X-Ray Studies on Phospholipid Bilayers. II. Polymorphic Forms of Dipalmitoyl Phosphatidylethanolamine

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Z. Naturforsch. 37c, 1157–1160 (1982); received June 2/August 16, 1982

Phospholipid, Bilayers, X-Ray Diffraction, Dipalmitoyl Phosphatidylethanolamine, Membrane

Oriented films and powder samples of the phospholipid L-α-dipalmitoyl phosphatidylethanolamine (DPPE) were studied by X-ray fiber diffraction techniques. The specimens were photographed under a complete range of hydration at room temperature. Two polymorphic forms were found. One of them was present in oriented films. It showed a close packing of DPPE molecules lying parallel to the normal to the bilayer plane. The other form, observed in the powder samples, had the molecules tilted by about 25°. Both forms were characterized by their unit cell dimensions, space groups, molecular conformations and packing arrangements.

Introduction

In a previous paper, the structural studies of the synthetic phospholipid L-α-dimyristoyl lecithin (DML) by X-ray fiber diffraction methods were reported [1]. It was found that three different types of oriented specimens of DML, photographed under exactly the same conditions including temperature and relative humidity, gave rise to three different types of diffraction patterns. They corresponded to three polymorphic forms of DML, which were described. We have extended these fiber structural studies to synthetic alkyl phosphatidylethanolamines, which have only been studied as single crystals by X-ray diffraction [2, 3].

Oriented films and powder samples of dipalmitoyl phosphatidylethanolamine (DPPE) were studied under a wide range of hydration at a constant temperature of 21 °C ± 2 °C.

Materials and Methods

Synthetic L-α-Dipalmitoyl Phosphatidylethanolamine, A grade, from Calbiochem (lot 902769) was used without further purification. Oriented films were grown from chloroform solutions by slow evaporation at low temperatures and collected on fine metallic rings. Powder samples, taken directly from the original container, were also studied. Both types of specimens were X-ray photographed in humidity controlled cameras as described elsewhere [4]. Flat plate cameras provided with fine glass collimators (Ø = 0.25 mm) and nickel-filtered CuKα radiation were used. Powder samples were also photographed in Debye-Scherrer cameras. The specimens were previously kept for several weeks in closed containers at constant relative humidities of 0, 33, 43, and 86%. The last three humidities were achieved by using saturated salt solutions of MgCl2·6H2O, KSCN and KCl respectively. Water contents of the samples, once at equilibrium, were determined by gravimetry; their densities were measured by flotation. Molecular models were built from CPK space-filling components (1.25 cm = 1 Å).

Results

The most crystalline X-ray diagrams of oriented films, i.e., those that showed the highest number of well defined reflections, were obtained at 33% r.h. They presented about two dozen equatorially orientated reflections and about twenty additional reflections. Fig. 1. X-ray diagram of an oriented film of DPPE equilibrated at 33% r.h. D = 55.5 mm.
distributed along six layer lines. Fig. 1 shows such a pattern. All the observed reflections were consistent with an orthogonal unit cell of dimensions $a = 8.23\ \text{Å}$, $b = 10.18\ \text{Å}$ and $c = 55.75\ \text{Å}$. At 33% r.h. there were 0.6 molecules of water per molecule of DPPE. The theoretical density ($d_t$), considering four molecules of DPPE per cell, was calculated to be $1.00\ \text{g/cm}^3$, which agreed very well with the experimentally determined value ($d_x$) of 1.02 g/cm$^3$. 

The unit cell dimensions increased with humidity without losing its orthogonal character on the whole range going from 0% r.h. to 86% r.h.

Table I presents the unit cell dimensions, degrees of hydration and both theoretical and experimental densities for oriented films of DPPE at each relative humidity.

In order to expose the oriented films to the highest possible hydration they were either cut in small strips or powdered and introduced into 1.5 mm diameter glass capillaries and filled with distilled water. Successive X-ray diagrams were taken for about a week. The results indicated that, besides a decrease in the number and quality of the reflections (after eight days only about half a dozen reflections were observed) the cell dimensions were affected very little. In fact, the major $c$ axis only increased by about 1 Å to a value of nearly 57 Å.

Table I. Unit cell parameters for oriented films of DPPE.

<table>
<thead>
<tr>
<th>Relative humidity [%]</th>
<th>0</th>
<th>33</th>
<th>43</th>
<th>86</th>
</tr>
</thead>
<tbody>
<tr>
<td>$a$ [Å]</td>
<td>8.05±0.02</td>
<td>8.23±0.02</td>
<td>8.32±0.02</td>
<td>8.50±0.05</td>
</tr>
<tr>
<td>$b$ [Å]</td>
<td>9.92±0.02</td>
<td>10.18±0.02</td>
<td>10.24±0.02</td>
<td>10.24±0.05</td>
</tr>
<tr>
<td>$c$ [Å]</td>
<td>55.50±0.01</td>
<td>55.75±0.01</td>
<td>55.98±0.02</td>
<td>56.00±0.02</td>
</tr>
<tr>
<td>H$_2$O/DPPE</td>
<td>0</td>
<td>0.6</td>
<td>2.7</td>
<td>2.9</td>
</tr>
<tr>
<td>$d_t$ [g/cm$^3$]</td>
<td>1.04</td>
<td>1.00</td>
<td>1.04</td>
<td>1.02</td>
</tr>
<tr>
<td>$d_x$ [g/cm$^3$]</td>
<td>1.02</td>
<td>1.02</td>
<td>1.02</td>
<td>1.00</td>
</tr>
</tbody>
</table>

On the other hand, powder specimens of DPPE showed somewhat different results. In fact, samples taken directly from their original container without further treatment were X-ray photographed at 0, 33, 43, and 86% r.h. The most crystalline diagram was also observed at 33% and is shown in Fig. 2. Table II presents a summary of the obtained results.

A comparison of these unit cell dimensions with those obtained with oriented samples under the same conditions of relative humidity and temperature shows some relevant differences. The long $c$ axes, for instance, on the average are about 7 Å shorter than in the oriented films, while the $a$ axes are about 4 Å longer. It was also observed that the lengths of the $c$ axes of powder samples decreased with the increase of hydration, while the $a$ axes showed the opposite effect. The experimental densities, which remained practically constant at 1.05 g/cm$^3$, generally showed good agreement with the theoretical values.

Table II. Unit cell parameters for powder samples of DPPE.

<table>
<thead>
<tr>
<th>Relative humidity [%]</th>
<th>0</th>
<th>33</th>
<th>43</th>
<th>86</th>
</tr>
</thead>
<tbody>
<tr>
<td>$a$ [Å]</td>
<td>12.09±0.04</td>
<td>12.15±0.02</td>
<td>12.57±0.01</td>
<td>13.10±0.02</td>
</tr>
<tr>
<td>$b$ [Å]</td>
<td>7.60±0.03</td>
<td>7.58±0.02</td>
<td>7.56±0.03</td>
<td>7.60±0.02</td>
</tr>
<tr>
<td>$c$ [Å]</td>
<td>49.35±0.05</td>
<td>49.10±0.04</td>
<td>48.00±0.04</td>
<td>47.30±0.03</td>
</tr>
<tr>
<td>H$_2$O/DPPE</td>
<td>0</td>
<td>0.3</td>
<td>0.8</td>
<td>1.0</td>
</tr>
<tr>
<td>$d_t$ [g/cm$^3$]</td>
<td>1.02</td>
<td>1.03</td>
<td>1.03</td>
<td>1.01</td>
</tr>
<tr>
<td>$d_x$ [g/cm$^3$]</td>
<td>1.05</td>
<td>1.05</td>
<td>1.05</td>
<td>1.05</td>
</tr>
</tbody>
</table>
Powder samples of DDPE were also mixed with excess of water as described before. After two months the only noticeable changes compared to the pattern observed at 86% r.h. were a lower number of reflections of less crystallinity. The cell dimensions, in fact, remained practically unchanged.

In order to check whether the observed differences between powder samples and oriented films of DPPE were due to the solvent used to prepare the latter, powder was recrystallized from chloroform. The procedure was the same for growing oriented films except for the time allowed for the evaporation of the solvent, which was shorter. Film-like powder aggregates were obtained. They were X-ray photographed at the same time as the untreated powder samples, at room humidity (60% r.h.). The two patterns were practically the same. Both cell dimensions were $a = 12.57 \text{ Å}$, $b = 7.56 \text{ Å}$, and $c = 48.0 \text{ Å}$. The recrystallized samples were also exposed to excess of water in glass capillaries as described for powder specimens. Again, similar results were obtained.

**Discussion**

From the described results it is possible to conclude that the synthetic phospholipid DPPE presents two polymorphic forms. One of the forms was observed in powder samples — both untreated and recrystallized from chloroform — while the other form was present in oriented films grown from the same solvent. Both forms showed their highest crystallinity at 33% r.h. At this humidity and at a temperature of about 20 °C the powder samples had an orthogonal unit cell of $a = 12.15 \text{ Å}$, $b = 7.58 \text{ Å}$, and $c = 49.10 \text{ Å}$. Under the same conditions, the oriented-film samples also presented an orthogonal cell but the dimensions were $a = 8.23 \text{ Å}$, $b = 10.18 \text{ Å}$, and $c = 55.75 \text{ Å}$. These differences of the two forms of DPPE were more or less kept in a broad range of hydration.

Molecular model studies were carried out to visualize the possible conformations and modes of packing of DPPE in the two polymorphic forms. These studies were based on the conformational restrictions imposed by their unit cell dimensions and the optimization of electrostatic and hydrophobic interactions. They were also based on the electron-density profile calculated for the homologous phospholipid dimyristoyl phosphatidylethanolamine (DMPE). In fact, multilayers of DMPE — which has two methylene groups less than DPPE in its hydrocarbon chains — were prepared and studied similarly to DPPE. The results obtained from both phospholipids were closely related [5].

With respect to the “oriented-film form” the most satisfactory model was the one shown in Fig. 3. This molecular model presents the following features:

a) In each DPPE molecule the hydrocarbon chains are almost parallel, fully extended and very closely packed. The polar groups are oriented almost perpendicular to the length of the hydrophobic chains.

b) The molecules are regularly stacked along the $a$ axis parallel to each other and to the normal to the bilayer plane. Hydrophobic interactions between the hydrocarbon chains and electrostatic attractions between opposite charges of neighboring polar groups are present.

c) Bilayers are formed through molecular interactions at the ends of the hydrocarbon chains in such a way that both monolayers are related by two-fold rotation axes normal to the $ac$ plane.

d) Inter bilayer electrostatic interactions might be present as opposite charges of the neighboring polar groups face each other. This, perhaps, explains the negligible changes of the $c$ axes under variation of the hydration.

e) The 10.18 Å vertical $b$ axis of DPPE at 33% r.h. in this form implies a “sandwich” type of packing, i.e., if the bilayer shown in Fig. 3 is numbered the first, the third bilayer will be located 10.18 Å exactly above it. The second bilayer, which lies somewhere in a plane along the $b$ axis is equally oriented like the other two but is somewhat shifted along the $a$ axis. Additional hydrophobic and electrostatic interactions with the neighboring first

![Fig. 3. Bidimensional molecular model of DPPE in the "oriented-film form"](image-url)
and third bilayers might also develop in this packing arrangement.

f) The only elements of symmetry present in this model are the two-fold rotation axes along $b$. The space group is, therefore, P2. The unit cell is pseudo C-centered monoclinic with orthogonal axes and contains four DPPE molecules. The observed reflections are consistent with this space group.

Molecular models were also built for the DPPE "powder form". The most satisfactory one was that presented in Fig. 4.

The major crystallographic axis $c$ is about 49 Å in the "powder form" at 33% r.h. This length, which corresponds to the bilayer width, is nearly 7 Å shorter than in the "oriented-film form". This necessarily implies that the molecules are not oriented perpendicularly with respect to the bilayer plane but are inclined. The angle of tilt with respect to the normal to the bilayer was found from model calculations to be nearly 25°. About the same angle was calculated from the expression $S \cos \phi = 2 \Sigma$. $S$ is the molecular area at the bilayer surface, $\phi$ the angle of tilt and $\Sigma$ the cross section of each hydrocarbon chain, which is 20.4 Å$^2$ when they are perpendicular to the bilayer surface [6]. The value of $S$ could be calculated from the relation $S = \frac{2M \bar{v}}{NC}$, in which $M$ is the molecular weight of the lipid, $\bar{v}$ its specific volume, $N$ Avogadro's Number and $c$, the crystallographic axis. This angle of tilt $\phi$ of DPPE molecules was found to increase with hydration in the "powder form", reaching nearly 30° at 86% r.h. while in the "oriented-film form" it remained practically unchanged.

Apart from the inclination of the molecules in the "powder form", their hydrocarbon chains are more separated and the polar groups are more tilted as compared to the "oriented-film form". However, the "powder form" also has a pseudo C-centred, orthogonal unit cell with monoclinic P2 symmetry and four molecules per unit cell.

Polymorphism has also been reported for a racemic crystalline DPPE [7]. Three microcrystalline polymorphs were identified by electron diffraction studies. The major form had cell dimensions $a = 7.76$ Å, $b = 10.03$ Å, and $c = 56.0$ Å, which are very similar to those obtained for the "oriented-film form". A previous paper reported two polymorphic forms for DPPE, but they were obtained from different solvents; both had their long axes (the only reported) near 56 Å [8]. Given the molecular flexibility of phospholipids, it is now evident that slight changes in the way of preparing or even handling the specimens, can result in different conformation and/or packing arrangements. This property, of course, should be understood on the basis of the role played by phospholipids in the phenomenon of transport across membranes, where a high degree of molecular flexibility of the lipid matrix is required.

Acknowledgements

The authors acknowledge gratefully the support of research grants from Vicerrectoria de Investigación, Universidad de Concepción (2.15.40) and Volkswagen Foundation (11-1744), and to Dra. H. Cid for helpful discussions on the manuscript.