranes of the thermoacidophilic *Bacillus acidocaldarius* hopanoids, a group of pentacyclic triterpeneoids, are accompanied by lipids with iso-branched and  $\omega$ -cyclohexyl fatty acids. The interaction between these membrane components, i.e. the hopanoids, which were shown to have a cholesterol-like function, and the lipids with the  $\omega$ -cyclohexyl fatty acids, are thought to provide an optimal packing in the membrane, thus enabling the bacteria to withstand higher temperatures (ref. 1-7). We have synthesized a number of phospholipids with different iso-branched and  $\omega$ -cyclohexyl fatty acids having different head groups as well. The thermotropic behavior of model membranes of these phospholipids was studied by differential scanning calorimetry (DSC) and differential scanning densitometry (DSD). In addition we studied the behavior of various binary mixtures of these compounds and of lipid/cholesterol mixtures (8,9).

### Methods

DSC-measurements were performed using a DASM-1 calorimeter as described before (10). The lipid concentrations used were between 1 and 4 mg/mL. The heating rate was 0.75 deg/min. DSD-measurements were made using a vibrating tube densitometer with sample and reference cell (11). Lipid concentrations were between 10 and 20 mg/mL, the heating and cooling rates were 20 deg/h.

## Results and Discussion

Phospholipids with branched chain or  $\omega$ -cyclohexyl fatty acids show a marked "odd-even" effect in their thermotropic behavior. While this is less pronounced for lipids with iso-branched chains - for these lipids the odd-even effect is only evident for the gel-gel transitions -  $\omega$ -cyclohexyl phospholipids display a marked alternation for the temperatures and enthalpies for the main gel to liquid-crystalline phase transition (8,12,13).

In the case of iso-branched phospholipids the structure of the various gel phases of odd and even-numbered phosphatidylcholines differ due to different packing constraints. For instance, the odd-numbered lipid 17iPC forms an almost crystalline dehydrated  $L_C$ -phase with the chains perpendicular to the membrane surface, while the even-numbered 20iPC forms an  $L_C$ -phase with tilted chains (14). Both PCs convert to an  $L_\beta$ -phase at higher temperature before the transition to the liquid-crystalline phase. The temperature of this gel-gel transition shows the odd-even effect mentioned above, the even-numbered PCs having the higher pre-transition temperatures. In the case of 18iPC this gel-gel transition merges with the main transition to the liquid-crystalline phase. We have also synthesized three different phosphatidylethanolamines with iso-branched fatty acids, namely 17iPE, 18iPE, and 19iPE. The PEs show a more complicated thermotropic behavior than their choline analogues. At least two different transitions can be detected at temperatures below the main transition, both of them corresponding to gel-gel transitions. At high temperatures close to 100°C a fourth transition is detectable in 18iPE and 19iPE. This thermotropic event is probably a conversion from the liquid-crystalline  $L_\alpha$ -phase to an inverted  $H_{II}$ -phase which is also observed in straight chain PEs, and particularly with PEs containing unsaturated fatty acids (15). Table I and Table II summarizes the DSC data for PCs and PEs with iso-branched fatty acid chains.

**Table I**

Thermodynamic parameters for iso-branched phosphatidylcholines (iPC) as determined by DSC.

lipid	$T_{pre}$ (°C)	$\Delta H_{pre}$ (kcal/mol)	$T_m$ (°C)	$\Delta H_m$ (kcal/mol)
17iPC	15.2	4.0	27.5	8.1
18iPC			34.8*	16.5*
19iPC	29.8	3.0	43.7	11.9

\*data for pre- and main transition, as separation of peaks not possible

**Table II**

Thermodynamic parameters for iso-branched phosphatidylethanolamines (iPE) as determined by DSC.

lipid	$T_{pre}^*$ (°C)	$\Delta H_{pre}^*$ (kcal/mol)	$T_m$ (°C)	$\Delta H_m$ (kcal/mol)	$T_{H_{II}}$ (°C)	$\Delta H_{H_{II}}$ (kcal/mol)
17iPE	25-30	1 - 1.7	43.5	5.6	>100	-
18iPE	26-29	1 - 2	49.3	5.7	90	0.5
19iPE	33-37	2 - 4	56.2	7.8	86-90	0.5

\*pre-transition values depend on the thermal history of the sample

From a comparison of the transition values of iso-branched PCs and PEs it is apparent that in these lipids the head group influence is smaller than in phospholipids with straight chains. For instance, the difference in  $T_m$ -values between dipalmitoyl-phosphatidylcholine (DPPC) and dipalmitoyl-phosphatidylethanolamine (DPPE) is ca. 23 degrees whereas for the comparable phospholipids 17iPE and 17iPC the difference amounts to only 16 degrees. On the other hand a marked decrease in  $\Delta H$  is observed for the branched-chain PEs compared to the corresponding PCs. This is again different than in straight-chain phospholipids. In this case PEs and PCs have almost the same transition enthalpies (10). At present these differences in the thermotropic behavior are not completely understood, in particular as no structural information exists on the gel phases of iso-branched PEs.

The data from differential scanning densitometry indicate no drastic differences between the molecular volumes of DPPC and DPPE on one hand and 17iPC and 17iPE on the other hand. The differences correspond in both cases roughly to the the volume of two additional  $\text{CH}_3$ -groups. Table III shows a comparison of DSD-data for straight and iso-branched PCs and PEs.

**Table III**

Molecular volumes (in  $\text{\AA}^3$ /molecule) of straight-chain and iso-branched PCs and PEs.

lipid	T = 10 °C	T = 65 °C
DPPC	1105	1255
17iPC	1156	1300
DPPE	1052	1142
17iPE	1078	1193

Inspection of Table III shows, however, that the differences in molecular volume between PCs and PEs in the gel state is generally lower than in the liquid-crystalline state. This is puzzling as other spectroscopic data from deuterium nmr measurements indicate, that the hydrocarbon region of PEs in the gel as well as in the liquid-crystalline phase is generally more tightly packed than that of the corresponding PCs (16). The DSD data seem to indicate the opposite, at least for the gel phase. It is likely, though, that changes and differences in hydration of the head group are responsible for these effects observed by DSD.

$\omega$ -Cyclohexyl-phospholipids show a strong odd-even effect in their thermotropic behavior (8, 13). The odd-numbered PCs show a large hysteresis of the main transition and have higher transition enthalpies than the even-numbered analogues. These even-numbered PCs have in addition gel-gel transitions not present in the odd-numbered PCs. We have synthesized some phosphatidylglycerols (PGs) with  $\omega$ -cyclohexyl fatty acids and one phosphatidylethanolamine of this lipid class. PGs with  $\omega$ -cyclohexyl fatty acids behave more or less similar to the corresponding PCs, having almost the same transition temperatures and enthalpies. This is different with the PE. In this case a drastic decrease in  $\Delta H$  is observed. In addition the temperature increase of the main transition due to the ethanolamine head group is only 8 degrees for 13cyPC as compared to a value of 23 degrees for DPPE compared to DPPC. The cause for this is

unknown at present. As mentioned above a similar though smaller reduction in temperature difference between the main transition of PCs and PEs is also observed for lipids with branched chains. In this case it seems likely that because the iso-branched PCs form a  $L_{\beta}$ -phase below the main transition the influence of head group interactions is reduced. For straight-chain phospholipids the differences in  $T_m$  arise from the different structures of the gel phases below the main transition, namely  $P_{\beta}$ - or  $P_{\beta}$ - with tilted chains for the PCs vs.  $L_{\beta}$ - for the PEs. The gel phases of the isobranched PCs and PEs may both have perpendicular chains. The same may apply to the gel phase structures of PCs and PEs with  $\omega$ -cyclohexyl fatty acids, though in these cases nothing is known about structural characteristics of the gel phases. Table IV shows the thermodynamic data for phospholipids with  $\omega$ -cyclohexyl fatty acids.

**Table IV**

Thermodynamic parameters for phospholipids with  $\omega$ -cyclohexyl fatty acids as determined by DSC.

lipid	$T_m$ ( $^{\circ}\text{C}$ )	$\Delta H$ (kcal/mol)
13cyPC	34.5	12.0
13cyPG	31.4	13.4
13cyPE	42.5	6.2
14cyPC	34.0	8.9
14cyPG	33.3	12.0
15cyPC	45.6	17.5
15cyPG	44.6	13.8

The data from differential scanning densitometry support the assumption that one reason for the strong odd-even effect is the much tighter packing of the odd-numbered species, which show the higher transition enthalpies. Table V summarizes some data on molecular volumes for phospholipids with  $\omega$ -cyclohexyl fatty acids.

**Table V**

Molecular volumes (in  $\text{\AA}^3$ /molecule) of  $\omega$ -cyclohexyl phospholipids in comparison to straight-chain phospholipids and iso-branched lipids

lipid	$T = 20$ $^{\circ}\text{C}$	$T = 60$ $^{\circ}\text{C}$
DPPC	1143	1250
DSPC	1211	1330
13cyPC	1227	1336
19iPC	1263	1395
14cyPC	1301	1386
15cyPC	1321	1443

It is evident from inspection of Table V that the odd-numbered PCs have very tightly packed gel phases, much tighter than the even-numbered analogues and the n-acyl phospholipids like DPPC or DSPC. Per additional methylene group one would expect an increase in molecular volume of roughly  $24-24 \overset{\circ}{\underset{3}{\text{Å}}}$  (17). The difference in molecular volume between DSPC with a chain of 18 C-atoms and 13cyPC (19 C-atoms) is only  $17 \overset{\circ}{\underset{3}{\text{Å}}}$ , though one would expect a difference of ca.  $50 \overset{\circ}{\underset{3}{\text{Å}}}$ , as the lipids have two chains. Remarkably 19iPC with the same number of C-atoms as 13cyPC has a larger molecular volume ( $\Delta V = 36 \overset{\circ}{\underset{3}{\text{Å}}}$ ) than the 13cyPC. The difference between 13cyPC and 15cyPC ( $\Delta V = 94 \overset{\circ}{\underset{3}{\text{Å}}}$ ) corresponds to an increase of ca.  $24 \overset{\circ}{\underset{3}{\text{Å}}}$  per  $\text{CH}_2$ -group, a value also observed before for other lipids. 14cyPC, however, is less tightly packed, the increase in molecular volume being  $74 \overset{\circ}{\underset{3}{\text{Å}}}$  compared to 13cyPC. These packing differences between odd- and even-numbered cyPCs are retained in the liquid-crystalline phase. Inspection of Table V also shows that the cyPCs have more condensed liquid-crystalline phases than the corresponding straight-chain or iso-branched phospholipids. This finding is relevant to explain the reduced permeabilities of membranes composed of these lipids as compared to normal n-acyl phospholipids (6,7).

We have also investigated binary mixtures of phospholipids of the iso-branched and  $\omega$ -cyclohexyl fatty acid type with n-acyl phospholipids like DPPC and DSPC. The DSC data show the extreme packing requirements introduced by the  $\omega$ -cyclohexyl rings. The phase diagrams of mixtures of DPPC with iso-branched phospholipids like 17iPC, 18iPC, and 19iPC as well as binary mixtures of 17iPC with 18iPC or 19iPC indicate almost ideal miscibility in the gel and liquid-crystalline phases. This is different for mixtures with  $\omega$ -cyclohexyl phospholipids. Neither mixtures of two different  $\omega$ -cyclohexyl phospholipids like 13cyPC with 14cyPC nor binary mixtures of either one of these phospholipids with 17iPC or DPPC show complete miscibility. Instead the phase diagrams indicate large miscibility gaps in the gel phase (8). The different packing requirements for straight or iso-branched chains and those with  $\omega$ -cyclohexyl rings prevent the formation of solid solutions. This also occurs when two lipids with  $\omega$ -cyclohexyl fatty acids of different length are mixed. The form of the phase diagrams determined by DSC indicate eutectic behavior of these binary mixtures. At certain mixing ratios minima in the transition temperature are observed. Such eutectic behavior has been proposed for other lipid mixtures based on theoretical arguments but has never been observed before experimentally (18). The immiscibility in the gel phase should also have some consequences for the mixing behavior in the liquid-crystalline phase. However, DSC measurements alone can give no direct information on non-ideal behavior in the liquid-crystalline phase.

In natural membranes like the membranes of the thermoacidophilic *B. acillus acidocaldarius* the lipids carrying  $\omega$ -cyclohexyl fatty acids are mixed with hopanoids, pentacyclic ring compounds, which have a cholesterol-like function (3,5). We have investigated the thermotropic behavior of these  $\omega$ -cyclohexyl phospholipids in mixtures with cholesterol by DSC and DSD. The DSC data indicate that addition of cholesterol up to 50 mol% prevents the gel to liquid-crystalline phase transition as observed before for other lipids. Differences are observed in the concentration dependence. More cholesterol is needed to broaden the phase transition than in bilayers of for instance DPPC. Obviously the length of the cholesterol ring system is such that it fits into the

hydrophobic region above the  $\omega$ -cyclohexyl ring at the end of the chains, thus forming a relatively tight arrangement. The  $\omega$ -cyclohexyl rings thus fill the void volume in the middle of the bilayer normally created by the cholesterol side chain. This effect has been proposed before (2). DSD data support this suggestion. The concentration dependence of the mean molecular volume of 13cyPC/cholesterol mixtures is such, that negative deviations from ideal behavior are observed, indicating a condensation effect on the bilayer. This condensation is much stronger than in *n*-acyl phospholipids like DPPC. The particular packing arrangements of lipids with  $\omega$ -cyclohexyl rings with molecules with rigid ring systems like the hopanoids or cholesterol thus lead to a bilayer membrane with optimal interactions between the hydrophobic moieties. For the bacterial membrane this will give an optimal adaption to the great environmental stress it has to withstand.

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