FORMATION OF CRYSTALLINE ARRAYS OF CHLOROPHYLL *a/b* – LIGHT-HARVESTING PROTEIN BY MEMBRANE RECONSTITUTION

JADE LI
Department of Biochemistry, Columbia University, New York, New York 10032 U.S.A.

CAROL HOLLINGSHEAD
Rosenstiel Basic Medical Science Research Center, Brandeis University, Waltham, Massachusetts 02254 U.S.A.

ABSTRACT The structure of the major protein constituent of photosynthetic membranes in higher plants, the chlorophyll *a/b*-light harvesting complex (LHC), was studied by x-ray diffraction and electron microscopy. The LHC was purified from Triton X-100 solubilized thylakoid membranes of the pea, and contained 6 mol of chlorophylls *a* and *b* per mole of a polypeptide of 27,000 molecular weight. X-ray diffraction showed that in the presence of 10 mM $MgCl_2$, purified LHC forms planar aggregates that stack with a period of 51 Å. Within each layer, LHC molecules pack with a center-to-center distance of 85 Å but without long-range order. However, when LHC is incorporated into single-walled vesicles of plant lecithin, the addition of NaCl above 10 mM, or $MgCl_2$ above 2 mM, led to the formation of plaques of hexagonal lattices, with a lattice constant of 125 Å. The large domain size and high degree of order in the plane of the membrane are evident from the sharp lattice lines observed to 7 Å resolution on the equator of the x-ray pattern. Freeze-fracture electron micrographs demonstrated an aligned stacking of the lattices in adjacent membranes, resulting in crystallinity in the third dimension over short distances. Micrographs of negatively stained membranes revealed a hexagonal lattice of the same lattice constant, formed by surface-exposed parts of the LHC molecules which are probably responsible for the ordered stacking of lattices. In both the LHC aggregates and in the reconstituted membrane lattices the diffracted x-ray intensities at 10-Å spacing on the equator indicate that the LHC molecule contains paralled α-helices or β-sheets that are oriented perpendicular to the planar arrays.

INTRODUCTION

Roughly half of the protein mass of the photosynthetic membranes in higher plants and green algae is present in one species of integral membrane protein, the light-harvesting chlorophyll *a/b* protein (LHC). This is a noncovalent but stoichiometric association of specific membrane polypeptide(s) with both chlorophylls *a* and *b* (Thornber et al., 1967; Thornber, 1975, 1979). It organizes one-half of the total cholorphylls in these membranes into complexes that serve as the major antennae for collecting solar energy to drive photosynthesis (Anderson, 1975). Transfer of excitation energy from the antenna to photosystem II leads to oxygen evolution and the reduction of certain intermediates to start the downhill electron transport coupled to ATP production. Part of the energy is also distributed to photosystem I, responsible for NADP reduction.

Thylakoid membranes can be solubilized by a number of detergents under mild conditions that preserve the absorption, fluorescence and circular dichroic characteristics of LHC in the membrane (Anderson, 1975). Under nondissociating conditions the solubilized LHC appears in electrophoresis as a green band of apparent mol wt 29,000. In this form, LHC has been identified as a major constituent of all chlorophyll *b*-containing membranes (Anderson and Levine, 1974; Chua et al., 1975; Giddings et al., 1980). Thornber (1975) calculated that it accounts for 50% of the total protein mass in these membranes as well as 50% of the total chlorophyll, including all of the chlorophyll *b*. In different organisms, the protein moiety has been variously reported to consist of one polypeptide, or two homologous polypeptides of mol wt 21,000–28,000 (Chua et al., 1976; Burke et al, 1978). These polypeptides from several higher plants and two green algae were found to have similar amino acid composition, being rich in proline and hydrophobic residues and poor in histidine (Thornber et al., 1967; Apel, 1977; Thornber, 1979).

In addition to harvesting solar energy, LHC has been implicated by studies of chloroplast development as a membrane adhesion factor, responsible for the close apposition of the outer surface of thylakoid membranes (Arntzen et al., 1976), which gives thylakoids the "stacked" morphology. In mature chloroplasts, LHC is localized in the stacked membrane areas (Anderson and

Levine, 1974). Miller et al. (1976) and Armond et al. (1976) proposed that LHC forms the outer parts of the large (160-Å Diam) intramembrane particles which are also restricted to these areas (Goodenough and Staehelin, 1971). Izawa and Good (1966) showed that thylakoid stacking requires a minimum cation concentration of 2–5 mM divalent cations or 50–100 mM monovalent cations. Lower cation concentrations lead to the reversible unstacking of thylakoid membranes, which can be restacked by increasing the cation concentrations. This cation regulation is mediated through protein carboxyl groups exposed at the membrane surface (Berg et al., 1974; Staehelin et al., 1976) and has been correlated with the presence of intact LHC in the membrane (Steinback et al., 1979). The reversible thylakoid stacking is accompanied by a reversible segregation of the large intramembrane particles into the stacked area (Ojakian and Satir, 1974; Staehelin, 1976). The mechanism by which changes in membrane stacking alter these lateral interactions between components in the hydrophobic phase remains to be elucidated.

Recently LHC has been purified from Triton X-100-solubilized thylakoid membranes of the pea by sucrose gradient fractionation followed by cation-induced precipitation (Burke et al., 1978). The purified pigment protein is shown to have fluorescence and aggregation properties reflecting its roles as antenna pigment and a membrane adhesion factor. Using freeze-fracture electron microscopy, McDonnell and Staehelin (1980) showed that the detergent-solubilized LHC forms sheets upon addition of cations at low concentrations, and the sheets form stacks when the monovalent or divalent cation concentrations are raised to levels required for thylakoid stacking. When the purified LHC is reconstituted into phospholipid vesicles, intramembrane particles of 80Å Diam can be observed (Steinback et al., 1978; McDonnell and Staehelin, 1980; Mullet and Arntzen, 1980). The reconstituted vesicles were found to simulate thylakoid stacking with a similar cation requirement, and the 80-Å intramembrane particles were seen to aggregate into the stacked areas of the vesicle membrane. In both the cation-precipitated LHC sheets and in the stacked, reconstituted vesicles, the aggregates of LHC appear as hexagonal planar arrays with a particle-to-particle distance of 80 Å in the plane. However, the polarity of the LHC molecule with respect to the sheets or vesicle membranes could not be determined by microscopy (McDonnel and Staehelin, 1980).

These observations suggest that purified LHC may be induced to form ordered arrays in two- and three-dimensions, and that the structure of LHC in these systems is immediately relevant to their function in the parent membrane. The ordered arrays are well suited to structural analysis by both x-ray diffraction and electron microscopy which will ultimately yield a detailed picture of the structure of this membrane-bound pigment protein. Although until now three-dimensional structures have been determined only for pigment proteins of rather rare occurrence, in each case the information obtained was valuable to the understanding of the basis of their function. For instance, the 2.8 Å resolution structure of the water-soluble bacteriochlorophyll *a* protein from the green photosynthetic bacterium, *A. aestuarii*, revealed the irregular orientation of the seven bacteriochlorophylls in each subunit of the trimer and the complete encasement of the seven pigment molecules by the 15 strand β-sheet of the polypeptide. The x-ray coordinates of the bacteriochlorophylls were used to calculate the coupling among the chromophores and its effect on the absorption spectrum in the visible region. Comparison of this calculated spectrum to that observed from the holoprotein served to emphasize the contribution of the protein environment in perturbing the absorption spectrum of the pigment from that measured in organic solvents (Fenna and Matthews, 1975; Matthews et al., 1979). The electron density maps of two phycobiliproteins at 5 Å resolutions elucidated the arrangement of different polypeptides in these multimeric complexes and allowed a direct comparison of the quarternary structure of the two related proteins (Fisher et al., 1980). Finally, the structure of bacteriorhodopsin derived from x-ray diffraction and electron microscopy showed that this membrane proton pump contains seven α-helices that span the membrane (Blaurock, 1975; Unwin and Henderson, 1975; Henderson and Unwin, 1975). The last example demonstrated that detailed structural information about integral membrane proteins is obtainable, provided these proteins can be placed in ordered arrays. We report here our initial progress in the structural studies of purified LHC placed into ordered arrays by membrane reconstitution.

MATERIALS AND METHODS

LHC was isolated from two-week-old pea seedlings according to the procedure of Burke et al. (1978), except that after disruption of the membranes with Triton X-100 (Sigma Chemical Corp., St. Louis, MO) the preparation was continued under dim light to avoid photooxidation of the pigments. The LHC was located on the sucrose gradient as the intensely fluorescent band, and precipitated with 10 mM $MgCl_2$ plus 100 mM KCl. This precipitate and precipitates formed by addition of salts to the dialyzed LHC (below) are the cation-induced aggregates examined by x-ray diffraction and electron microscopy. For reconstitution experiments, the precipitated LHC was redissolved in 10 mM Tricine (Sigma)-NaOH, pH 7.8, containing 10 mM KCl, 10 mM EDTA, and 1.5% (vol/vol) Triton X-100. It was dialyzed extensively against the same solution to remove sucrose and excess salts, and then dialyzed against this solution minus the EDTA before storage at −80°C.

The chlorophyll content of the LHC was determined following extraction into 80% aqueous acetone according to Arnon (1949). Protein was determined according to Lowry et al. (1951). For SDS-gel electrophoresis, the KCl was replaced by NaCl to precipitate the LHC; the pellet was taken up in the sample buffers according to McDonnell and Staehelin (1980). These were: for electrophoresis under nondissocating condition, 0.0625 M Tris-HCl, pH 6.5, 0.2% SDS, 0.1% mercaptoethanol; for the dissociating condition, 0.0625 M Tris-HCl, pH 6.8, 2% SDS, 2% mercaptoethanol. Gels for the nondissocating condition were run at 4°C.

The procedure for reconstitution of LHC with phospholipid was based on that of McDonnell and Staehelin (1980). Plant lecithin (Supeloco

Inc., Bellefonte, PA) was used without repurification. The lipids were dried from $CHCl_3$ and taken up to 10 mg/ml in 18 mM Na phosphate, pH 8.0. The suspension was sonicated under nitrogen for 30 min in a cleaning bath sonicator (Ladd Research Industries, Burlington, VT), and consisted of mostly single-walled vesicles (Gerritsen et al., 1978). Aliquots of thawed LHC of known protein concentration were added to aliquots of the vesicles with vortexing to give protein:lipid ratios (wt/wt) ranging from 1:20 to 4:1 in different samples. Typical sample volume was 0.5 ml. The sample was frozen in liquid nitrogen and thawed in water at room temperature for three cycles. It was then stirred gently at room temperature for 90 min. After addition of washed BioBeads SM-2 (Bio Rad Labs, Richmond, CA) to an excess of 1.0g/0.07g of Triton present (Holloway, 1973), the sample was stirred for another 45 min. The beads were removed and the sample was freeze-thawed for three cycles and sonicated for 10 s. Finally NaCl was added to concentrations between 0 and 140 mM, or $MgCl_2$ was added to concentrations between 0 and 15 mM. In control experiments, LHC was substituted with the Tricine storage buffer.

For x-ray diffraction, the LHC aggregates and the reconstituted membranes were oriented by sedimentation into Beem capsules (size 00, Polysciences, Inc., Warrington, PA) at 100,000 *g* for 2 h followed by gradual dehydration of the pellet in the x-ray capillary. Diffraction patterns were taken with a double mirror camera, with the incident beam parallel to the face of the pellet.

For freeze-fracture electron microscopy, the LHC aggregates, and the reconstituted membranes were pelleted at 9,000 *g* for 10 min and the pellets were infiltrated with 30% (vol/vol) glycerol overnight before freezing. The reconstituted membranes were also examined after negative staining with 1% uranyl acetate. All micrographs were taken on a Philips 301 electron microscope (Philips Electronics, Inc., Mahwah, NJ). Optical diffraction patterns of the micrographs were used to select highly ordered areas for computing filtered images. The filtered images were computed on a PDP 11-40 computer interfaced with Optronics P-1000 Photoscan (Optronics International, Inc., Chelmsford, PA), and a Grinnell graphics terminal (Grinnell Systems, Inc., Santa Clara, CA). Programs for the two-dimensional Fourier transformation were kindly provided by Dr. D. J. DeRosier.

RESULTS

Purification

The chlorophyll *a/b*-light harvesting protein (LHC) purified from pea thylakoid membranes showed a weight ratio of 5:1 protein (Lowry et al., 1951)-to-chlorophyll (Arnon, 1949). On SDS-gel electrophoresis under nondissociating conditions (see Methods), the purified LHC migrates as a single band of 29,000 mol wt. Under dissociating conditions, a major band of apparent mol wt 27,000 and a minor band of slightly lower mol wt were observed. The protein:chlorophyll ratio and the apparent mol wts imply a stoichiometry of six mol chlorophyll/mol of the major polypeptide. The chlorophyll *a:b* ratio was found to be 1.0. Therefore, within experimental error, the purified LHC contained 3 mol each of chlorophylls *a* and *b*/mol of a polypeptide of mol wt 27,000, in agreement with the stoichiometry calculated by Thornber et al. (1967; Thornber, 1979).

Cation-Induced Aggregates

The cation requirement for LHC aggregation into sheets and stacks of sheets, reported by McDonnell and Staehelin (1980), has been confirmed. X-ray diffraction patterns (Fig. 1 *a*) of the LHC precipitated in 10 mM $MgCl_2$ indicate that the LHC aggregates into planar arrays that stack with a period of 50.9 ± 3.3 Å. The sharpness of the strong meridional reflections of this repeat, observed to 10 Å resolution, demonstrates the high regularity of the stacking distance and hence the strong adhension between the layers of LHC molecules. On the equator, the first three reflections from a hexagonal lattice of lattice constant 84.9 ± 1.3 Å are present. However, these reflections are broad, and higher order reflections in the hexagonal series were not detected. Therefore these reflections arise from a hexagonal close packing of the LHC molecules within the planar aggregates, with a center-to-center distance of ~ 85 Å, but there is no long-range order in the plane.

Electron micrographs of replicas of frozen-fractured LHC aggregates showed extensive stacking of the planar aggregates, with more than 50 layers present in some stacks (Fig. 1 *b*). Each layer is made of closely packed particles of ~ 80-Å dia. The packing appears hexagonal in small domains of 20–30 particles, but no long-range order can be discerned (Fig. 1 *c*). These observations confirmed the findings from x-ray diffraction at low resolutions.

Reconstituted LHC Membranes

Using a modified procedure of McDonnell and Staehelin (1980), LHC solubilized in Triton X-100 has been incorporated into single-walled vesicles (Gerritsen et al., 1978) of plant lecithin. At LHC:lipid ratios ranging from 1:20 to 4:1 (wt/wt) in different experiments, incorporation appeared to be complete, because when cations were added no structure resembling the LHC aggregates could be detected by either x-ray diffraction or electron microscopy. Stacking of the LHC membranes was brought about by addition of NaCl to concentrations > 60 mM, or $MgCl_2$ to > 2 mM, as was reported by McDonnell and Staehelin (1980). In the absence of LHC the lipid vesicles do not stack at these salt concentrations.

When the reconstituted LHC membranes at protein:lipid ratios > 2:1 were exposed to NaCl at concentrations > 10 mM, or $MgCl_2$ > 2 mM, x-ray diffraction patterns taken with the beam parallel to the surface of the oriented membranes showed sharp lattice lines on the equator to 7 Å spacing, and two distinct series of reflections on the meridian (Fig. 2 *a*). The equatorial lattice lines are never observed in the absence of LHC; therefore they are due to a lattice in the plane of the membrane, formed by the incorporated LHC. The observation of sharp lattice lines to 7 Å resolution limit, which is a small spacing compared with the close-packing distance of 85 Å between LHC molecules in their planar aggregates, shows that in the reconstituted membranes the arrays of LHC are truly crystalline, as opposed to a planar close packing with random azimuthal orientations. The sharpness of the lattice lines is comparable to that in the x-ray patterns of

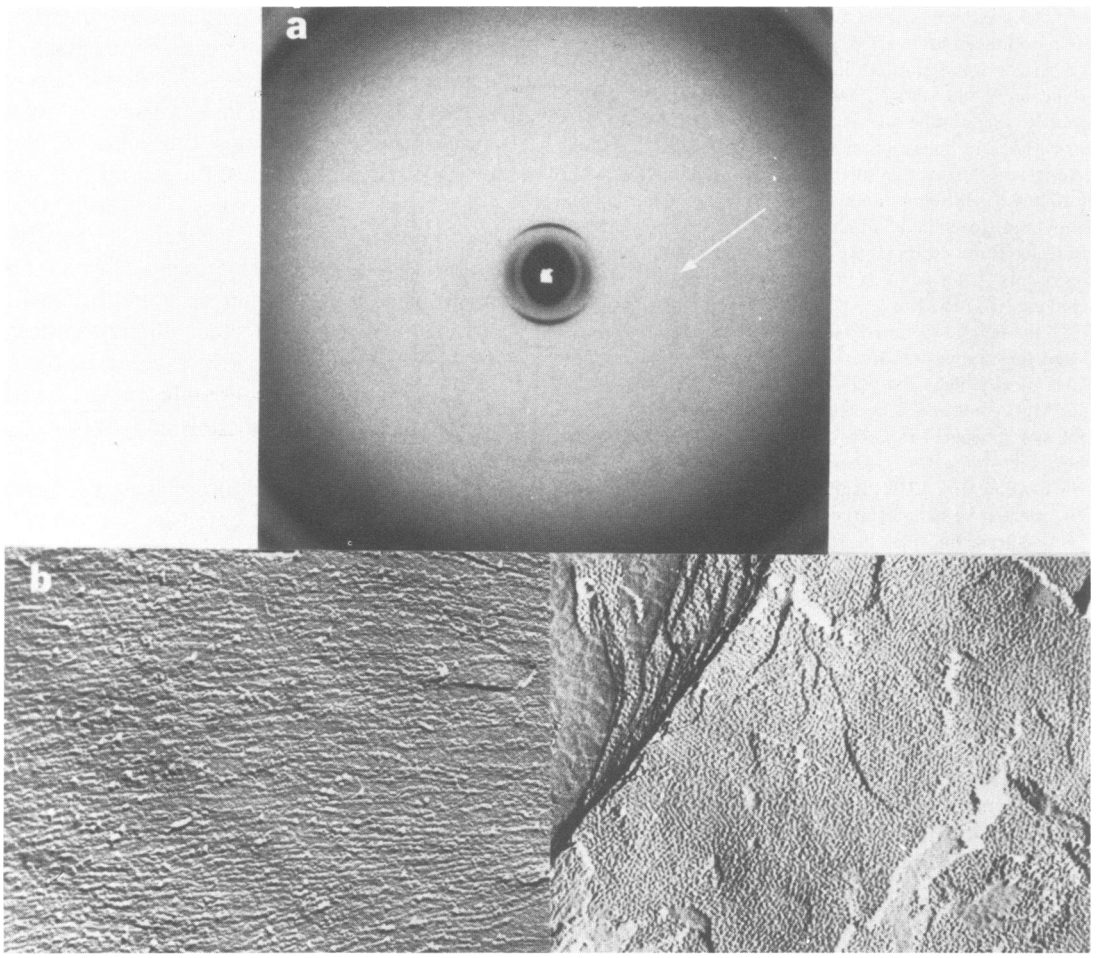

FIGURE 1 X-ray diffraction pattern and freeze-fracture electron micrographs of oriented aggregates of LHC. *a*, x-ray pattern taken with the beam parallel to the sheets of LHC aggregates. Three orders of a 51-Å repeat period appear on the meridian; they are due to the stacking of LHC sheets. The first three orders of diffraction from a planar hexagonal lattice are observed on the equator, and are due to the packing of LHC within the sheet. The band of diffuse intensity on the equator at 10-Å spacing (arrow) indicates the presence of parallel α-helices or β-sheets that are oriented perpendicular to the LHC layers. *b*, micrograph of cross-fractured LHC aggregates showing the tight, regular stacking of LHC sheets, which gives rise to the strong meridional diffraction. *c*, micrograph of LHC aggregates fractured parallel to one sheet. The hexagonal ordering of particle packing is maintained only over small domains. Consequently the equatorial series of hexagonal reflections in *a* are diffuse and do not extend beyond 36-Å spacing.

hydrated purple membranes (cf. Blaurock, 1975). This indicates that each of the ordered domains of the LHC membrane lattice contains a very large number of unit cells.

The spacings of the first few observed lattice lines were 108.3, 64.7, 50.4, 42.5, 37.3, 30.9, and 23.7 Å. These spacings are within 5% of those expected for lattice lines out to the (3, 2) order of a planar hexagonal lattice of lattice constant 125 Å. The slight discrepancy may be due to disorientation of different membranes in the x-ray pellet. This causes a corresponding rotation of the lattice lines about the center of the diffraction pattern, so that the apparent lattice line positions of the higher orders are shifted to higher radii. Around 10 Å spacing on the equator, the intensities of the lattice lines are comparatively strong. (This is more obvious in diffraction patterns from thicker pellets, which however show poorer membrane orientation.) The strong intensity in this region indicates the presence of parallel-packed secondary structure in the protein, either α-helices or β-sheets (Blaurock, 1975; Cohen et al., 1980), that are oriented perpendicular to the face of the reconstituted membrane. Thus the orientation of the LHC molecules with respect to the planar arrays is similar in both the cation-induced aggregates and in the crystalline planar lattice formed in the reconstituted membranes.

The series of meridional reflections of 70 Å repeat can be identified as due to the stacking of the LHC planar lattices because its high degree of orientation is similar to that of the lattice lines seen on the equator. The repeat period indicates that the stacking unit in the pellet is a single layer of membrane lattice. The stacking of the lattices is generally not in register, since the intensities on the lattice lines are continuously varying rather than being

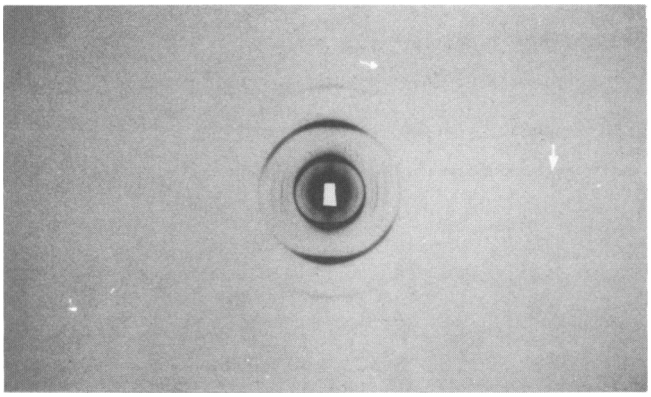

FIGURE 2 *a* X-ray diffraction pattern from an oriented pellet of reconstituted LHC membranes. The strong but disoriented (arced) reflections of 55-Å repeat are due to a pure lipid phase in the pellet. Reflections of a 70-Å period, originating from the stacked LHC membranes, are oriented on the meridian but are obscured by the lipid diffraction in this photograph. On the equator, sharp oriented lattice lines due to a highly ordered planar lattice of lattice constant 125 Å (see text) can be observed to 7-Å spacing (arrow).

FIGURE 2 *b* Electron micrographs of frozen-fractured LHC membranes. The apparently hexagonal lattice of particles is extensive and highly ordered in the plane of the membrane. There is three-dimensional order in the stacking of the membrane lattices. As the fracture passes obliquely through several stacked membranes the rows of particles on adjacent layers appear precisely aligned, and the boundaries of the plaques of lattices are in register.

sampled at the 70-Å period which is the distance between planar lattices. The other series of meridional reflections, of 55 Å repeat, is much less oriented, and can be attributed to a separate lipid phase in the x-ray specimen, because it was also observed in diffraction from the LHC-free controls.

Electron micrographs of the frozen-fractured x-ray specimens showed regions of apparently hexagonal lattice of particles on the fracture faces, contiguous with smooth, particle-free areas characteristic of pure lipid bilayers (Fig. 2 *b*). The plaques of lattices were flat, while the smooth areas have greater curvature. These observations are consistent with the x-ray data, indicating that both a highly oriented LHC membrane lattice and a less oriented lipid phase are present in the x-ray specimens. In comparison, the reconstituted vesicles in the absence of added salts showed intramembrane particles that are dispersed over the smooth matrix. This suggests that the LHC lattice is formed by lateral migration of LHC molecules within the lipid bilayer to form specific intermolecular contacts. The plaques of lattices are generally larger and more ordered in samples treated with $MgCl_2$ than those with NaCl.

Three-dimensional arrays of limited extent appear to be present in the pellets of reconstituted LHC membranes. Freeze-fracture micrographs showed that occasionally the planar lattices on adjacent membranes are aligned with each other. Where this ordered stacking occurs, the boundaries of the plaques of lattices in successive membranes appear to lie in register (Fig. 2 *b*), i.e., a small three-dimensional crystal is formed of the stacked LHC lattices which are contiguous with the lipid bilayer.

Reconstituted LHC membranes at LHC:lipid ratios of 2:1 and 4:1 have been examined by microscopy after negative staining. Apparently hexagonal lattices of 125-Å lattice constant have been observed in membranes suspended in 10 mM NaCl, a concentration insufficient to cause stacking. Optical diffraction patterns of the micrographs contained reflections to the (2, 1) order at 36-Å spacing. In samples suspended in 15 mM $MgCl_2$ the membranes are stacked, but micrographs have been taken from the occasionally unstacked, stained area (Fig. 3). The lattices under these conditions are more ordered and the optical diffraction patterns of the micrographs showed reflections out to the (3, 1) order, 27-Å spacing. The intensities in the optical diffraction patterns appear to have *6mm* symmetry (International Tables for X-ray Crystallography), implying that the structure has 622 rotational symmetry. Computer-filtered images of the negatively stained membrane lattice in 15 mM $MgCl_2$ have been obtained. These images show (Fig. 3, inset) a central stain-excluding area surrounded by six oblong stain-excluding areas; the outer stain-excluding features are shared between the neighboring unit cells. However, this view is probably the superposition of images of the "front" and "back" surfaces of the membrane lattice. The symmetrically arranged, surface-exposed parts of the LHC molecule visualized by negative staining probably form contacts with their counterparts in an adjacent membrane to give rise to the ordered stacking of the two-dimensional lattices which was observed by freeze-fracture microscopy.

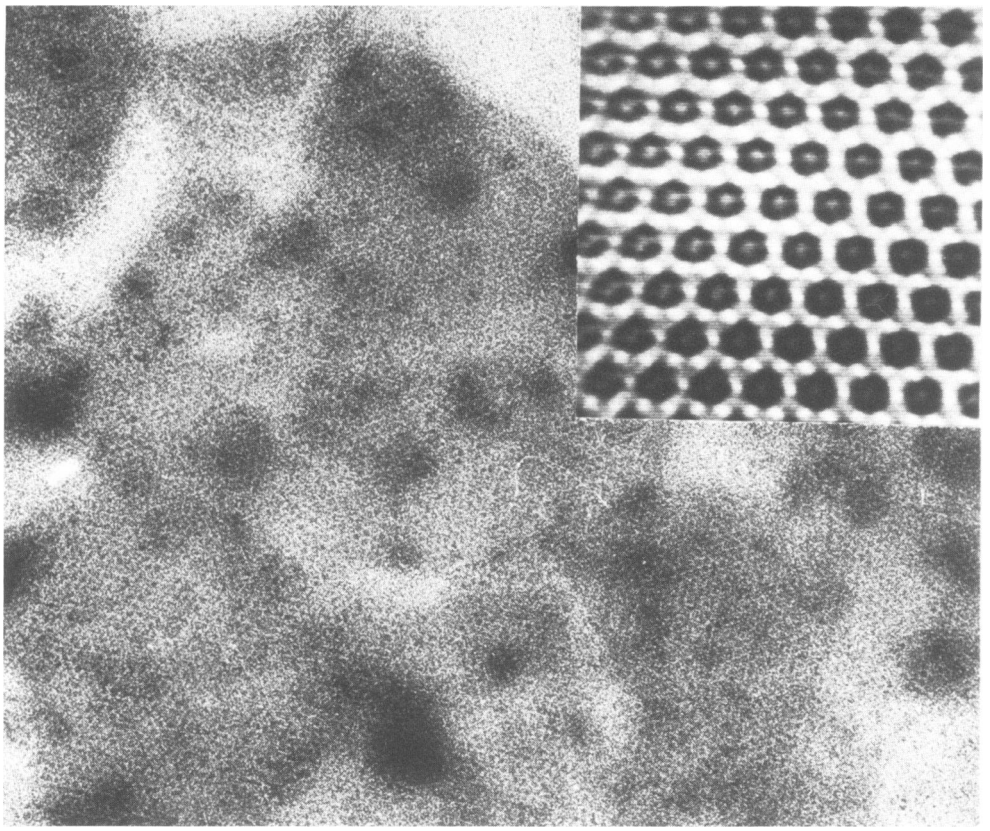

FIGURE 3 Electron micrograph of negatively stained, reconstituted LHC membrane. The extensive two-dimensional lattice formed by the surface-exposed parts of the membrane-bound LHC molecules is apparent. Optical diffraction patterns of this micrograph exhibit reflections out to the (3, 1) order, at 27-Å spacing. The inset is a computer-filtered image of the negatively stained membrane surface-lattice. This image shows a central stain-excluding area surrounded by six oblong stain-excluding areas. The outer stain-excluding features are shared between neighboring units of the lattice. Hexagonal symmetry was not imposed in the computation of this image.

DISCUSSION

We have obtained direct evidence that the planar arrays formed by the LHC incorporated into phospholipid bilayers are crystalline at the molecular level, because these arrays diffract x-rays in the direction parallel to the membrane plane to a spacing significantly smaller than the distance (85 Å) between LHC molecules at closest packing. The crystallinity implies that the polarity of insertion of LHC into the bilayer cannot be random, although randomness was inferred from earlier electron microscopic observations (McDonnell and Staehelin, 1980; Mullet and Arntzen, 1980). However, both unidirectional and periodic, bidirectional insertions are compatible with our x-ray data. Both modes of arrangement have been observed, for example, in the planar crystalline arrays of cytochrome oxidase that were prepared by different detergent treatments of the same parent membrane (Henderson et al., 1977; Fuller et al., 1979).

The optical diffraction pattern of the image of the negatively stained LHC membrane lattice showed *6mm* symmetry. *6mm* means that there is a six-fold rotational axis perpendicular to the plane of the image, and there are two sets of mirror lines at 30° to each other and intersecting at the six-fold axis. The spacings of the observed x-ray lattice lines are consistent with a hexagonal planar lattice. However, since biological structures cannot have intrinsic mirror symmetry, the electron microscopic image must have acquired the mirror symmetry due to the projection onto the membrane plane of elements related by a two-fold rotational axis contained in that plane. Assuming that the same lattice is being observed by both x-ray diffraction of the pellet of reconstituted membranes and by electron microscopy of the negatively stained membranes, the 70 Å periodicity of the meridional x-ray reflections indicates that the stacking unit is one layer of membrane lattice, which would mean that each layer of the lattice contains a two-fold rotational axis. Therefore, the combined evidence from x-ray diffraction and negative staining electron microscopy implies that the LHC membrane lattice is made up by periodic, bidirectional insertion of the LHC protein. If this deduction is correct, the arrangement of LHC molecules can be compared to the lattice of cytochrome oxidase obtained by Fuller et al. (1979) by deoxycholate extraction of the mitochondria inner membrane. It is interesting to note that the ratio of the lattice constant of

the LHC membrane lattice to the distance of hexagonal close packing in the LHC aggregates is $\sqrt{2}$, so the ratio of "unit cell" areas is two. This indicates that the unit cell of the LHC membrane lattice can contain twice as many molecules as can that of the LHC aggregate, which would be consistent with the presence of a two-fold axis in the plane of the lattice. More precise measurements are required to determine the symmetry unambiguously.

Regardless of the nature of arrangement of LHC in the membrane lattice, the greater, 70-Å thickness of the membrane lattice compared to both the 55-Å stacking period of the LHC-free lipid phase and the 51-Å period of the cation induced LHC planar aggregates shows that the LHC is inserted into the bilayer with a significant portion exposed at the surface. Because it was shown that the ability of the cation-induced sheets to stack is impaired by pronase treatment of either the thylakoid membranes prior to isolation of LHC (McDonnell and Staehelin, 1980), or the sheets themselves (Mullet and Arntzen, 1980), the orientation of the LHC in the sheets and the thylakoid membranes is the same. The 51-Å distance between tightly stacked, planar aggregates is therefore a measure of the height of the LHC molecule perpendicular to the thylakoid membrane. This height is sufficient to span the bilayer. The increased thickness of the membrane lattice compared to the height of LHC implies that LHC protrudes from the bilayer surface by at least 10 Å.

In the cation-induced aggregates of LHC, one can calculate the vol/mol under planar hexagonal close packing from the 51-Å thickness and the 85-Å center-to-center distance. Based on a subunit stoichiometry of six mol chlorophyll/polypeptide chain of mol wt 27,000 obtained by SDS-gel electrophoresis, this volume can accommodate six to seven of such subunits. Therefore particles of ~ 80-Å Diam seen by freeze-fracture electron microscopy represents a multimeric form of the light-harvesting complex.

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REFERENCES

Anderson, J. M. 1975. The molecular organization of chloroplast thylakoids. *Biochim. Biophys. Acta.* 416:191–235.

Anderson, J. M., and R. P. Levine. 1974. The relationship between chlorophyll protein complexes and chloroplast membrane polypeptides. *Biochim. Biophys. Acta.* 357:118–126.

Apel, K. 1977. Chlorophyll-proteins from *Acetabularia mediterranea*. *Brookhaven Symp. Biol.* 28:149–161.

Armond, P. A., C. J. Arntzen, J.-M. Briantais, and C. Vernotte, 1976. Differentiation of chloroplast lamellae. Light harvesting efficiency and grana development. *Arch. Biochem. Biophys.* 175:54–63.

Arnon, D. I. 1949. Copper enzymes in isolated chloroplasts. Polyphenoxidase in *Beta vulgaris*. *Plant Physiol.* 24:1–5.

Arntzen, C. J., P. A. Armond, J. -M. Briantais, J. J. Burke, and W. P. Novitsky. 1976. Dynamic interactions among structural components of the chloroplast membrane. *Brookhaven Symp. Biol.* 28:316–337.

Berg, S., S. Dodge, D. W. Krogmann, and R. A. Dilley. 1974. Chloroplast grana membrane carboxyl groups: their involvement in membrane association. *Plant Physiol.* 53:619–627.

Blaurock, A. E. 1975. Bacteriorhodopsin: a transmembrane pump containing α-helix. *J. Mol. Biol.* 93:139–158.

Burke, J. J., C. L. Ditto, and C. J. Arntzen. 1978. Involvement of the light-harvesting complex in cation regulation of excitation energy distribution in chloroplasts. *Arch. Biochem. Biophys.* 187:252–263.

Chua, N.-H., G. Blobel, P. Siekevitz, and G. E. Palade. 1976. Periodic variations in the ratio of free to thylakoid-bound chloroplast ribosomes during the cell cycle of *Chlamydomonas reinhardtii*. *J. Cell Biol.* 71:497–514.

Cohen, F. E., M. J. E. Sternberg, and W. R. Taylor. 1980. Analysis and prediction of protein β-sheet structures by a combinatioral approach. *Nature (Lond.).* 285:378–382.

Fenna, R. E., and B. W. Matthews. 1975. Chlorophyll arrangement in a bacteriochlorophyll protein from *Chlorobium limicola*. *Nature (Lond.).* 258:573–577.

Fisher, R. G., N. E. Woods, H. E. Fuchs, and R. M. Sweet. 1980. Three-dimensional structures of C-phycocyanin and B-phycoerythrin at 5-Å resolution. *J. Biol. Chem.* 255:5082–5087.

Fuller, S. D., R. A. Capaldi, and R. Henderson. 1979. Structure of cytochrome *c* oxidase in deoxycholate-derived two-dimensional crystals. *J. Mol. Biol.* 134:305–327.

Gerritsen, W. J., A. J. Verkleij, R. F. A. Zwaal, and L. L. M. van Deenen. 1978. Freeze-fracture appearance and disposition of band 3 protein from the human erythrocyte membrane in lipid vesicles. *Eur. J. Biochem.* 85:255–261.

Giddings, T. H., N. W. Withers, and L. A. Staehelin. 1980. Supramolecular structure of stacked and unstacked regions of the photosynthetic membranes of *Prochlorn sp.*, a prokaryote. *Proc. Natl. Acad. Sci. U.S.A.* 77:352–356.

Goodenough, U. W., and L. A. Staehelin. 1971. Structural differentiation of stacked and unstacked chloroplast membranes. *J. Cell Biol.* 48:594–619.

Govindjee. 1975. Bioenergetics of Photosynthesis. Academic Press, New York.

Henderson, R., R. A. Capaldi, and J. S. Leigh. 1977. Arrangement of cytochrome oxidase molecules in two dimensional vesicle crystals. *J. Mol. Biol.* 112:631–648.

Henderson, R., and P. N. T. Unwin. 1975. Three-dimensional model of purple membrane obtained by electron microscopy. *Nature (Lond.).* 257:27–32.

Holloway, P. W. 1973. A simple procedure for removal of Triton X-100 from protein samples. *Anal. Biochem.* 53:304–308.

International Tables for X-ray Crystallography. 1969. Kynoch Press, Birmingham. Vol. I. 24.

Izawa, S., and N. E. Good. Effects of salt and electron transport on the conformation of isolated chloroplasts. *Plant Physiol.* 41:544–552.

Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265–275.

McDonnell, A., and L. A. Staehelin. 1980. Adhesion between liposomes mediated by the chlorophyll *a/b* light-harvesting complex isolated from chloroplast membranes. *J. Cell Biol.* 84:4–56.

Matthews, B. W., R. E. Fenna, M. C. Bolognesi, M. F. Schmid, and J. M. Olson. 1979. Structure of a bacteriochlorophyll *a* protein from the green photosynthetic bacterium *Prosthechloris aestuarii*. *J. Mol. Biol.* 131:259–285.

Miller, K. R., G. J. Miller, and K. R. McIntyre. 1976. The light-harvesting chlorophyll protein complex of photosystem II. Its location in the photosynthetic membrane. *J. Cell Biol.* 71:624–638.

Mullet, J. E., and C. J. Arntzen. 1980. Simulation of grana stacking in a model membrane system: medjation by a purified light-harvesting

pigment-protein complex from chloroplasts. *Biochim. Biophys. Acta.* 589:100–117.

Ojakian, G. K., and P. Satir. 1974. Particle movements in chloroplast membranes: quantitative measurements of membrane fluidity by the freeze-fracture technique. *Proc. Natl. Acad. Sci. U.S.A.* 71:2052–2056.

Staehelin, L. A. 1976. Reversible particle movements associated with unstacking and restacking of chloroplast membranes *in vitro*. *J. Cell Biol.* 71:136–158.

Staehelin, L. A., P. A. Armond, and K. R. Miller. 1977. Chloroplast membrane organization of the supramolecular level and its functional implication. *Brookhaven Symp. Biol.* 28:278–315.

Steinback, K. E., J. J. Burke, J. E. Mullet, and C. J. Arntzen. 1978. The role of the light-harvesting complex in cation-mediated grana formation. *In* Chloroplast Development. G. Akoyunoglou et al., editors. Elsevier/North Holland Press. Amsterdam, The Netherlands. 389–400.

Thornber, J. P. 1975. Chlorophyll-proteins: light-harvesting and reaction center components of plants. *Ann. Rev. Plant Physiol.* 26:127–158.

Thornber, J. P. 1979. Plant chlorophyll-protein complexes: recent advances. *Photochem. Photobiol.* 29:1205–1216.

Thornber, J. P., J. C. Stewart, M. W. C. Hatton, and J. L. Bailey. 1967. Studies on the nature of chloroplast lamellae. II. Chemical composition and further physical properties of two chlorophyll-protein complexes. *Biochemistry.* 6:2006–2014.

Unwin, P. N. T., and R. Henderson. 1975. Molecular structure determination by electron microscopy of unstained crystalline specimens. *J. Mol. Biol.* 94:425–440.

DISCUSSION

Session Chairman: Donald M. Engelman *Scribes*: Debra A. Thompson and Maria D. Suárez-Villafane

MANNELLA: Have you looked at the negative staining with something like phosphotungstate as well as the uranyl acetate?

LI: No, but I will. I realize that by using positively charged or a negatively charged stain, one might get different images that could give additional information.

WALLACE: You have indicated the presence of a 10-Å equatorial reflection that you ascribe to side-to-side packing of either α-helices or β-pleated sheets. Do you see any meridonal reflections at either 4.7 or 5.4 Å to indicate the presence of, or to distinguish between, those two features?

LI: We have not yet looked at the reflections in that region because that we have not taken the diffraction pattern of the specimen in a glass capillary.

HUI: Can you see the unit structure in the freeze fracture electron micrographs? If you cannot, did you try optical filtering?

LI: No, I didn't. That is also a possibility.

HUI: I keep wondering whether the structure you can see in the freeze fracture would be the same as the one seen by negative staining.

LI: I cannot make any predictions about that because the two techniques may not be looking at the same part of the molecule.

ENGELMAN: Is there any spectroscopic evidence concerning secondary structure? I'm particularly interested in the issue of α-helicity in the proteins.

LI: I don't think there is any evidence. CD measurements have been made on these chlorophyll proteins in the visible range since the problem of interest was chlorophyll coupling (Canaani and Fauer. 1978. *Biochim. Biophys. Acta.* 501:545–551; Zan Matter. 1977. *Biochim. Biophys. Acta.* 462:642–658).

EISENBERG: Electron microscopists have seen a variety of lattices in sectioned chloroplasts with different intermolecular spacings. Does your lattice correspond to any of those which have been reported?

LI: No, but there is some relationship. There are three types of lattices which have been seen by electron microscopists looking at intact chloroplasts. The first type, on the outer surface of the thylakoid membrane, has been unambiguously shown to be the coupling factor, peripheral protein (Howell and Moudrinakis. 1967. *J. Mol. Biol.* 27:323–333). If you wash the membrane with EDTA you can completely remove the protein (as determined by gel electrophoresis, or by activity) (Strotmann et al. 1973. *Biochim. Biophys. Acta.* 314:202–210.). You can restore the binding of these molecules to the chloroplast just by incubating the coupling factor with the membrane (Miller and Staehelin. 1976. *J. Cell. Biol.* 68:30–47). RuDP carboxylase is also found on the outer surface and can be removed by a similar washing procedure (Strotman et al., 1973; Miller and Staehelin, 1976). But the lattice appears to be due to the coupling factors (Howell and Moudrinakis, 1967).

The second type of lattice that has been seen is made up of large intramembrane particles that are found only in the stacked region. It is believed that the light-harvesting complex represents a part of these particles because, during development as the amount of light-harvesting complex increases, the particle size, but not the particle number, also increases and the size seems to increase in discrete steps (Armond et al. 1976. *Arch. Biochem. Biophys.* 175:54–63). These particles are also seen in mutants which do not have chlorophyll *b* and do not have light-harvesting complex, but they seem to be slightly smaller. Occasionally large particles are seen in the lattices, both by freeze fracture and by freeze-etching, and these observations have been used to imply that the particles span the membrane. However, the lattice dimensions are slightly smaller in the mutants lacking this protein (Miller et al. 1976. *J. Cell Biol.* 71:624–638).

The third type of lattice that has been seen is in the lumen of the thylakoid (Miller, unpublished observations), and I don't know what that is. Only the second type is related to the lattice we study.

MANNELLA: The bilayer spacing you see by the x-ray diffraction was 55 Å in the reconstituted system. That is fairly tight. Do you know the protease sensitivity of the particles? For example, if you treat them with trypsin do you still see the same arrays?

LI: I have not done that experiment, but other laboratories have done the protease treatment both on the cation-precipitated sheets and on the reconstituted membrane. After protease treatment a very small part of the molecular weight is removed: some laboratories say 1,000, some say 2,000. That treatment effectively abolishes the restacking because you have to unstack the system to do that experiment. In abolishing the restacking, one also abolishes the lattice formation.