Structure of the Ripple Phase $P_{\beta'}$ in Hydrated Phosphatidylcholine Multimembranes

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Small- and wide-angle x-ray-diffraction measurements on the $P_{\beta'}$ phase of fully hydrated *DL*-β,γ-dipalmitoyl-α-phosphatidylcholine were performed in highly oriented lipid-water multibilayer samples. The data extend previous results on the ripple structure of this phase, as obtained by x-ray diffraction and scanning tunneling microscopy, and for the first time provide evidence that the $L_{\beta'} \rightarrow P_{\beta'}$ phase transition is characterized by a change in hydrocarbon-chain tilt direction, from the orthorhombic *b* into the *a* direction.

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Since the basic studies on lecithin structure by Luzzati and co-workers,[1] many structural investigations have been performed on the different phases exhibited by aqueous dispersions of lipids and in particular on the lamellar lyotropic liquid-crystal phases consisting of hydrated lipid bilayers. In fact, great interest has arisen in recent years concerning the exact physical nature of these phases. In particular, very recently two outstanding experiments were completed by using scanning tunneling microscopy[2] (STM) and high-resolution x-ray diffraction[3] on these systems, which are of fundamental interest from both physical and biological points of view. Moreover, a model was recently developed,[4] in the mean-field approximation, which accounts for many of the observed physical properties of the lamellar lyotropic liquid-crystal phases. In the STM experiment the three-dimensional contours of the ripple phase $P_{\beta'}$ of dimyristoylphosphatidylcholine (DMPC) were imaged for the first time with a very high spatial resolution, namely, better than a nanometer. A ripple wavelength of 13 nm and an amplitude of 4.5 nm were obtained. This information constitutes an elegant confirmation, and refinement, of what was known before by other techniques. But in addition to that, a puzzling unforeseen result was obtained: A fine structure of apparently periodic nature crosses the ripples roughly orthogonally to the ripple direction. The authors conclude that it is difficult to say whether these additional ripples are related to the molecular structure of the $P_{\beta'}$ phase or whether it is some unknown artifact. In order to answer this question it appears crucial to know more about the hydrocarbon-lattice orientation and tilt direction. In new x-ray-diffraction experiments[3] on the similar lipid DMPC, it was shown that the $L_{\beta'}$ phase consists in fact of three phases, of 2D nature, each distinguished by the direction of the chain tilt with respect to the 2D bond direction.

This paper reports a structural investigation by x-ray diffraction of the ripple phase $P_{\beta'}$ of fully hydrated *DL*-β,γ-dipalmitoyl-α-phosphatidylcholine (DPPC) highly oriented samples. The main result obtained is the observation, for the first time, that the $L_{\beta'}$-$P_{\beta'}$ phase transition is characterized by a 30° (modulo 60°) change of the lattice tilt direction, in addition to the well-known lattice symmetry transition from orthorhombic to hexagonal. This observation appears to support the hypothesis that the secondary ripple structure in $P_{\beta'}$ seen by STM (Ref. 2) is more related to a real physical fact than to artifacts. We used oriented lipid-water multibilayer samples by improving our orientation method of multisandwiching.[5,6]

Fluka DPPC of 99.8% purity was added to the same amount of distilled water and stored for one week at 4 °C in order to allow for equilibrium hydration. A 1-mg droplet of the suspension is laid on a 6-μm Mylar sheet of 2 cm diameter, all on a flat support of controlled 60 °C temperature. Rapid covering by another Mylar sheet is followed by pressing this with a preheated glass slide by $\simeq$1 N until the suspension fills an area of 1 cm^2. After repeating this procedure 40 times the stack of alternating Mylar and suspension is $\approx$0.8 mm thick. The lipid membranes have now oriented themselves parallel to the many Mylar surfaces which are the limiting walls in the steric interaction of the bilayers. The shear gradient created by the flux of material during pressing is another alignment parameter besides the water and temperature distribution. At room temperature the received stack is stiff enough to be cut into stripes of 1 by 8 mm, each of which is sealed in a x-ray quartz capillary containing a droplet of water (without any direct contact to the sample), in order to guarantee 100% saturated atmosphere (which might account for biological relevance).

X-ray diffraction is carried out with a pinhole-type camera (Kiessig) resolving 0.3- to 30-nm reflections from Ni-filtered Cu $K\alpha$ radiation. The primary beam of 0.3 mm diameter crosses the sample parallel to the Mylar films. Taken from a standard fine-focus x-ray tube, the diffracted intensities were recorded simultaneously on two planar films for small and wide angles within a few hours. Several samples were inspected at rising and falling temperatures between 15 and 46 °C, accompanied

by the well-known changes in the layer repeat distance, between 6.6 nm at 25 °C and 7.1 nm at 41 °C, with reproducibility of better than 0.2 nm from sample to sample. The one with best orientation had a mosaic spread of less than 1.5°.

Small-angle diffraction patterns of the $P_{\beta'}$ phase at 41 °C reveal the two-dimensionally resolved ripple structure by several satellite spots besides the meridian [Fig. 1(a), with reduced brightness inside the inset]. The ripple period is 20 nm at a layer period of 7.1 nm, which is of the same order of magnitude as the 16.5-nm value obtained by Alecio, Miller, and Watts[7] and identical to our earlier estimation.[6] Similar to the first two-dimensional ripple pattern of Alecio, Miller, and Watts, we find a residual intensity of 6.9-nm spacing on the meridian, as clearly separated in the third-layer line of Fig. 1(a) [the corresponding ($\bar{3}0$) reflection is missing due to the inclined sample]. Although the (20) reflection of $P_{\beta'}$ is present, (30) is absent. The ⟨32⟩ reflections can only be detected on the densitometric trace of the third-layer line (parallel to the equator) in Fig. 1(c). Figure 1(b) indicates all detected reflections (solid circles). Within the resolution of the spot sizes the two-dimensional lattice of the ripple structure has orthorhombic symmetry ($\gamma = 90° \pm 2°$).

Figure 2(a) reports the wide-angle x-ray-diffraction patterns obtained from the $L_{\beta'}$ and $P_{\beta'}$ phases. For the first time, eight separated first-order wide-angle reflections have been observed from the $P_{\beta'}$ phase of DPPC, four above and four below the equator. Six reflections, two on the equator, occur in the $L_{\beta'}$ phase as reported earlier (only the symmetric left and right halves are shown). This clearly indicates that the related $L_{\beta'}$-$P_{\beta'}$ phase transition is characterized by a change in the tilt direction of the hydrocarbon chains with respect to the lattice orientation in the membrane plane. The superimposed parallel density traces along the meridional direction of the wide-angle diagram result in Figs. 2(b) and 2(c) where the maxima of the envelopes determine the positions of the separated reflections, which in the $L_{\beta'}$ phase all have equal weights, while in the $P_{\beta'}$ case the outer reflections have half the intensity of the inner ones according to their multiplicity as assigned by indices. The splitting angles θ are 13° and 26° in the $P_{\beta'}$ phase at 41 °C. The $L_{\beta'}$ phase at 25 °C gives $\theta_1 = 0°$ and $\theta_2 = 24.5°$. As discussed earlier,[5] the splitting angles relate to the hydrocarbon-chain inclination angle φ according to the rules of rotation patterns of misaligned single crystals. All the first-order reflections of the hydrocarbon lattice in the $P_{\beta'}$ phase have an equal spacing of 4.2 Å, and thus hexagonal symmetry. In the $L_{\beta'}$ phase the (200) reflections on the equator relate to 4.23 Å while the other ones correspond to 4.10 Å. This clearly indicates an orthorhombic symmetry, which differs by 3% from being hexagonal. Although the phase transition from $L_{\beta'}$ to $P_{\beta'}$ undergoes a change in lattice symmetry from orthorhombic to hexagonal, we keep the equivalent orthorhombic indexing for simplicity. The choice of indexing will become more evident in the following.

The c axis of the hydrocarbon lattice is parallel with the chain axis and the unit-cell length is two c bonds. The splitting geometry of the inclined hydrocarbon lattice depends on the direction of the tilt axis in the a^*-b^* plane of the reciprocal lattice [Fig. 3(a)]. This axis spans an angle ψ with a^*. The projections of the first-order reciprocal-lattice ($hk0$) points along the tilt axes result in different numbers and weights of reciprocal-lattice points and positions on the projection axis depend-

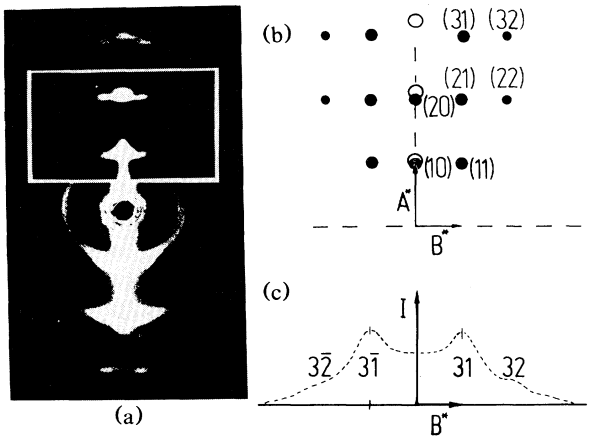

FIG. 1. Small-angle x-ray diffraction of the $P_{\beta'}$ phase of fully hydrated DPPC. (a) Oriented diffraction pattern of ripples of 20-nm period. (b) Detected reflections of two-dimensional orthorhombic symmetry (solid circles). Residual $L_{\beta'}$ period is shown by open circles. (c) Densitometric trace along the third-layer line.

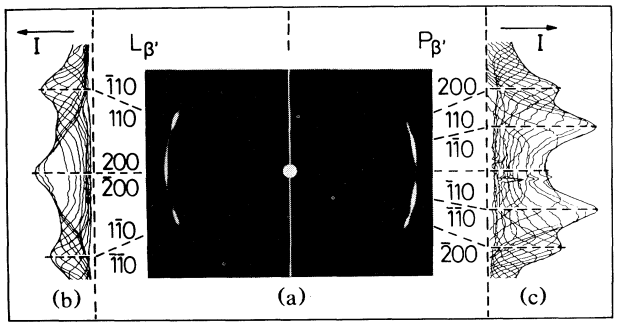

FIG. 2. Wide-angle x-ray diffraction of oriented DPPC. (a) Left: $L_{\beta'}$ phase with three arcs (of six); right: $P_{\beta'}$ phase with four arcs (of eight). Only half of the symmetric patterns are shown. (b) Multiple vertical densitometric traces of separated CH_2-lattice reflections due to inclined chains in $L_{\beta'}$. (c) Scans of $P_{\beta'}$ with different weights in envelope. All traces are taken from the indicated halves of the patterns, but indices refer to the complete set.

ing on ψ. They give the weights and number (modulo 2) of observable reflections when the lattice is tilted with respect to the membrane normal and then rotated about it. The two projections along a^* and b^* of Fig. 3(a) correspond to six equal reflections of the $L_{\beta'}$ phase (vertical line) and the eight $P_{\beta'}$ reflections of different weights (top line). Their splitting geometry is given in Fig. 3(b) in the (Ewald) plane of reflection, where the equator is parallel to the average bilayer orientation. If the hydrocarbon lattice were untilted, the chain axis c^* would be vertical and coincide with the membrane normal. The clockwise inclination of the lattice about the axis perpendicular to the drawing plane creates the tilted directions c_t^*, a_t^* and the reciprocal-lattice points on the inclined dot-dashed line. The crosses are out of the reflection plane, but as the reciprocal lattice of this tilted arrangement is to be rotated about the (vertical) bilayer normal (as the sample has no preferential orientation about this axis), this causes the crossed positions to meet the plane on the dashed circle (open symbols). Clearly, the splitting angles θ_n are related to the tilt angle φ of the CH_2 lattice, depending on the orientation of the tilt axis ψ, as

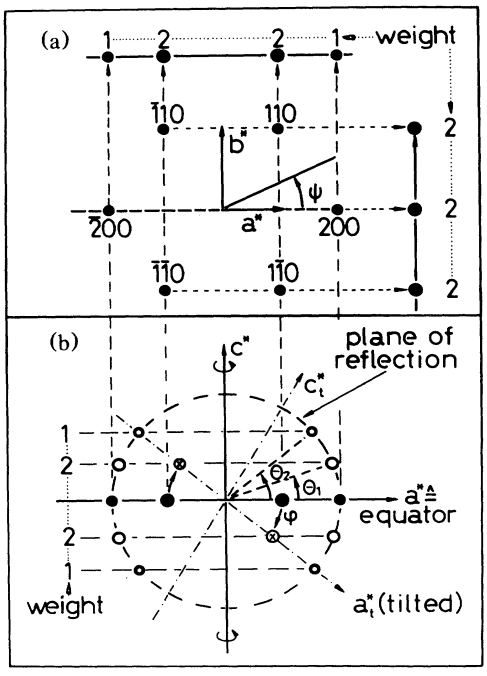

FIG. 3. Reciprocal CH_2-lattice inclination geometry. (a) Orthorhombic (face-centered) lattice points of a^*-b^* plane with projections along a^* and b^* (the corresponding tilt axes of $L_{\beta'}$ and $P_{\beta'}$) determine number and weights of expected reflections, depending on the angle ψ between tilt axis and a^*. (b) Ewald plane of reflection with projection along b^* axes on equator (solid circles, $P_{\beta'}$) inclined by φ and finally rotated about c^* gives splitting angles θ_1, θ_2 and positions of observed reflections (open circles).

given earlier:[5]

$$\sin\theta_n = (\sin\varphi)\sin(\psi \pm \alpha), \quad (1)$$

with $\alpha = \arccos(d_{110}/2d_{200})$ or $\alpha = 0$. In hexagonal cases as in Fig. 3(b) ($P_{\beta'}$), α is 60° or 0. α solely accounts for an "orthorhombic distortion" from hexagonal symmetry. If $\psi = 0$, Eq. (1) yields six solutions; if $\psi = 30°$ or 90°, eight reflections occur, and twelve in general.

All three cases were observed in a slightly different situation (DMPC instead of DPPC) in three new lyotropic phases of $P_{\beta'}$ in Ref. 3, namely, $L_{\beta F}$, $L_{\beta L}$, and $L_{\beta I}$. There is a coincidence in the tilt direction of the high-water-content $L_{\beta I}$ and our $L_{\beta'}$ phase, while $L_{\beta F}$ corresponds to the $P_{\beta'}$ tilt (eight reflections): In $L_{\beta'}$ adjacent chains are inclined towards next-nearest neighbors (b direction), and in $P_{\beta'}$ towards the gap between two next-nearest neighbors. The tilt angle φ of the hydrocarbon lattice in the $P_{\beta'}$ phase equals the larger splitting angle $\theta_2 = 26°$, which is clear from Fig. 3 or Eq. (1) [$\sin(\psi + \alpha) = 1$].

In the $L_{\beta'}$ phase all six reflections are of similar weight as $\psi = 0$. With the equatorial spacing $d_{200} = 4.23$ Å and $d_{110} = 4.10$ Å, the splitting angle $\theta_2 = 24.5°$ yields the tilt angle $\varphi = 28°$, by applying Eq. (1). Our tilt angles are in strikingly good agreement with Ref. 3, where 27.4° and 29.8° are reported for the respective phases $L_{\beta F}$ and $L_{\beta I}$ in DMPC. (A periodically undulated membrane cannot have *one* tilt angle between the chain axis and the locally changing surface normal. The angle is to be defined between the c axis of the hydrocarbon lattice and the average bilayer normal.) It is remarkable that the tilt direction and tilt angle of the $P_{\beta'}$ phase of DPPC are those of the lower-water-content $L_{\beta'}$ phase of DMPC. The enlarged surface of rippled bilayers provides a larger (local) surface area per lipid headgroup but there is a reduction of this area by a smaller (global) tilt angle φ. Following Smith *et al.*[3] we suspect a new directional type of dipole-dipole headgroup interaction.

Referring to the ripple structure, if we assume for a moment that the secondary ripples in Ref. 2 are not an artifact, the two wave vectors exist simultaneously and are mutually orthogonal. Obviously the tilt direction cannot coincide with both classes of wave vectors at the same time. We felt encouraged to refer to our former models, which discussed cases with the tilt direction of CH_2 chains perpendicular to the wave vector of the ripples.[6] These models, on the basis of limiting surface net planes, have the advantage of symmetric shape and of a global CH_2 chain tilt with all chains parallel to each other as in the $L_{\beta'}$ phase, but of uniform thickness of the bilayer. If the normal projection of the rippled membrane were not of uniform electron density along the wave vector as in the well-accepted (asymmetric) model of Tardieu, Luzzati, and Reman,[1] the periodic changes in thickness should be observable in equatorial (01) reflections from the well-oriented small-angle x-ray patterns, but they are not present in our samples nor report-

ed by Alecio, Miller, and Watts.[7] Nevertheless, Mortensen *et al.*[8] found a thermal dependence of [presumably (01) and (02)] neutron-diffraction intensities of unoriented DMPC—17-wt%-H_2O samples in the range from 12.3 to 14.2 nm, without mentioning (hk) reflections. Furthermore, the Zasadzinski images reveal a pitch of the ripple slope of 40° to 60°. If CH_2 chains were uniformly tilted by 30° in the plane of the wave vector and the average membrane normal, as proposed by Tardieu, Lezzati, and Reman,[1] they would stand almost parallel to the membrane surface of either one of the two slopes depending on whether the tilt goes to left or right. This is most improbable. Since the uniform CH_2 chain tilt of 26° in the a direction cannot be neglected, we propose that the tilt direction (the crystallographic a axis) is roughly perpendicular to the main ripple wave vector, and more likely aligned with the wave vector of the secondary (weaker) undulations. Depending on the preparation techniques and the "history" of the lipid samples different characteristics of the $P_{\beta'}$ phase seemingly develop, which account for some contradictions in the literature.

The following conclusions can be derived.

(i) In saturated humidity, well-oriented diffraction patterns of the $P_{\beta'}$ phase in DPPC with several satellite reflections of the type (hk) confirm the occurrence of orthorhombic symmetry in the 2D ripple structure of this phase. The obtained value of 20 nm for the ripple wavelength compares well with the values previously obtained. The CH_2 lattice has a uniform tilt angle. The (main) ripple wave vector is most likely not parallel with the tilt direction of the CH_2 lattice.

(ii) Evidence was obtained for the first time that at the $L_{\beta'}$-$P_{\beta'}$ phase transition there is a change in the CH_2-lattice tilt direction from the orthorhombic b direction into the a direction corresponding to hexagonal ⟨110⟩ directions. The new findings support the possibly physical nature of the secondary ripple structure recently observed in scanning tunneling microscopy.

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