FREEZE-FRACTURE STUDIES ON BARLEY PLASTID MEMBRANES V. *VIRIDIS-n³⁴*, A PHOTOSYSTEM I MUTANT

by

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Keywords: Chloroplast, electron microscopy, photosystem I, *P700*-chlorophyll *a*-protein 1, rotary shadowing

Thylakoids of chloroplasts isolated from the nuclear gene barley mutant, *viridis-n³⁴*, were examined by electron microscopy of rotary shadowed freeze-fracture replicas. This mutant was known to be partially deficient in the *P700* chlorophyll *a*-protein 1, which is the reaction centre of photosystem I. The ultrastructure of the four fracture faces was characterised in terms of particle density and size distribution. When compared with wild-type thylakoids, the major difference was a reduction in the average size of the PFu particles, due to the specific loss of many of the large, heavily shadowed particles from this face. This was accompanied by a similar reduction in the number of pits in the complementary EFu face. It is concluded that the *P700* chlorophyll *a*-protein 1 is localised in the large particle population of the PFu face, and that these particles form the pits on the EFu face during freeze-fracturing. Loss of this chlorophyll protein causes a reduction in particle size, without significant change in particle density. This is in marked contrast to earlier results (13,15) with the light-harvesting chlorophyll *a/b*-protein 2 complex and the reaction centre of photosystem II containing chlorophyll *a*-protein 3, the loss of which causes a loss of particles on the PFs and EFs faces respectively.

1. INTRODUCTION

The ultrastructural organization of the photosynthetic membrane of chloroplasts has been extensively analysed by freeze-fracture electron microscopy. Four different fracture faces can be distinguished on the basis of the size and density of their freeze-fracture particles, corresponding to EF and PF faces of stacked and unstacked

regions of the thylakoid membrane (3,13,14). It is likely that the freeze-fracture particles, which are located within the membrane, are composed largely of protein, consisting of several different polypeptides in stoichiometric amounts (13). They thus represent both structural and functional units within membranes.

The nature and composition of the different freeze-fracture particles can be deduced by examining thylakoids of chloroplasts from mutants. The absence of one or more polypeptides from mutant membranes can be correlated with differences between the freeze-fracture ultrastructure of wild-type and mutant thylakoids (8,13,15). Using the chlorophyll *b*-less mutant, *chlorina-f2*, it has recently been shown that the light-harvesting chlorophyll *a/b*-protein 2 is localised in the majority of particles on the PFs face (13). This paper describes the ultrastructure of rotary shadowed freeze-fractured thylakoids from the well-characterised barley mutant *viridis-n³⁴*, which is partially deficient in photosystem I activity (4,5,9,16,17).

2. MATERIALS AND METHODS

Seedlings were grown for 7 days in continuous white light and chloroplasts isolated by homogenization and differential centrifugation as previously described (12). Isolated, unfixed thylakoids were freeze-fractured or freeze-etched using a Balzers BAF 301 (Balzers, Liechtenstein) unit fitted with a rotary stage, and measurements of particle size and density were made according to the procedures described earlier (12,13). Electron micrographs were taken with a Philips EM400 operated at 80 kV and calibrated with catalase crystals (Balzers Union) using a lattice spacing of 87.5 Å.

3. RESULTS

Thin-section electron microscopy of *viridis-n³⁴* chloroplasts revealed that the thylakoids were organised into stacked regions (grana) and unstacked regions (stroma lamellae). In some chloroplast profiles, some of the stroma lamellae appeared to have collapsed, so that the intrathylakoidal space was no longer visible (Figure 1). With this exception, the appearance of mutant thylakoids resembled that of wild-type chloroplast membranes.

Four different freeze-fracture faces could be distinguished for *viridis-n³⁴* thylakoids (Figure 2). The ultrastructure of the EF and PF faces from granal regions (Figure 4) closely resembled that of the corresponding faces of wild-type thy-

lakoids (Figure 3). The same was true for the EFu face, although the pits that characterise this face were not as numerous, nor as prominent as found for wild-type (Figures 5 and 6). Visual inspection of *viridis-n³⁴* PFu fracture faces revealed subtle, but significant differences when compared with the PFu face of wild-type thylakoids. The rotary shadowed PFu face of wild-type thylakoids was characterised by numerous large, well-shadowed particles (Figure 7), and this feature helps to distinguish it from the PFs face (12,13). The PFu face of *viridis-n³⁴* thylakoids contained fewer of these large particles, although the total particle density was not greatly altered (Figure 8). Occasionally the fracture plane passed through a collapsed stroma lamella region, corresponding to similar features seen in thin-sectioned material.

The true surfaces of *viridis-n³⁴* thylakoids revealed by freeze-etching were similar to those of wild-type. The PSu was covered with large particles, often in clusters, and the ESs was characterised by large numbers of tetrameric particles. The ESu appeared to be covered with particles of low surface relief, as was found for wild-type (13), but it was difficult to determine whether there were any significant differences from wild-type, corresponding to those found on the PFu face.

Comparisons between corresponding fracture faces of wild-type and mutant thylakoids were made by objectively measuring particle density and size distribution for each face (14). The differences in particle density were small, except for the EFu face of *viridis-n³⁴* thylakoids, which had fewer particles per square micron than that of wild-type (Table I). The average size of the freeze-fracture particles of *viridis-n³⁴* was between 20 - 30 Å smaller than those on corresponding faces of wild-type, with the exception of the PFs face (Table II). The data for *viridis-n³⁴* particle size and shape are plotted as three-dimensional histograms in Figure 9, and may be compared with those for wild-type previously published (14). Statistical comparison of particle sizes for corresponding faces was made using the χ^2 test. Highly significant differences ($p < 0.0005$) were found for the PFu, EFu and EFs faces, and a smaller difference ($p < 0.05$) for the PFs face (Table III).

Figure 1. Thin section of a chloroplast in a seedling leaf of *viridis-n³⁴*.

The thylakoids are differentiated into well-organised grana (g) and stroma (s) lamellae which are similar to those found in wild-type, with the exception that some stroma lamellae appear to have collapsed (arrows) and have no intrathylakoid space. (Bar = 0.5 μ m). $\times$ 54,000.

Table I

Freeze-fracture particle densities of wild-type and *viridis-n³⁴* thylakoids

S.E. = standard error of the mean

Material	Face	No./ μ m ² $\pm$ S.E.	Area measured (μ m ²)
wild-type*	EFu	347 $\pm$ 6	6.69
	PFu	4553 $\pm$ 25	1.28
	EFs	1624 $\pm$ 11	1.83
	PFs	6257 $\pm$ 33	1.02
<i>viridis-n³⁴</i>	EFu	200 $\pm$ 6	4.04
	PFu	4331 $\pm$ 28	0.83
	EFs	1455 $\pm$ 19	1.03
	PFs	6219 $\pm$ 32	0.70

* Data from SIMPSON (1979).

Table II

Freeze-fracture particle sizes of wild-type and *viridis-n³⁴* thylakoids

S.D. = standard deviation

Face	Axis	Average size $\pm$ S.D. ($\text{\AA}$)	
		wild-type*	<i>viridis-n³⁴</i>
EFu	small	105 $\pm$ 15	83 $\pm$ 14
	large	126 $\pm$ 18	96 $\pm$ 15
PFu	small	93 $\pm$ 15	74 $\pm$ 14
	large	112 $\pm$ 18	92 $\pm$ 19
EFs	small	117 $\pm$ 13	94 $\pm$ 12
	large	155 $\pm$ 20	127 $\pm$ 21
PFs	small	74 $\pm$ 12	74 $\pm$ 10
	large	93 $\pm$ 14	90 $\pm$ 14

* Data from SIMPSON (1979)

Figure 2. Low magnification survey freeze-fracture image of *viridis-n³⁴* thylakoids.

Four distinct freeze-fracture faces can be distinguished and bear the same morphological relationship as that found for wild-type thylakoids. (Bar = 0.5 μ m). $\times$ 100,000.

Figure 3. High magnification micrograph of the EFs and PFs faces of wild-type grana. (Bar = 0.2 μm). $\times 248,000$.

Figure 4. Freeze-fracture image of the EFs and PFs faces of *viridis-n³⁴* grana.

The EFs face particles are slightly smaller and less densely packed than those of the wild-type, whereas there is no significant difference for the PFs face. (Bar = 0.2 μm). $\times 248,000$.

Figure 5. The EFu face of wild-type thylakoids is characterised by a low particle density on a pitted background. (Bar = 0.2 μ m). $\times$ 248,000.

Figure 6. The EFu face of *viridis-n³⁴* thylakoids has even fewer particles than the wild-type, the pits are less well-defined and there appears to be fewer per square micron. (Bar = 0.2 μ m). $\times$ 248,000.

Figure 7. High magnification freeze-fracture appearance of the wild-type Pfu face.

Note the presence of large dark particles (arrows) and small, less clearly shadowed particles (arrow heads). (Bar = 0.2 μ m). $\times$ 248,000.

Figure 8. Particles on the Pfu face of *viridis-n*³⁴ have almost the same density as wild-type thylakoids but are much smaller on average, mainly due to a reduction in the number of the large, heavily shadowed particles. (Bar = 0.2 μ m). $\times$ 248,000.

Table III

Statistical analysis of particle size data
(df = degrees of freedom)

Face	χ^2 value	df	significance level
EFu	210.4	16	<0.0005
PFu	186.3	33	<0.0005
EFs	271.0	36	<0.0005
PFs	38.6	23	<0.05

4. DISCUSSION

The single nuclear gene mutant *viridis-n³⁴* in barley has been extensively characterised with respect to thylakoid composition and electron transport properties (4,5,9). In comparison with wild-type, there is a partial deficiency in the amount of the *P700*-chlorophyll *a*-protein I which contains the reaction centre of photosystem I. Associated with this, there is also a deficiency in *P700* (4) and three low molecular weight polypeptides, two of which are probably iron-sulphur proteins functioning on the reducing side of photosystem I (9). The composition of mutant thylakoids is otherwise similar to wild-type with respect to polypeptides, chlorophyll-proteins (4,5,9) and cytochromes (9). Photosynthetic electron transport through photosystem II (from H_2O to ferricyanide/phenylenediamine) is also normal, but the flow of electrons through photosystem I is between 3 and 30% that of wild-type (4,9), depending on the choice of electron donors and acceptors used for the measurements. The primary effect of the mutation in *viridis-n³⁴* therefore appears to be restricted to a partial (between 10 and 40%) deficiency in the reaction centre chlorophyll-protein of photosystem I and three low molecular weight polypeptides associated both structurally and functionally with photosystem I (9).

The localisation of photosystem I to a specific population of particles in freeze-fractured thylakoids is complicated by the significant size differences of particles on three of the four freeze-fracture faces when compared with the corresponding wild-type faces (Table III). In granal regions, no significant change in particle size or density was found for the PFs, while on the EFs face, the average size of the particles decreased

by about 25 Å, with a 10% decrease in particle density (Tables I and II).

The ultrastructural changes in stroma lamellae were much more dramatic, particularly on the PFu face, where the particle density is high. In wild-type thylakoids, the PFu face is characterised by 4553 particles/ μm^2 , of which 3213 ± 30 ($\pm$ S.E.) or 70% are large and well-defined after rotary shadowing. Thylakoids from *viridis-n³⁴* chloroplasts have approximately the same total particle density on their PFu face, although there is a higher degree of subjectivity in deciding whether a feature is a particle or not for the purposes of counting, due to the low surface relief of so many of the particles. When only the large, well-defined particles are counted, a value of 1372 ± 31 ($\pm$ S.E.) per square micron is obtained, representing 43% of the large particle density on the wild-type PFu face. Interestingly, HILLER et al. (1980) have determined that the amount of the *P700*-chlorophyll *a*-protein I in *viridis-n³⁴* is 41% of wild-type, using SDS-polyacrylamide gel electrophoresis with minimal denaturation of chlorophyll-proteins. This may be a more valid measure than electron transport rates for correlating with freeze-fracture ultrastructural differences.

The EFu face of *viridis-n³⁴* also contained fewer particles (57% of the wild-type density, Table I), as well as a reduction in the number of pits, as judged from visual inspection. A value of 1420 ± 49 ($\pm$ S.E.) per square micron for the density of EFu pits was measured, but the uncertainties in counting such features make this value less reliable than, for example, particle densities. It would seem, however, from the similar numerical values for the EFu pits and PFu large particle densities (3298 ± 46 and 3213 ± 30 for wild-type versus 1420 ± 49 and 1372 ± 31 for *viridis-n³⁴*) that they are complementary features. They are probably the best candidate for the localisation of the *P700*-chlorophyll *a*-protein I in the thylakoid. Their size (93×112 Å or larger) is consistent with that of photosystem I particles (106 Å) isolated by detergent fractionation and inserted into artificial liposomes (10). If it is assumed that each large PFu particle contains one photosystem I reaction centre, and that each EFs particle contains one photosystem II reaction centre (18), the ratio of PSII/PSI can be calculated from the ra-

Figure 9. Three-dimensional frequency distribution plots of freeze-fracture particle size measured from rotary shadowed replicas of *viridis-n*³⁴ thylakoids.

These should be compared with the corresponding plots for wild-type (14). The *viridis-n*³⁴ PFu size distribution lacks many of the large particles found in the wild-type.

tio of EFs/PFu. The particle densities for the EFu, EFs and destacked EF faces (3,12,13) indicate that 63% of the thylakoid area in barley chloroplasts is stacked. Thus, the ratio of EFs/PFu is $1624 \times 0.63 / 3213 \times 0.37$, which is 0.86. This value is consistent with a PSII/PSI ratio of 0.77 reported for mature pea chloroplasts (6).

The localisation of the *P700*-chlorophyll *a*-protein 1, and hence photosystem I reaction centres, in the large PFu particles is also consistent with the results from thylakoid fractionation using French press (11) and digitonin (2). These studies have shown that the stroma lamellae are enriched in photosystem I activity, and that the grana lamellae are enriched in photosystem II. The freeze-fracture ultrastructure of the photosystem I maize mutant 1481, which contains 20% of the *P700*-chlorophyll *a*-protein 1 found in wild-type (7), has recently been investigated by MILLER (1980). Using unidirectionally shadowed replicas, the average size of the PFu particles was found to be 16 Å smaller than those of the wild-type, and no significant size differences were measured for PFs, EFu or EFs particles. It was therefore concluded that the loss of this chlorophyll-protein caused a reduction in the size of the PFu particles, and that, in vivo, photosystem I might be confined to these particles in stroma lamellae. A similar hypothesis has been proposed on the basis of recent results from phase partition enrichment of partition regions (1). The results from the analysis of the *viridis-n*³⁴ mutant cannot exclude the possibility that some photosystem I reaction centres are located in granal regions. The *chlorina-f2* data indicate that up to 20% of the PFs particles found in wild-type thylakoids do not contain the light-harvesting chlorophyll *a/b*-protein 2 (13), and may, therefore, be photosystem I reaction centres. The loss of such particles from the PFs would reduce the average particle size by only 3 Å, which would be difficult to detect. The cause of the size reduction of the EFs particles in *viridis-n*³⁴ is not known, but is unlikely to be due to the loss of the *P700*-chlorophyll *a*-protein 1. The localisation of photosystem I within the thylakoid will be checked by examining the freeze-fracture ultrastructure of other photosystem I mutants of barley, such as *viridis-h*¹⁵ and *viridis-zb*⁶³, which completely lack the *P700*-chlorophyll *a*-protein 1 (9), or double mutants

obtained by crossing photosystem I mutants with *chlorina-f2*.

ACKNOWLEDGEMENTS

The support and encouragement of Professor DITER VON WETTSTEIN throughout the course of this work is gratefully acknowledged. I wish to thank JØRGEN PEETZ for computer programming, NINA RASMUSSEN for drawing the figures and JEAN SAGE for cutting the thin sections. Financial assistance was provided by the Commission of European Communities contract ESD-013-DK Solar Energy Program.

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