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X-ray diffraction studies of the structural organisation of prolamellar bodies isolated from *Zea mays*

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Abstract Transmission electron microscopy (TEM) indicates that maize prolamellar bodies (PLBs) are built up of tetrapodal units based on a highly convoluted but continuous lipid bilayer exhibiting diamond cubic (Fd3m) symmetry. Such lattices are often described in terms of infinite periodic minimal surfaces (IMPS) exhibiting zero net curvature and dividing the system into two identical subvolumes. If so, X-ray diffraction measurements would be expected to index on a double-diamond (Pn3m) lattice with a unit cell length half that of the TEM lattice. Our measurements index on a Fd3m lattice with a similar repeat distance to the TEM images. The PLB membrane is thus inherently asymmetric, probably as the result of the distribution of membrane protein.

Key words: Prolamellar body; X-ray diffraction; Schwartz surface; Infinite minimal periodic surface; Membrane organisation

1. Introduction

Analysis of thin-section electron micrographs indicates that the majority of PLBs are built up of tetrapodal units based on a highly convoluted but continuous lipid bilayer assembled into a network of interconnected tubes with the units occupying analogous positions to the tetravalent carbon atoms of the diamond lattice [1]. The lipid bilayer thus divides the structure into two continuous interlocking aqueous compartments. Bicontinuous cubic lattices of this type have been reported for a variety of surfactant, membrane lipid and block co-polymer systems [2–6] but are normally thought to be extremely rare in living systems. Landh [7], however, has recently suggested that they may be of more importance than previously recognised and play a role in the organisation of the smooth endoplasmic reticulum, inner mitochondrial and various plasma and endosomal membrane systems.

The possibility of dividing fluid systems into two non-intersecting subvolumes separated by a sheet consisting of oriented amphiphilic molecules forming an IMPS was first recognised by Scriven [3]. This suggestion led to considerable debate as to whether the structure of bicontinuous cubic phases in general, and PLBs in particular, can be described in terms of such surfaces. Larsson et al. [4] pointed out that the X-ray patterns for the viscous isotropic (cubic) form of glycerol monooleate (GMO) indicated a body centred cubic lattice in which the lipid bilayers might be arranged as in an octahedral Schwartz P-surface. Longley and McIntosh [5] subsequently showed that in the presence of excess water GMO yields an X-ray diffraction pattern indexing on a primitive cubic lattice (Pn3 or Pn3m). They interpreted this in terms of two interlinked networks of water, each having diamond symmetry, separated by a continuous lipid bilayer following a tetrahedral Schwartz D-surface. The presence of two such identical lattices, as they pointed out, results in an overall structure which is described by a primitive cubic lattice with a unit cell length half of that of the parent diamond structures. Linstedt and Lilljenberg [8] have suggested that the PLB membrane may also be an example of a Schwartz D-surface.

The aims of this study were two-fold. First to determine whether PLBs isolated from *Zea mays* were sufficiently well-organised to give rise to an X-ray diffraction pattern. If so, to then determine the relationship between the repeating unit giving rise to this pattern and that seen by TEM, with the view to determining whether the lipid bilayer in PLB can be appropriately described in terms of a Schwartz D-surface.

2. Materials and methods

Maize seedlings (*Zea mays* L. cv. LG 20.80 Alarik, Weibulls, Landskrona, Sweden) were grown in a fertilised peat and soil mixture for 8–9 days at 27°C. The leaves were first surface-sterilised in 2% sodium hypochlorite, 0.25% Tween 80 and 1% NaCl and then thoroughly rinsed with distilled water. The PLB membranes were isolated from a crude membrane pellet by sucrose gradient centrifugation as described by Widell-Wigge and Selstam [9] with the sole modification that the isolation medium was supplemented with 0.2% BSA and 5 mM cysteine. Isolated PLB membranes were stored at −20°C in pH 7.6 buffer containing 20 mM tricine, 10 mM HEPES, 50 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA and 0.3 mM NADPH. Thawed samples were prepared for X-ray diffraction measurements first by dilution with a large excess of pH 7.6 buffer containing 20 mM tricine, 10 mM HEPES, 50 mM KCl, 0.3 mM NADPH to reduce the sucrose content to close to zero. The PLBs were then re-concentrated by centrifugation at $46\,000 \times g$ for 10 min to yield a final concentration of about 10 mg lipid per 100 μl.

The pelleted membranes were then transferred to a 1 mm thick sample cell with thin mica windows which was mounted directly on the heating element of a modified THM 600 thermally controlled microscope stage (Linkam Tadworth UK) connected to a liquid N_2 pump. Sample temperature was monitored using a thermocouple located in the sample cell. Small angle X-ray scattering (SAXS) measurements were made on Station 2.1 of the Daresbury synchrotron using a standard Daresbury quadrant detector and a camera length of 3.5 m. The system was calibrated using the first nine orders of wet rat-tail collagen (repeat distance 67 nm) and the data acquisition system was set to yield a series of consecutive 5 s duration diffraction patterns.

A small aliquot of the PLB sample prepared for the diffraction measurements was fixed by suspension in 0.1 M sodium cacodylate buffer (pH 7.4) containing 2.5% glutaraldehyde, stained with osmium and sectioned for TEM measurements. TEM measurements were made using a Philips EM301G electron microscope calibrated using a standard cross-grating replica.

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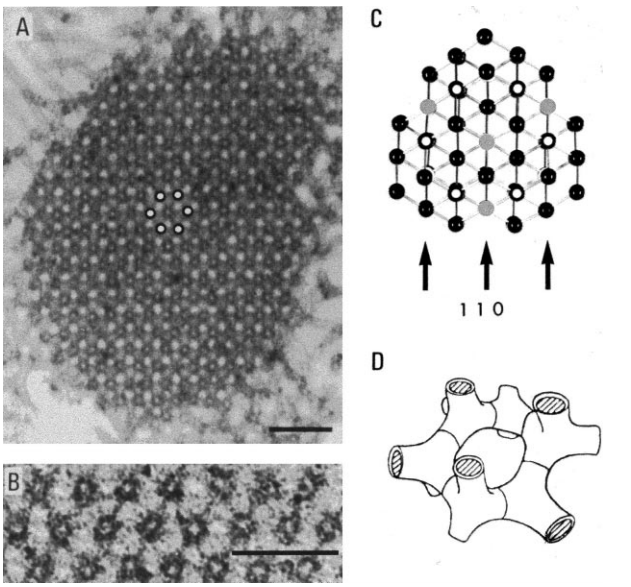

Fig. 1. A, B: Cross-sectional views of PLBs isolated from etioplasts of dark grown *Zea mays* seedlings. The structure is built up of tetrapodal units made up from a highly convoluted continuous lipid bilayer assembled to form a diamond cubic lattice. The hexagonal pattern is created by intersecting sets of 110 and 220 planes of this lattice viewed along the 11 axis of the unit cell as illustrated in C. A diagrammatic representation of a set of six interconnected tetrapodal units, is shown in D. The sections are cut parallel to the 111 plane and show the heavily stained arms of the set of tetrapodal units projecting perpendicularly from this plane (highlighted in A and C). The corresponding set of units with arms pointing vertically down into the lattice are lightly stained. The third set of units making up the hexagonal pattern are associated with lower 111 planes. They are absent in A but visible in the slightly thicker section shown in B. The magnification bars correspond to 100 nm.

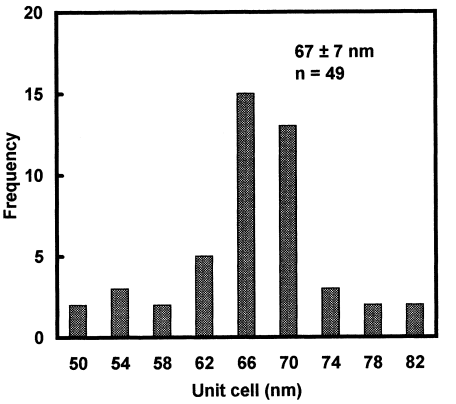

Fig. 2. Histogram showing the unit cell sizes of 49 different PLBs measured from electron micrographs of the type shown in Fig. 1. The unit cell sizes were estimated on the basis that the spacing of the 110 planes forming the hexagonal structures was $a/\sqrt{2}$. Every PLB in a given field was examined in turn and measurements made on those PLBs suitably oriented to yield cross-sectional views showing hexagonal symmetry.

3. Results and discussion

TEM electron micrographs of PLB isolated from dark grown maize (*Zea mays*) seedlings chosen to reveal the hexagonal arrays typical of the 11 plane of the diamond (Fd3m) lattice making up the PLB are presented in Fig. 1A and B. A diagrammatic representation of a group of six tetrapodal units of the type making up the PLB is shown in Fig. 1D. The fact that these units occupy analogous positions to those of the carbon atoms of the diamond lattice is readily apparent on reference to the model of the diamond lattice shown in Fig. 1C.

A major problem encountered in diffraction measurements performed on PLB is the inherent heterogeneity of their unit cell dimensions. This reflects the fact that the population of leaf cells within the etiolated seedlings from which the PLBs are isolated are of differing age and hence of slightly different stages of development. The PLBs in the samples used in this study, as indicated by the measurements shown in Fig. 2, were characterised by a range of diamond cubic unit cell lengths with a mean value of a=67 nm and a standard deviation of 7 nm. A typical PLB of about 1 μm diameter contains 2000–3000 of such units.

A typical small angle X-ray scattering (SAXS) pattern is shown in the upper trace of Fig. 3. It consists of three reasonably sharp maxima located at $S = 0.0251$, 0.0413, and 0.0561 nm^{-1} (d= 39.4, 24.2, and 17.8 nm) together with two much broader maxima centred at about $S = 0.10$ and 0.16 nm^{-1} (d = 10.0 and 6.25 nm), originating from the PLB. These maxima are superimposed on a non-coherent scattering background originating from the suspension medium and membrane debris that co-sediments with the PLB. Dilution measurements showed that the position of the maxima were independent of sample concentration confirming that they reflected PLB organisation.

Baseline correction for the diffraction component of the total pattern was carried out using a novel freeze-subtraction technique. The SAXS pattern of PLB samples was continuously monitored as the sample was cooled from 20° to −25°C at a rate of 5°C min^{-1}. The SAXS pattern remained essentially unchanged until the supercooled samples froze at about −10°C. Lorentz-corrected patterns for unfrozen and frozen samples are shown as the upper two traces in Fig. 3. They clearly consist of two essentially independent components; a highly variable component in the low angle region and a more constant component at wider angles.

A particularly interesting feature of these measurements, as

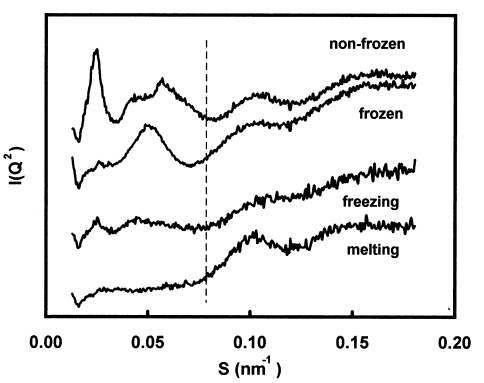

Fig. 3. Typical Lorentz-corrected SAXS patterns of *Zea mays* PLB preparations measured immediately prior to freezing (−5°C), shortly after freezing (−15°C), during the freezing process (−10°C) and at the point of remelting (0°C). The dashed line separates the regions of the pattern arising from diffraction by the PLB lattice and scattering from the tetrapodal units making up this lattice. See text for detailed explanation.

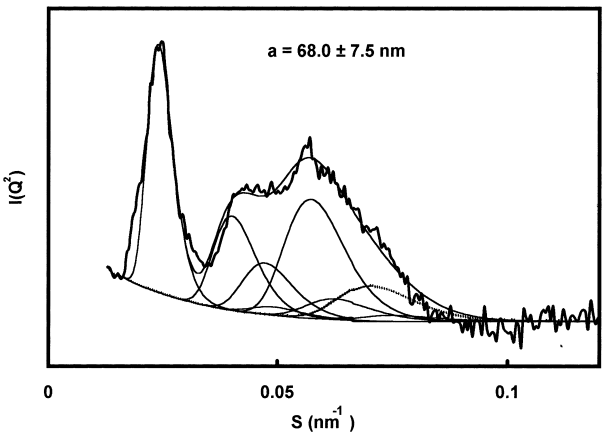

Fig. 4. PLB diffraction pattern calculated as the difference between the SAXS patterns for the non-frozen sample and that collected during sample melting shown in Fig. 3. The pattern is modelled as a set of Gaussian curves centred on the main diffraction planes of a diamond cubic lattice. The small deviation from linearity of the baseline in the narrow angle region reflects changes in the non-coherent scattering background on freeze-thawing.

illustrated in the third trace in Fig. 3, is that the variable component is almost completely eliminated in the short time period (10–20 s) over which freezing takes place. We attribute this to a transitory suppression of those maxima associated with the diffraction pattern of the quasi-crystalline lattice of the PLB brought about by the migration of water from the PLB to the developing ice-front. This perturbation of the long-range order of the PLB has relatively little effect on the wider angle maxima associated with the X-ray scattering pattern of the individual tetrapodal units from which the PLBs are constructed or the non-coherent scattering background and they remain essentially unchanged. Local temperature heterogeneities in the rapidly freezing sample mean that the diffraction components are not completely eliminated in the freezing process. If, however, the frozen sample is reheated, a similar but more complete loss of the diffraction components is seen as the ice in the sample melts at 0°C and water migrates back into the PLB. This is illustrated in the lower trace in Fig. 3. In this case, the diffraction pattern is effectively completely eliminated while the wider angle X-ray scattering and non-coherent scattering components again remain unchanged.

The difference pattern obtained by subtraction of the SAXS pattern for the melting sample from that of the initially unfrozen sample shown in Fig. 4 can, therefore, be attributed to diffraction arising from the internal lattice of the PLB. This pattern, as illustrated in the figure, can be represented as the sum of a set of Gaussian components indexing in the anticipated sequence for a diamond cubic (Fd3m) structure $a/\sqrt{3}$, $a/\sqrt{8}$, $a/\sqrt{11}$, $a\sqrt{12}$, $a/\sqrt{16}$, $a/\sqrt{19}$, $a/\sqrt{24}$, $a/\sqrt{27}$, ... [10] with a mean unit cell length of a=68.0 nm and a standard deviation of 7.5 nm. The choice of Gaussian components reflects the observed distribution of unit cell dimensions shown in Fig. 2. In the absence of knowledge of the form factor of the repeating unit, all contributions of different unit cell lengths within this distribution have been allocated equal weight. Apart from some minor differences in the region of the $a/\sqrt{3}$ maximum the agreement with experiment is extremely good.

The close agreement of the SAXS and TEM results in terms of both the mean unit cell length and observed standard deviation provides a high degree of confidence that the basic structure of maize PLB is best described in terms of a diamond cubic structure. The existence of a sharp diffraction maximum at about 40 nm effectively eliminates the possibility that the structure can be described as a Schwartz D-surface as the unit cell length of the corresponding double-diamond (Pn3m) lattice would be expected to be half that of the parent diamond cubic structure seen by TEM.

The protein composition of PLB is extremely simple with protochlorophyllide oxidoreductase (PCOR) by far the dominant species [11,12]. PCOR is known to be membrane-associated although its method of attachment is still unclear [12,13]. The deviation of the lipid bilayer in PLB from the Schwartz D-surface seen in model lipid systems probably reflects a breakdown in symmetry as the result of an asymmetric distribution of this protein with respect to the lipid bilayer resulting in changes in electron density distribution across the lipid bilayer and/or the relative dimensions of the two subvolumes of the lattice. Our measurements provide no information on the net curvature of the PLB membrane but it is likely that it is characterised by a constant non-zero curvature and constitutes one of the family of unbalanced constant potential energy surfaces described by Landh [8].

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