FREEZE-FRACTURE STUDIES ON BARLEY PLASTID MEMBRANES. III. LOCATION OF THE LIGHT-HARVESTING CHLOROPHYLL-PROTEIN

by

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The thylakoids of chloroplasts from wild-type barley and the nuclear gene mutant *chlorina-f2* were examined by freeze-fracturing and rotary shadowing. The advantages of rotary shadowing were: the shape of freeze-fracture particles was revealed, particles on membrane surfaces were seen in greater detail, estimation of particle density was more reliable, and measurements could be made of membrane thickness. When wild-type and *chlorina-f2* thylakoid ultrastructure were compared, the most significant difference was the large reduction in the number of particles on the PFs face of the mutant. In addition, EFs particles were about 12% smaller in the mutant, whereas the appearance of the EFu, PFu and PSu was substantially the same as for wild-type thylakoids. These ultrastructural differences are correlated with the complete absence of the light-harvesting chlorophyll *a/b*-protein from *chlorina-f2* thylakoids. Freeze-fracturing of purified light-harvesting complex in vesicles revealed particles similar in size and shape to those missing from the PFs face of *chlorina-f2* but present on the PFs face of wild-type. It is concluded that in wild-type granal thylakoids, the light-harvesting chlorophyll *a/b*-protein is located in particles which are closely associated with EFs particles, but which cleave with the PFs face during freeze-fracturing.

Abbreviations: EFs = endoplasmic fracture (stacked), EFu = endoplasmic fracture (unstacked), ES = endoplasmic surface, ESs = endoplasmic surface (stacked), ESu = endoplasmic surface (unstacked), PFs = protoplasmic fracture (stacked), PFu = protoplasmic fracture (unstacked), PSu = protoplasmic surface (unstacked), S.D. = standard deviation, S.E. = standard error of the mean, Tricine = N-(tris-(hydroxymethyl)-methyl)glycine.

1. INTRODUCTION

The structure of the chloroplast thylakoid membrane has been examined by a variety of physical and chemical techniques. These include X-ray diffraction, the use of chemical and fluorescent probes, spin-labels, circular dichroism and other spectroscopic methods, antibody labelling and electron microscopy. One of the powerful electron microscopic techniques is freeze-fracturing which, due to its unique ability to cleave the lipid bilayer along the internal hydrophobic plane, reveals structures within the membrane at a supramolecular level. The membrane surface can be studied by freeze-etching, which enables the visualisation of structures on the membrane without chemical fixation or the damage caused by surface tension forces generated by air drying of material examined by negative staining.

When thylakoid membranes are freeze-fractured and examined with the electron microscope, many small particles are seen in the cleaved membrane. Evidence from reconstitution (15, 27, 41) and specific labelling experiments (32) supports the idea that many freeze-fracture particles are composed of protein. Such particles are too large to consist of a single polypeptide, so it is probable that freeze-fracture particles consist of several polypeptides (14). There is an increasing amount of evidence in favour of the concept that the structural units visualised by freeze-fracturing consist of polypeptides organised into discrete functional units. This concept has been incorporated into a recent model of the thylakoid membrane (4, 40).

Ultimately, one would like to determine the localisation of all the polypeptide components of thylakoids and assign them to the various freeze-fracture particles found on the different fracture faces. One approach to this problem is to isolate structural and/or functional components of thylakoids. These can then be inserted into artificial lipid bilayer vesicles and their freeze-fracture ultrastructure examined (4, 41). In this way, the appearance of specific membrane polypeptide components can be studied in a relatively simple model system. The opposite, and complementary technique is to investigate the freeze-fracture ultrastructure of thylakoids from mutants which specifically lack one or more components (36, 37, 45). Since there is an

extensive and genetically well-characterised collection of barley plastid mutants (44), this species is an obvious choice for such an investigation.

The nuclear gene mutant of barley known as *chlorina-f2* lacks the light-harvesting chlorophyll *a/b* protein 2 (Chl_{a/b}-P2) (25) and a small number of minor polypeptides (2, 10, 17, 24). Chl_{a/b}-P2 is a major component of wild-type thylakoids and is a hydrophobic polypeptide (42), so it would be expected that there would be sufficient mass of this polypeptide located at the freeze-fracture plane for its absence to be detected. There is circumstantial evidence which suggests that Chl_{a/b}-P2 is a component of the EF particles of grana (3, 39, 40). Thus, freeze-fracture studies of *chlorina-f2* thylakoids have tended to concentrate on the size of these EFs particles, and the results are conflicting (1, 5, 17, 18, 30). The most careful study indicates a slight reduction in EFs particle size of the mutant compared wild-type (30). This result is difficult to reconcile with the much larger change in EFs particle size reported during the greening of peas under conditions where one of the major polypeptides synthesised is Chl_{a/b}-P2 (3).

In order to resolve this apparent conflict, the freeze-fracture ultrastructure of all four fracture faces and both surfaces of *chlorina-f2* thylakoids was examined. These were compared with wild-type thylakoids isolated from plants of the same age and grown under the same conditions as the mutant (35). The isolation medium contained a higher concentration of divalent cations (Mg⁺⁺) to maintain stacking in the mutant (10, 41). In order to detect small changes in particle size, shape or density, the technique of rotary shadowing (7, 13, 26, 43) was used and the results are compared with those from conventional uni-directional shadowing.

2. MATERIALS AND METHODS

2.1. Plant material

Seeds of wild-type (*Hordeum vulgare* cv. Svalöfs Bonus) and mutant (*chlorina-f2*) barley were germinated in plastic trays containing vermiculite moistened with tap water. Seedlings were harvested after growing for 7 days at 20 °C in continuous white light (1,700 lux) as described previously (35).

2.2. Isolation of chloroplasts

Precooled leaves were homogenised in 3 volumes of grinding medium at 4 °C using a kitchen blender with replaceable razor blades (20). The grinding medium consisted of 0.4M-sucrose, 50mM-Tricine/NaOH, pH 7.9, 5mM-MgCl₂ for wild-type and 0.4M-sucrose, 50mM-Tricine/NaOH, pH 7.9, 25mM-MgCl₂ for *chlorina-f2*. The resulting slurry was filtered through 2 layers of 31 µm nylon gauze and centrifuged for 5 min at 1,400 g. The pellet was resuspended in a small volume of the appropriate grinding medium and an equal volume of 60 % glycerol in 5 or 25mM-MgCl₂ was added, followed by centrifugation for 5 min at 5,000 g. This pellet was used for freeze-fracturing. Preparation of samples for freeze-etching involved washing the initial chloroplast pellet 3 times in 5mM-Tricine/NaOH, pH 7.9 5mM-MgCl₂ (wild-type), or 10mM-MgCl₂ (*chlorina-f2*), before a final centrifugation for 10 min at 10,000 g.

2.3. Freeze-fracturing

Unfixed chloroplasts were frozen on gold planchettes in melting Freon 22 cooled by liquid nitrogen. Replicas were made with a Balzers BAF 301 freeze-etching apparatus (Balzers AG, Liechtenstein) equipped with a rotating cold stage that could be used for the preparation of both uni-directional and rotary shadowed replicas. Freeze-fracturing was performed at -110 °C and freeze-etching at -100 °C for 2 min at a vacuum of 2×10^{-6} torr or better. Subsequent treatment was as previously described (34). All uni-directionally shadowed micrographs are printed so that the direction of shadowing is from bottom to top, and the terminology used is that of BRANTON et al. (8).

2.4. Calculations for rotary shadowing

When freeze-fractured specimens are replicated with Pt/C by uni-directional shadowing, it is usual to deposit a metal film to a depth of 20 Å. The deposition process can be monitored by the change in the frequency of oscillation of a quartz crystal. The arrangement of the electron beam gun, specimen stage and quartz crystal monitor is shown in Figure 1. The change in frequency is related to the metal film thickness by the formula:

α : angle of shadowing

β : angle between centre line of evap. source and centre line of crystal

Figure 1. Diagram of the arrangement of the evaporation source, specimen and quartz crystal holder in the Balzers BAF301 freeze-etch unit.

$$\Delta f = \frac{k \cdot d_1 \cdot \cos \beta}{\sin \alpha}$$

Where Δf = frequency change (Hz); k = material constant (Hz/Å); d_1 = desired film thickness (Å); α = angle of shadowing; β = angle between centre line of evap. source and centre line of crystal. Usually, for uni-directional shadowing, $\alpha = 45^\circ$ and $\beta = 30^\circ$, and a frequency change of 170 Hz corresponds to a film thickness of 20 Å of Pt/C. If the angle of the quartz crystal holder is not altered, then $\beta = 75^\circ - \alpha$. If the shadowing angle α is changed to α' , then the frequency change ($\Delta f'$) needed to produce a 20 Å thick film is given by:

$$\begin{aligned} \Delta f' &= \frac{\Delta f \cdot \cos (75 - \alpha') \cdot \sin 45^\circ}{\sin \alpha' \cdot \cos 30^\circ} \\ &= \frac{138.8 \cdot \cos (75 - \alpha')}{\sin \alpha'} \end{aligned}$$

Determination of values of $\Delta f'$ for different values of α are given in Table I. In practice it is not useful to use angles below 20° because of shading of the quartz crystal by the coldstage, unless the angle of mounting of the crystal holder is altered. In this study, a shadowing angle of 22.5° was used, with a corresponding $\Delta f'$ of 220 Hz.

Table I

Relationship between shadowing angle (α) and frequency change ($\Delta f'$) for deposition of a 20 Å thick Pt/C film.

Shadowing Angle (α)	$\Delta f'$ (Hz)
6°	476
10°	338
15°	268
20°	233
22.5°	221
25°	211
30°	196
45°	170

Figure 2. Predicted appearance of a hemispherical particle after rotary shadowing.

Because the specimen is rotating during shadowing, Pt/C will be deposited on all sides of projecting freeze-fracture particles. The amount deposited on a hemisphere decreases from the base towards its top as the angle between the evaporation source and the particle surface decreases. Thus, as illustrated in Figure 2, a hemispherical particle after rotary shadowing is expected to appear as a dark ring surrounding a less dense central portion. The particle casts an annular shadow with a radius equal to the particle height divided by the sine of the shadowing angle (Figure 3). The thickness of the Pt/C film in this region should therefore be less than 20 Å. If the particle does not have vertical sides, there will be no sharp edge corresponding to the position of the boundary between the particle and the lipid layer (Figure 2). To determine the true particle diameter, it is necessary to calculate the width of the film at the base of the particle. In this case the true value (D) is related to the measured value by the equation:

$$D = y - 2x \quad \text{where } y = \text{measured diameter} \\ x = \text{film thickness at base (calculated)}$$

The value of 'x' can be calculated from a consideration of the amount of Pt/C deposited at the base of a particle during shadowing. For a uni-directionally shadowed particle, the film thickness on a horizontal surface, measured in the direction of shadowing is given by:

Figure 3. Diagram showing parameters used to calculate the width (x) of the platinum layer at the base of a uni-directionally shadowed hemispherical particle of height 'r', shadowing angle α and d_1 = thickness of platinum layer.

$$T = \frac{d_1}{\sin \alpha}$$

where d_1 = film thickness determined by quartz crystal monitor

During rotary shadowing, however, while the value of T is the same for flat areas as for uni-directional shadowing, the amount of Pt/C deposited at the base of a particle is less since the same amount of Pt/C must be evenly distributed over a larger area. The ratio of Pt/C deposited at the base of a hemispherical particle during rotary shadowing compared with uni-directional shadowing for the same length of time is given by:

$$\frac{\int_0^\pi K \sin \Theta \cdot d\Theta}{\int_0^{2\pi} K \cdot d\Theta}$$

where K = rate of Pt/C deposition

This formula yields:

$$\frac{[-\cos \Theta]_0^\pi}{[\Theta]_0^{2\pi}} = 1/\pi$$

That is, a rotary shadowed particle has a film thickness (T) measured in the direction of shadowing, which is $1/\pi$ of a particle shadowed without rotation. It follows from Figure 3 that the thickness of Pt/C at the base of the particle (x) is given by:

$$x = \frac{T \cdot \cos \alpha}{\pi} \\ = \frac{d_1}{\pi \cdot \tan \alpha}$$

For $d_1 = 20 \text{ \AA}$ and $\alpha = 22.5^\circ$, $x = 15.4 \text{ \AA}$. For a uni-directionally shadowed particle with $d_1 = 20 \text{ \AA}$ and $\alpha = 45^\circ$, $x = 20 \text{ \AA}$.

This means that a rotary shadowed particle with a measured diameter of $100 \text{ \AA}$ has a true diameter of $100 - 2 \times 15.4 = 69 \text{ \AA}$, i.e., its diameter would be overestimated by 45%.

2.5. Freeze-fracture particle measurements

Particle densities (number of particles per square micron) were measured on micrographs enlarged to $200,000 \times$ (EF faces) or $400,000 \times$

(PF faces) as previously described (34). Particle size and size distribution were determined with a digitiser-computer-plotter system (34) from micrographs taken at $100,00 \times$ and enlarged to $800,000$. For rotary shadowed preparations, the long and short axis (measured at right angles) of each particle were recorded, and plotted separately, and the semi-axis ratio calculated. No correction was made for the thickness of the Pt/C film.

3. RESULTS

Thylakoids of wild-type barley chloroplasts (Figure 4) are organised into stacked regions (grana) and unstacked regions. This is also true for the chloroplasts of *chlorina-f2* (Figure 5). It was found that the wild-type grana had an average diameter of $0.42 \pm 0.05 \text{ \mu m}$ ($\pm$ S.D.) and there were 10.4 ± 4.8 ($\pm$ S.D.) thylakoid discs per granum. This can be compared with *chlorina-f2* chloroplasts in which the grana had an average diameter of $1.01 \pm 0.29 \text{ \mu m}$ ($\pm$ S.D.) with 8.5 ± 3.4 ($\pm$ S.D.) thylakoid discs per granum (see Table VII).

The ultrastructure of rotary shadowed freeze-fractured thylakoids from wild-type barley is shown in Figure 6. Although the visual appearance of such a preparation differed considerably from uni-directionally shadowed thylakoids, the four different fracture faces could be identified on the basis of differences in particle size and density (see 35). They are shown at higher magnification in Figures 7–9. The particles on the EFs face (Figure 7) had the same appearance as shown diagrammatically in Figure 2. Each particle was bounded by a sharp edge which distinguished it from the lipid matrix. The centre of the particle was less electron dense, but there was not a clear boundary between this region and the platinum deposited at the base of the particle (see Figure 2). Visual inspection revealed that EFs particles varied not only in shape (from circular to elliptical), but also in size. The complementary PFs face (Figure 8) was densely covered with close-packed small particles. Most of these particles appeared to be uniformly sized but there were some particles that were slightly larger and more electron dense than the rest, as if they projected above the others.

Figure 4. Thin-sectioned chloroplast from wild-type barley seedling leaf grown for seven days in continuous light.

Thylakoid membranes are differentiated into stacked regions called grana (g) and unstacked regions (arrow-heads). $\times 75,000$ (Bar = $0.5\mu\text{m}$).

Figure 5. Thin-section of a chloroplast from *chlorina-f2* mutant of barley, grown under the same conditions as the wild-type in Figure 4.

Well-developed grana are clearly present at this stage. $\times 75,000$ (Bar = $0.5\mu\text{m}$).

Figure 6. Rotary shadowed freeze-fractured thylakoids from wild-type barley chloroplasts showing the relationship between the four different fracture faces.

Particles are visible as electron dense deposits which cast no shadow, in contrast to particles in unidirectionally shadowed replicas. $\times 100,000$ (Bar = $0.5\mu\text{m}$).

Figure 7. Enlargement of the EFs and EFu faces of wild-type barley thylakoids.

Note that particles of the EFs face vary in both size and shape and are more densely packed than those on the EFu face, with which it is continuous. The background of the EFu face is pitted with very small electron-transparent holes, in contrast to the smooth EFs face. $\times 150,000$ (Bar = $0.25\mu\text{m}$).

Figure 8. Micrograph showing the appearance of the PFs face of wild-type thylakoids and its relationship to the EFs face.

The particles on the PFs face are very densely packed. $\times 150,000$ (Bar = $0.25\mu\text{m}$).

Figure 9. The PFu face of wild-type thylakoids is covered with particles that are slightly larger and less densely packed than those on the PFs face. $\times 150,000$ (Bar = $0.25\mu\text{m}$).

Table II

Freeze-fracture particle densities of rotary shadowed wild-type and *chlorina-f2* chloroplast thylakoids. S.E. = standard error.

Material	Face	No./ $\mu\text{m}^2 \pm \text{S.E.}$	Area measured (μm^2)
Wild-type	EFu	347 ± 6	6.69
	PFu	4553 ± 25	1.28
	EFs	1624 ± 11	1.83
	PFs	6257 ± 33	1.02
<i>Chlorina-f2</i>	EFu	491 ± 7	7.34
	PFu	4092 ± 25	1.28
	EFs	2004 ± 11	5.59
	PFs	1124 ± 35	0.53

Table III

Freeze-fracture particles densities of uni-directionally shadowed wild-type and *chlorina-f2* chloroplast thylakoids. S.E. = standard error.

Material	Face	No./ $\mu\text{m}^2 \pm \text{S.E.}$	Area measured (μm^2)
Wild-type	EFu	428 ± 6	8.61
	PFu*	4378 ± 36	1.61
	EFs*	1670 ± 26	1.66
	PFs*	4662 ± 49	0.62
<i>Chlorina-f2</i>	EFu	558 ± 8	5.74
	PFu	3902 ± 22	1.54
	EFs	2103 ± 12	3.23
	PFs	1337 ± 28	0.77

* Values from SIMPSON (35).

The EFu face, which is continuous with the EFs, was characterised by a low density of particles that were smaller and rounder than those on the EFs (Figure 7). In contrast to the smooth background of the EFs, the EFu face was pitted with numerous small holes which

were seen as electron transparent spots. The PFu face (Figure 9) was covered with a large number of particles which were generally circular in outline and less densely packed than the particles on the PFs face.

The characteristics of rotary shadowed thyla-

koids were quantified by measuring particle size and numbers of particles per square micron for each of the four fracture faces (Tables II and IV). Comparable data for uni-directionally shadowed material are given in Tables III and V and in (35). The EFu and PFu particle densities as measured from rotary shadowed specimens were respectively 19% and 3% lower than the values from uni-directionally shadowed replicas. The density of particles on the PFu face was 4% higher for rotary shadowed material and 34% higher for the PFs face when compared with the corresponding uni-directionally shadowed face (Tables II and III). The particle density on the uni-directionally shadowed EFu face was much lower than previously reported (35). The higher value found in earlier work (736 versus 347 particles/ μm^{-2}) was probably due to movement of particles out of the EFs regions due to partial destacking under the lower ionic strength used (3mM- $\text{Ca}(\text{NO}_3)_2$ compared with 5mM- MgCl_2 in the present study).

The appearance of rotary shadowed freeze-fractured thylakoids from the mutant *chlorina-f2* can be seen at low magnification in Figure 10, which shows the relationship between three of the four fracture faces. The ultrastructure of the EFs and PFs faces shadowed uni-directionally is shown in Figure 11. The EFs face was covered with relatively large particles packed close together, lying on a smooth background. The PFs face had a small number of particles on a rough background containing pits and other irregularities (Figure 11). The EFs face, when examined by rotary shadowing (Figure 12), contained numerous particles which varied in both size and shape, ranging from elliptical to almost circular. The PFs face was characterised by a low density of approximately circular particles lying on an irregular background (Figure 12). It thus differed considerably from the equivalent face seen in wild-type thylakoids (Figure 8).

The EFu face of *chlorina-f2* was identical to the corresponding face of wild-type thylakoids both for uni-directional (Figure 13) and rotary (Figure 14) shadowing. The same was true for the PFu face (Figures 15 and 16). This information is given in quantitative terms in Tables III and V and in Figure 17, where the particle densities, particle size and size distribu-

Table IV

Freeze-fracture particle sizes of rotary shadowed wild-type and *chlorina-f2* chloroplast thylakoids. S.D. = standard deviation.

Face	Axis	Average size $\pm$ S.D. ($\text{\AA}$)	
		wild-type	<i>chlorina-f2</i>
EFu	small	105 $\pm$ 15	104 $\pm$ 14
	large	126 $\pm$ 18	121 $\pm$ 18
PFu	small	93 $\pm$ 15	91 $\pm$ 13
	large	112 $\pm$ 18	109 $\pm$ 16
EFs	small	117 $\pm$ 13	104 $\pm$ 13
	large	155 $\pm$ 20	138 $\pm$ 21
PFs	small	74 $\pm$ 12	90 $\pm$ 15
	large	93 $\pm$ 14	103 $\pm$ 17

tion of uni-directionally shadowed *chlorina-f2* thylakoids can be compared with data from wild-type thylakoids. The data for rotary shadowed *chlorina-f2* thylakoids is presented in Tables II and IV. The main difference between wild-type and mutant thylakoids was found on the PFs and EFs faces. The mutant PFs face had a greatly reduced number of particles (1124 / μm^2 versus 6257 / μm^2 for wild-type), and these particles were slightly larger than those on the wild-type PFs face (Tables IV and V). The EFs face of *chlorina-f2* thylakoids contained a higher density of particles than the wild-type (2004 / μm^2 versus 1624 / μm^2) and these particles were on average 12% smaller than the wild-type EFs face particles (Table IV). There were some small differences in the particle densities and sizes on

Table V

Freeze-fracture particle sizes of uni-directionally shadowed wild-type and *chlorina-f2* chloroplast thylakoids. S.D. = standard deviation.

Face	Average size $\pm$ S.D. ($\text{\AA}$)	
	wild-type	<i>chlorina-f2</i>
EFu	97 $\pm$ 17	102 $\pm$ 19
PFu	101 $\pm$ 27*	94 $\pm$ 16
EFs	144 $\pm$ 29*	126 $\pm$ 26
PFs	81 $\pm$ 13*	89 $\pm$ 18

* From SIMPSON (35).

Figure 10. Rotary shadowed freeze-fractured thylakoids of *chlorina-f2* chloroplasts showing the relationship between the PFs, EFs and EFu faces.

Note the large areas of fractured membranes from grana (EFs and PFs) in comparison with wild-type. $\times 100,000$ (Bar = $0.5\mu\text{m}$).

Figure 11. Uni-directionally shadowed freeze-fractured *chlorina-f2* thylakoids showing the appearance of the EFs and PFs faces.

The PFs face contains a low density of particles on a rough background. $\times 150,000$ (Bar = $0.25\mu\text{m}$).

Figure 12. Rotary shadowed freeze-fractured *chlorina-f2* thylakoids from the preparation used in Figure 11, showing the EFs and PFs faces.

These may be compared with the corresponding wild-type faces in Figures 7 and 8. $\times 150,000$ (Bar = $0.25\mu\text{m}$).

Figure 13. Uni-directionally shadowed freeze-fractured *chlorina-f2* thylakoids showing the appearance of the EFu face. $\times 150,000$ (Bar = $0.25\mu\text{m}$).

Figure 14. As in Figure 13, showing the EFu face after freeze-fracturing followed by rotary shadowing. $\times 150,000$ (Bar = $0.25\mu\text{m}$).

Figure 15. The PFu face from uni-directionally shadowed freeze-fractured *chlorina-f2* thylakoids. $\times 150,000$ (Bar = $0.25\mu\text{m}$).

Figure 16. The PFu face from the same material as in Figure 15 after rotary shadowing.

There is no significant difference between the EFu and PFu faces of wild-type and *chlorina-f2* thylakoids. $\times 150,000$ (Bar = $0.25\mu\text{m}$).

Table VI

Characteristics of rotary shadowed thylakoid surfaces of wild-type and *chlorina-f2*. S.D. = standard deviation. S.E. = standard error.

Surface	Axis	Particle size $\pm$ S.D. ($\text{\AA}$)	
		wild-type	<i>chlorina-f2</i>
PSu	small		99 ± 13
	large		120 ± 14
ESs	small	142 ± 11	122 ± 11
	large	191 ± 16	174 ± 15
No./ $\mu\text{m}^2 \pm$ S.E.		1450 ± 15	2054 ± 17
ESs array	small	130 ± 10	115 ± 10
	large	195 ± 11	178 ± 9
Centre-to-centre spacings		$152 \times 237 (\text{\AA})$	$145 \times 207 (\text{\AA})$

Table VII

Characteristics of wild-type and *chlorina-f2* grana. Values are $\pm$ standard deviation.

Parameter	wild-type	<i>chlorina-f2</i>
Diameter (μm)	0.42 ± 0.05	1.01 ± 0.29
No. of discs	10.4 ± 4.8	8.5 ± 3.4
Centre-to-centre spacing		
thin sections ($\text{\AA}$)	161.7 ± 5.4	196.9 ± 5.9
freeze-fracture ($\text{\AA}$)	272.5 ± 7.4	267.7 ± 13.8
Partition width ($\text{\AA}$)	193.8 ± 19.2	200.3 ± 16.4
Intrathylakoid space width ($\text{\AA}$)	79.3 ± 14.4	64.2 ± 12.1

Figure 17. Computer-drawn histogram plots of the size distribution of the particles on the four different fracture faces of uni-directionally shadowed *chlorina-f2* thylakoids (light lines) compared with those of the corresponding faces of wild-type thylakoids (heavy lines).

Numerical values are summarised in Table V.

Figure 18. The ESs of wild-type thylakoids after etching and rotary shadowing.

The tetrameric particles sometimes form arrays in stacked thylakoids. The average size of such particles is $130 \times 195 \text{ Å}$ with centre-to-centre spacings of $152 \times 237 \text{ Å}$. $\times 300,000$ (Bar = $0.1 \mu\text{m}$).

Figure 19. Rotary shadowed ESs array of *chlorina-f2* thylakoids.

Particles appear very similar to wild-type, having a tetrameric substructure and similar surface relief, with an average size of $115 \times 172 \text{ Å}$ and centre-to-centre spacings of $145 \times 207 \text{ Å}$. $\times 300,000$ (Bar = $0.1 \mu\text{m}$).

Figure 20. Rotary shadowed ESs and ESu of wild-type thylakoids.

The ESs is covered with tetrameric particles with an average size of $142 \times 191 \text{ \AA}$. The ESu has a particulate appearance suggesting the presence of numerous particles, but these are difficult to resolve clearly from one another. $\times 150,000$ (Bar = $0.25 \mu\text{m}$).

Figure 21. The ESs and ESu of *chlorina-f2* thylakoids after etching and rotary shadowing.

As found for wild-type, the ESs is covered with tetrameric particles, with an average size of $122 \times 174 \text{ \AA}$ and a density close to that of EFs particles. The appearance of the ESu indicates that it is covered with particles of a low surface relief. $\times 150,000$ (Bar = $0.25 \mu\text{m}$).

the EFu and PFu faces of mutant thylakoids compared with wild-type (Tables II and IV), but these were small in comparison with those found for the PFs and EFs faces.

Since it is thought that ESs particles correspond to the intramembranous EFs particles (28, 30), the nature of these particles on mutant and wild-type thylakoids was also investigated by rotary shadowing. These particles were sometimes found in arrays in stacked thylakoids of wild-type (Figure 18) and *chlorina-f2* (Figure 19). The centre-to-centre spacings of these arrays (Table VI) revealed that *chlorina-f2* ESs particles were spaced slightly closer together than those of wild-type, mainly in the longer dimension. The size of particles in *chlorina-f2* ESs arrays (Table VI), were 13% smaller than the wild-type, which was the same as the difference in size of the EFs particles. A similar size difference was found for ESs particles not in arrays, which were slightly wider than the particles in arrays (Table VI). The size of the ESs particles was much more uniform than the EFs particles, although the number of particles per square micron was close to that found for the EFs face (Tables II, IV and VI).

The majority of ESs particles, both in arrays and not arrayed, in wild-type and *chlorina-f2*

thylakoids, had a tetrameric substructure (Figures 18–21). The relationship between these particles and PFs particles can be seen in Figure 22 in which the rows of ESs particles coincide with the rows of grooves between PFs particles. The ESs of *chlorina-f2* thylakoids occasionally contained particles in a square lattice (Figure 23) similar to those on the ESs of wild-type thylakoids (35). A close examination of the ESu of wild-type (Figure 20) and mutant (Figure 21) thylakoids revealed no tetrameric particles. Instead there were a large number of particles of low surface relief, which were much smaller than those seen on the ESs. These particles were not seen in specimens shadowed at 45° from one direction (35, 39).

The PSu faces of wild-type and *chlorina-f2* thylakoids were essentially the same, being covered with many large and small particles (Figure 24). The large particles, thought to correspond to coupling factor 1 and ribulose-1,5-bisphosphate carboxylase molecules (39) were often clustered into groups of 5 to 15 particles. The average size of the large particles was determined to be about $100 \times 120 \text{ \AA}$ (Table VI).

When transversely fractured rotary shadowed thylakoids were examined, it was possible to distinguish the partition region from the intra-

Figure 22. Etched preparation of de-stacked thylakoids of wild-type barley demonstrating that the grooves between double rows of PF particles correspond with the rows of ES particles. $\times 300,000$ (Bar = $0.1 \mu\text{m}$).Figure 23. This type of lattice was occasionally seen on the ES of *chlorina-f2* thylakoids, and has previously been found on wild-type thylakoids (35) $\times 300,000$ (Bar = $0.1 \mu\text{m}$).Figure 24. Rotary shadowed PSu of *chlorina-f2* thylakoids.

The large particles, thought to be the coupling factor 1 and ribulose-1,5-bisphosphate carboxylase, measure about $100 \times 120 \text{ \AA}$ and often occur in small clusters. There are also many smaller particles present. $\times 300,000$ (Bar = $0.1 \mu\text{m}$).

Figure 25. Rotary shadowed transversely fractured grana of wild-type thylakoids.

This type of micrograph was used to determine the centre-to-centre separation of thylakoid discs, as well as the width of the partition and intrathylakoidal space. The electron dense particles, which are confined to one membrane only in appressed regions, may be plastically deformed freeze-fracture particles. It is possible that the surface irregularities, particularly of the unstacked membranes, are due to particles seen on the PSu and ESs. $\times 150,000$ (Bar = $0.25\mu\text{m}$).

Figure 26. As in Figure 25, showing rotary shadowed transversely fractured grana from *chlorina-f2* chloroplasts. $\times 150,000$ (Bar = $0.25\mu\text{m}$).

thylakoidal space (Figures 25 and 26). It was also possible, by looking along the partition, to distinguish the boundary between the two thylakoid membranes. Such micrographs were used to make measurements of the centre-to-centre spacing of the thylakoids in grana for the purpose of comparing wild-type and mutant, and thin-sectioned and freeze-fractured material (Table VII). In addition, the width of the partition and intrathylakoid space in freeze-fractured material was measured. It was apparent that the thylakoids were much closer together in thin-sectioned material, mainly due to a reduction of the partition width. There was no significant difference, however, in the spacings of freeze-fractured wild-type or *chlorina-f2* grana and values for membrane thickness, calculated from the partition width, was about $100\text{ \AA}$ in both cases (Table VII). The particles seen in these cross-fractured membranes (Figures 25 and 26) were usually confined to one half of the partition, and never extended across the entire width. These particles could, therefore, be EFs or PFs particles that have been fractured

Figure 27. The polypeptide composition of wild-type and *chlorina-f2* thylakoids compared with that of isolated light-harvesting complex.

The polypeptides were separated by SDS-urea-polyacrylamide gel electrophoresis (25). The major difference between wild-type and *chlorina-f2* is the absence of the band in the mutant pattern corresponding to the apoprotein of chlorophyll *a/b*-protein 2.

Figure 28. The structure of the isolated light-harvesting complex after Mg^{++} precipitation is shown by conventional fixation and thin-sectioning techniques.

In transverse section, this material has a lamellar structure and forms closely appressed stacks. When sectioned parallel to the plane of the lamella, regions of hexagonally packed particles are revealed (arrowhead). $\times 75,000$ (Bar = $0.5\mu m$).

in a plane at right angles to the plane of membrane cleavage.

A comparison of the polypeptide composition of purified thylakoids isolated from wild-type and *chlorina-f2* chloroplasts (Figure 27) shows that the major difference is the absence of the light-harvesting chlorophyll *a/b*-protein (25). It is possible to isolate this protein in reasonably pure form by detergent treatment of wild-type thylakoids (9). Characterisation of the light-harvesting complex by freeze-fracturing and

rotary shadowing should be useful to establish which particles they might correspond to in wild-type thylakoids, and to see if such particles are absent from *chlorina-f2* thylakoids. The polypeptide composition of purified light-harvesting complex is shown in Figure 27. The final step in the purification of this light-harvesting complex (LHC) involved Mg^{++} precipitation and centrifugation through a 0.5 M -sucrose cushion. The resulting pellet consisted of compact material at the bottom of the centrifuge tube with

Figure 29. The appearance of the light-harvesting complex preparation in Figure 28 is shown after freeze-fracturing and freeze-etching for 1 min at -100°C , followed by rotary shadowing.

The fracture face contains hexagonally arrayed particles with an average centre-to-centre distance of $122\text{\AA}$. The etched surface consists of hexagonally arrayed electron transparent holes. The rows of holes on this surface coincide with the grooves between rows of particles on the fracture face. The boundary between the fracture face and etched surface is easily recognised (arrowheads). $\times 300,000$ (Bar = $0.1\mu m$).

Figure 30. Light-harvesting complex vesicles (liposomes) from the soft pellet, showing areas of hexagonally arrayed particles and areas of lipid containing individual particles (arrows).

Particle arrays were seen with equal frequency on both convex and concave (asterisk) faces. $\times 100,000$ Bar = $0.5\mu m$).

softer material above it. The appearance of the hard pellet after fixation and thin sectioning is shown in Figure 28. It consisted of membrane-like lamellae which appeared to stack together in many places. When these lamellae were sectioned parallel to their surface, a hexagonal lattice was seen (Figure 28). The same material was examined by freeze-fracturing and rotary shadowing and it was found that the lamellae directed the fracture plane, revealing particles in hexagonal arrays (Figure 29). It can be seen that these lamellae were cleaved by freeze-fracturing since there is a discontinuity (Figure 28) where the fracture face meets the etched surface. The rows of particles on the fracture face coincided with the lines between rows of holes in hexagonal arrays on the etched surface.

The soft pellet contained numerous large vesicles which were visible under phase contrast light microscopy. When freeze-fractured, these vesicles contained particles in close-packed arrays and less densely packed particles in lipid rich areas (Figure 30). These arrays deviated by no more than 2° from a hexagonal lattice and the average centre-to-centre spacing in all three directions was $122.3 \pm 1.9 \text{ \AA}$ ($\pm$ S.D.). It can be seen from Figures 29 and 30 that there were two types of particles. The majority was of moderate electron density and had dimensions of 70.3 ± 7.3 by $94.6 \pm 11.0 \text{ \AA}$ ($\pm$ S.D.). The other type of particle was very electron dense with more sharply defined edges and had an average size of 77.4 ± 6.3 by $87.4 \pm 6.7 \text{ \AA}$ ($\pm$ S.D.). These particles were found both in arrays and in smooth areas by themselves. Particle arrays covered most of the area of the vesicles and were found on both convex fracture faces and concave fracture faces (Figure 30). The size of the particles in freeze-fractured light-harvesting complex vesicles correspond closely with that of the particles missing from the PFs face of *chlorina-f2* thylakoids.

4. DISCUSSION

Rotary shadowing, particularly of the EFs face, has shown that freeze-fracture particles of thylakoids range in shape from circular to elliptical, i.e., they have this cross-sectional shape in the plane of membrane cleavage. This

confirms earlier work (35) predicting elliptical shape of EFs particles in ordered arrays. The variety of particle sizes and shapes explains why it is not usually possible to resolve EFs particles into discrete size classes when their size distribution is plotted (Figure 17). Even when measurements are made on a population of particles known to have the same composition and size (those of the light-harvesting complex), the standard deviation of such measurements is about $6.5 \text{ \AA}$. To determine the precision of the digitiser, one hundred figure 0's on a sheet of Letraset were measured. These 7.7 mm high figures were imagined to be 800,000 times magnified and yielded values of $98.4 \pm 1.5 \text{ \AA}$ by $72.0 \pm 2.4 \text{ \AA}$ ($\pm$ S.D.), for the large and small diameters, respectively. Thus, the method is sufficiently accurate to resolve populations of uniformly sized freeze-fracture particles differing in size by $13 \text{ \AA}$ (i.e. 2 times $6.5 \text{ \AA}$).

The technique of rotary shadowing of etched surfaces enables the visualisation of surface particles with greater clarity than is possible with uni-directional shadowing. This is due partly to the lower shadowing angle (22.5° versus 45°) and partly to the fact that the particles are coated on all sides, so that detail is not obscured by the shadow. The tetrameric substructure of the ESs particles is particularly clear, and fine structure of the ESu has been revealed in greater detail than before. There are also suggestions of substructure in the large PSu particles (Figure 24), although not as clearly and uniformly as for ESs particles. Ribulose-1,5-bisphosphate carboxylase and coupling factor 1 are located on the PSu (39) and contain subunits with molecular weights of about 55,000 daltons (45).

No attempt was made in this study to describe fine structure of rotary shadowed particles on fracture faces. It seems likely that any such detail is destroyed by plastic deformation during freeze-fracturing of thylakoids, although it is thought that this deformation is less extensive in membranes where complementarity between the EF and PF faces is preserved (14, 22). Such fine structure has been reported for rotary shadowed particles of the PF face of the red blood cell membrane (26), but it is not clear to what extent it is caused by decoration, particularly under conditions of low shadow angle and a thin coating of platinum.

Figure 31. High magnification electron micrograph of the particles on the EFs face of *chlorina-f2* thylakoids after freeze-fracturing and uni-directional shadowing at 45° .

The shape of the shadow indicates that the particles have a rounded contour and are probably hemispherical, although judging from the length of the shadow (= particle height = $150 \text{ \AA}$), considerable plastic deformation must have occurred during freeze-fracturing. $\times 1,000,000$ (Bar = 25 nm).

Figure 32. Rotary shadowed EFs particles of *chlorina-f2* thylakoids.

Particle shape varies continuously between circular and elliptical, and each particle has the appearance shown schematically in Figure 2. $\times 1,000,000$ (Bar = 25 nm).

It is often difficult to make accurate measurements of particle density on densely populated fracture faces from uni-directionally shadowed replicas. Particles may not be visualised if they are of low surface relief and/or lie in the shadow of larger particles. Rotary shadowing overcomes this problem to some extent as demonstrated by the increased density found on the PFs face of rotary shadowed wild-type thylakoids compared with uni-directionally shadowed material (Tables II and III). There is still a degree of subjectivity in deciding whether a feature is a particle for the purposes of counting. The lower shadowing angle used in rotary shadowing allows the holes in the EFu face of the thylakoid to be visualised easily (compare Figures 13 and 14). The density of these holes was estimated to be 3298 ± 46 ($\pm \text{S.E.}$), which is about two-thirds of the density of the particles on the PFu face. It remains to be determined whether all particles

on the PFu face have complementary holes, or only a fraction of them.

There is a lower limit to the shadowing angle that can be usefully employed during rotary shadowing. In particular, shading of one particle by others becomes more and more pronounced as the angle is decreased. Although it is not possible to make accurate estimates of particle heights from uni-directionally shadowed replicas, since it is difficult to determine the angle of the fracture plane with respect to the evaporation source, an approximate value of $150 \text{ \AA}$ seems reasonable for EFs particles (see Figure 31). The shadow length is equal to the particle height divided by the sine of the shadowing angle, which for EFs particles shadowed at 22.5° is $390 \text{ \AA}$. Particles on the EFs face are closer together than $390 \text{ \AA}$ so that the background of the EFs face is less electron dense than the EFu (Figure 7) due to a thinner layer of platinum

resulting from shading by overlapping shadows of EFs particles.

When the plane of an area of a rotary shadowed specimen deviates from the horizontal, the particles cast a shadow on one side, and have much less platinum deposited on that side (see areas in Figures 24 and 30). It is therefore easy to determine visually whether a surface is horizontal or not. The values obtained from rotary shadowed replicas for the particle densities of EFs and EFu faces are slightly lower than corresponding values from uni-directionally shadowed material (Tables II and III). The probable explanation is that areas used from uni-directionally shadowed thylakoids were not always horizontal, causing a reduction in the size of the projected area and an apparent increase in measured particle density. One can use shadow length as a criterion for tilting in the direction of the evaporation source, and focusing differences to determine tilting of the specimen at right angles to the evaporation gun, but an error of only 10° in both directions, causes a 3% overestimation of particle density.

Visual selection of transversely fractured grana oriented horizontally in rotary shadowed material allows the accurate determination of the repeat distance between thylakoid discs and the width of the thylakoid membrane in material that has not been subjected to chemical fixation, dehydration, embedding and thin-sectioning. The value of approximately 270 Å for the repeat distance and 200 Å for the partition thickness is consistent with earlier freeze-fracture results, and values measured from aqueous-plastic-embedded material (see 21). Since the lipid bilayer is 40 Å thick, or maximally 60 Å in freeze-fractured membranes (21), and a grana thylakoid membrane is 100 Å thick, the protein projects at least 40 Å out of the bilayer. Further experiments are needed to decide if the particles seen in transversely fractured membranes correspond to EFs and PFs particles fractured at right angles to the normal freeze-fracture plane (Figures 25 and 26).

A comparison of particle sizes in corresponding faces of uni-directionally and rotary shadowed thylakoids revealed surprisingly small differences (Tables IV and V). In the case of *chlorina-f2* thylakoids and for the EFu face of wild-type, these comparisons were made from rotary

shadowed and uni-directionally shadowed replicas prepared from the same material. Sizes obtained for rotary shadowed material were about 7% greater than uni-directionally shadowed thylakoids, except for the EFs face, where particles were about 5% smaller in rotary shadowed replicas. It had been assumed that determination of shadow width at the base of the particle at right angles to the direction of shadowing in uni-directionally shadowed material would provide an accurate measure of particle width (39). Calculations for rotary shadowed particles (Section 2.4) indicated that the size of rotary shadowed particles would be overestimated by about 30 Å. These calculations were based upon the assumption that, among other things, particles were approximately hemispherical after freeze-fracturing. An examination of the shape of the shadow of uni-directionally shadowed *chlorina-f2* EFs particles (Figure 31) indicates that this assumption is justified, and the appearance of these particles after rotary shadowing (Figure 32) is consistent with that predicted for a hemispherical particle (Figure 2). It is possible that shading effects may cause a reduction in the thickness of platinum deposited at the base of a particle, particularly on the PFu and PFs faces. This phenomenon may be responsible for the slight reduction in width of ESs particles in arrays (see Table VII). An investigation of platinum-shadowed gold particles indicated that measurement of shadow width of uni-directionally shadowed particles overestimates the true diameter by between 40 and 70 Å, depending on particle size (33). This was due to growth of platinum crystals on the gold particle. The present study shows that uni-directionally shadowed freeze-fracture particles have the same diameter as the corresponding rotary shadowed ones. This suggests that values for particle widths from uni-directionally shadowed replicas may be overestimated by about 30 Å on faces that are not densely populated. On the other hand, the size of the large particles on the PSu (100×120 Å) is close to the reported values for coupling factor 1, which is a sphere with a diameter of 110 Å (31) and ribulose-1,5-bisphosphate carboxylase, which is a cube with 120 Å long sides (46). The relationship between particle size as measured from freeze-etched preparations and by physical methods needs to

Figure 33. Diagrammatic representation of the freeze-fracture behaviour of the thylakoid membrane from grana of wild-type barley chloroplasts.

The light-harvesting complex is visualised as being localised in particles distinct from the EFs particles, but closely associated with them. During freeze-fracturing the light-harvesting complex and EFs particles are separated by the fracture plane and cleave with opposite halves of the thylakoid membrane.

be determined using lipid bilayers into which a single purified protein of known dimensions has been inserted.

The *chlorina-f2* mutant lacks chlorophyll *b* and consequently has no chlorophyll *b*-containing proteins (6, 25). Although the original paper describing the ultrastructure of the chloroplasts of this mutant reported the presence of grana (16), many authors cite this mutant as an instance where the absence of the light-harvesting chlorophyll *a/b*-protein is correlated with the absence of grana. This seems to have resulted from a desire to establish a causal relationship between light-harvesting chlorophyll *a/b*-protein and stacking in thylakoids, since early studies of detergent fractionated thylakoids indicated that the light-harvesting chlorophyll *a/b*-protein was located in the grana (see ANDERSON for a review). It has been shown by others (10, 16, 30) and by the present investigation using plants of the same age and grown under the same conditions as the wild-type control, that grana are present in *chlorina-f2* (Figure 5). In seven-day old seedlings, most of the

grana in *chlorina-f2* chloroplasts are at least twice the diameter of wild-type grana, and they have a slightly lower number of discs (Table VII).

The striking difference between the freeze-fracture ultrastructure of wild-type and *chlorina-f2* thylakoids is in the appearance of the PFs face, both in uni-directionally and rotary shadowed preparations (Figures 11 and 12). In the latter there is a reduction in the number of particles from 6257 to 1124 per μm^2 (Table II). It is concluded that since chlorophyll *a/b*-protein 2 is such a major polypeptide in wild-type thylakoids, it is located in the PFs particles absent from the *chlorina-f2* mutant (Fig. 33). It is known that PFs particles align themselves with respect to EFs particles when such particles form arrays (35, 38), and are probably in intimate contact with them. The major difference between the interpretation in Figure 33 and that of ARMOND et al. (3) is that the freeze-fracture plane follows a hydrophobic region between the light-harvesting complex and the EFs particle so that each cleaves with opposite sides of the mem-

brane. This is consistent with the method for isolating the light harvesting complex in which Triton X-100 is used to disrupt the hydrophobic association between this protein and the other membrane components (9). Furthermore, the size of the freeze-fracture particles in light-harvesting complex vesicles is close to that of the particles missing from the *chlorina-f2* PFs face, but present on the wild-type PFs face.

It is likely that the EF particles of thylakoids are the structural equivalents of photosystem II units, although the evidence is indirect (3, 4, 40). Since fluorescence studies indicate a close association between the light-harvesting chlorophyll *a/b*-protein and photosystem II reaction centres (4, 23), many models of the structure of the thylakoid membrane place the light-harvesting chlorophyll *a/b*-protein in the EFs particles (3, 4, 12, 21, 40). Most of the evidence to support this model comes from a study of the greening in continuous light of plants grown under intermittent light (3). It was found that EFs particles increased in size in a stepwise manner which was correlated with the appearance of the light-harvesting chlorophyll *a/b*-protein during greening. The major change in the freeze-fracture ultrastructure during greening of these plants was the increase in EFs particle size, and the major change in the polypeptide composition was an increase in the amount of the light-harvesting chlorophyll *a/b*-protein. This was interpreted as an addition of light-harvesting chlorophyll *a/b*-protein subunits to a photosystem II 'core' particle (3, 18). Apart from difficulties in resolving particle size groups in a population of elliptical particles shadowed from one direction, there was an implicit assumption that the PFs particles that appeared during greening were the same as, or were derived from, particles present on the PF face before greening commenced. In addition, it is not clear from scans of the SDS-polyacrylamide gels published, that the light-harvesting chlorophyll *a/b*-protein was the only component which showed a relative increase during greening.

A model in which the light-harvesting chlorophyll *a/b*-protein was located in EFs particles was apparently supported by early freeze-fracture studies of the light-harvesting chlorophyll *a/b*-protein deficient *chlorina-f2* thylakoids which were reported to lack large EFs

particles (1, 5). These studies were perhaps complicated by the instability of these membranes in low salt conditions. The results from MILLER et al. (30) and the present paper, clearly demonstrate the presence of EFs particles in *chlorina-f2* thylakoids. The SDS-urea polyacrylamide gel in Figure 27 shows that the mutant thylakoids contain almost all of the 60 polypeptides resolved in wild-type thylakoids. For greening of pea thylakoids after intermittent illumination, an EFs particle size increase of 100% was found (3), which is comparable to the 80% increase determined for greening of dark-grown barley thylakoids (34, 35). In contrast the EFs particles of *chlorina-f2*, which completely lacks the light-harvesting chlorophyll *a/b*-protein, are only 12% smaller than wild-type (Table IV).

The diagram in Figure 34 is a hypothetical representation of the organisation of the light-harvesting complex and EFs particles within the grana membrane. The figure is drawn to scale, taking into consideration values obtained for partition thickness, particle spacings in EFs, PFs and light-harvesting complex arrays, and particle sizes at the ESs and fracture plane. This arrangement may offer a mechanism for energy transfer which is known to occur between at least four photosystem II units (11, 19). One of the problems in placing photosystem II reaction centres in EF particles is that they appear to be too far apart to allow energy transfer, but if this process can occur across two appressed thylakoids via the light-harvesting chlorophyll *a/b*-protein located in PFs particles, this objection is overcome (see also ARNTZEN (4)). It seems that the light-harvesting chlorophyll *a/b*-protein does not mediate energy transfer within the plane of a single thylakoid membrane, since it does not occur in de-stacked thylakoids, as deduced from the kinetics of fluorescence induction in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (see 19). No measurements of this kind have been published for stacked thylakoids of *chlorina-f2*, so it is not known if energy transfer occurs across the grana in this mutant, although the fluorescence induction kinetics of *chlorina-f2* and wild-type barley leaves are qualitatively the same (23). Energy transfer between photosystem II units has been demonstrated in non-stacked membranes, however, in the III-C mutant of *Cyanidium cald-*

Figure 34. Model of the thylakoid membrane from the grana of wild-type barley chloroplasts.

The size and shape of the EFs and PFs particles have been calculated from measurements of EFs particles and those in light-harvesting complex vesicles, which yield the cross-sectional size at the fracture plane. In addition, the centre-to-centre distance of light-harvesting complex particles in arrays has been used as the maximum width of the PFs particle at the PSs. The relationship between EFs and PFs particles has been deduced from the appearance of particles arrays in barley thylakoids (35). Light-harvesting complex particles may make contact at the PSs, but are not shown as spanning the membrane until more data have been obtained. It is possible that the appearance of the light-harvesting complex particles at the ESs corresponds to the etched surface seen in Figure 29. It is assumed that the particles in light-harvesting complex vesicles correspond to the particles missing from the PFs face of the *chlorina-f2* mutant.

rium, which lacks both chlorophyll *b* and phycobilisomes (11). In this mutant (47), as in *chlorina-f2*, there is also an increase in the density of EF particles compared with the corresponding wild-type, so the particles may be close enough for direct energy transfer.

Studies with the isolated light-harvesting chlorophyll *a/b*-protein (10, 41) and the *chlorina-f2* mutant show that the light-harvesting chlorophyll *a/b*-protein is involved in membrane stacking, but is not essential for grana formation, since *chlorina-f2* thylakoids form grana. Grana are also present in mutants which are deficient in photosystem II activity and have a greatly reduced density of EFs particles, but a normal PFs face and large amounts of light-harvesting chlorophyll *a/b*-protein (29, 36, 37). The formation of grana with few EFs particles is difficult to explain if the light-harvesting chlorophyll *a/b*-protein is located in EFs particles. The slightly smaller size (about 20 Å) of the *chlorina-f2* particles, both at the ESs and EFs

planes (Tables IV and VI) may be due to the absence of another chlorophyll *b*-containing protein, chlorophyll *a/b*-protein 1 (25), which may be involved in transferring excitons from the light-harvesting chlorophyll to the reaction centre of photosystem II. The particles remaining on the PFs face of *chlorina-f2* thylakoids may correspond to photosystem I units.

Although some models show the light-harvesting complex as spanning the membrane (3, 4, 21), there is only evidence that it is exposed at the outer surface (ESs) (9, 41). In liposomes containing light-harvesting complex, holes between particles are seen on the surface (Figure 29), while the arrays on the PS of thylakoids (28, 35) may be revealing the surface appearance of the light-harvesting complex on the other side of the membrane. This is taken into consideration in Figure 34, although new data may prove that the light-harvesting complex does indeed span the membrane. The molecular composition of light-harvesting complex particles has not been

determined. However, a correlation between the size of freeze-fracture particles in the purple membrane of *Halobacterium halobium* and the electron diffraction map of this membrane has shown that each particle of 120 Å diameter consists of several polypeptides, with a total molecular weight of about 300,000 (14). It is not valid to make a direct comparison between this membrane and light-harvesting complex liposomes since sufficient data are not yet available, but light-harvesting complex particles may contain about eight molecules of the light-harvesting chlorophyll *a/b*-protein, each of which contains approximately 14 molecules of chlorophyll (9).

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