FREEZE-FRACTURE STUDIES OF PHOTOSYNTHETICALLY DEFICIENT "SUPERGRANAL" CHLOROPLASTS IN TISSUE CULTURES CONTAINING VIRUS-LIKE PARTICLES

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INTRODUCTION
Since the division of the light reactions of photosynthesis into two separate photoacts by Hill and Bendall (1960), several investigators have attempted to identify morphological units within chloroplast membranes which correspond to the two separate photosystems. Arntzen et al. (1969) indicated that the membrane fragments enriched in photosystem I (PS I) activity exhibited only small (110 Å, diameter) particles on fracture faces while membranes enriched in photosystem II (PS II) activity showed primarily larger particles (175 Å, diameter) on their fracture faces. Goodenough and Staehelin (1971) examined freeze-fractured replicas of wild type and mutant Chlamydomonas reinhardti and found that the distribution of the large (160 ± 10 Å) particle was related to the stacking of the membranes and was not related to their photosynthetic capacity.

We have observed chloroplasts that have "supergrana" consisting of many stacked membranes in tissue cultures containing a virus-like particle (Sjolund and Shih, 1970). These superstacked chloroplasts contain normal levels of chlorophyll, but they are deficient in both CO₂ incorporation and O₂ evolution. Although these chloroplast membranes are aggregated into large stacks, freeze-fracturing reveals that they lack the 175-Å particle found in control chloroplast membranes.

1 Abbreviations used in this paper: DCMU, 3,3,4-dichlorophenol, -1,1-dimethylurea; PS I, photosystem I; PS II, photosystem II.

MATERIALS AND METHODS
The callus tissue used in this investigation was initially derived from an excised cotyledon of the California shield leaf, Streptanthus tortuosus (Kell.) var. orbiculatus (Greene) Hall (Cruciferae). It was maintained by subculturing for 5 yr prior to the discovery of a virus-like particle in the cells (Sjolund and Shih, 1970). The tissue containing this virus-like particle will be subsequently referred to as cell line STV. The callus tissue cultures used in this investigation as a source of control tissue for comparison with the STV cell line were derived from seeds of Streptanthus tortuosus (Kell.) var. orbiculatus (Greene) Hall supplied by Dr. A. Kruckeberg of the University of Washington, Seattle, Washington. This line of tissue will subsequently be referred to as cell line 6045, control cells.

Tissue was prepared for electron microscopy by fixation in Karnovsky's fixative (Karnovsky, 1965), embedded in Spurr's plastic (Spurr, 1969), thin sectioned with a diamond knife, and examined with either a Zeiss EM-9A or Hitachi HU-12 electron microscope.

Material for freeze-fracturing was fixed in 0.2% glutaraldehyde and infiltrated in 25% glycerol for 12 h. Freeze-fracturing, etching, and replication were done using a Balzers freeze-etch device (Moor and Mühlethaler, 1963). Oxygen evolution was measured using a Gilson medical electronics oxygraph (Gilson Medical Electronics, Inc., Middleton, Wis.) equipped with a Clark electrode. The cells were illuminated with 3,600 foot candles of light. Where 3,3,4-dichlorophenol, -1,1-dimethylurea (DCMU) was employed as an inhibitor, it was used at a concentration of 5 X 10⁻⁵M.

Carbon dioxide incorporation was measured using NaH¹⁴CO₃. Whole cells were suspended in an inorganic medium, placed in a small flask, and equili-
Figure 1  Thin sectioned chloroplasts from 6045 (control) cells. Arrow indicates grumum. Bar equals 0.25 µm. X 49,500.

Figure 2  Freeze-fractured replica from control chloroplast. Large (175 Å) particles are seen on the B face and smaller (120 Å) particles are seen on the C face. Broad arrow indicates direction of metal evaporation. Bar equals 0.1 µm. X 150,000.
Figure 3  Thin sectioned supergranal chloroplast showing membrane configuration in the supergranum (SG). Bar equals 0.25 µm. × 49,500.

Figure 4  Freeze-fractured replica of a supergranal chloroplast similar to that in Fig. 3. One supergranum is fractured both at right angles to the membrane stack and through the membrane faces. Bar equals 0.25 µm. × 90,000.
ibrated in the light (7,600 foot candles) or dark at 25°C for 5 min. The reaction was started by the addition of NaH14CO3 (25 µCi/10 ml of suspended cells). Samples were withdrawn and placed on a planchet containing an equal volume of concentrated acetic acid. The samples were dried, weighed, and counted using a gas-flow counter. Chlorophyll determinations were done by the method of Arnon (1949).

RESULTS

Chloroplast Structure in Control Cells

The chloroplasts of cell line 6045 (control) resemble those of most higher plants and are characterized by having several (2-10) small grana consisting of three to eight thylakoids interconnected by stroma lamellae (Fig. 1). Replicas of freeze-fractured control (6045) chloroplasts reveal membrane faces and two size classes of particulate structures (Fig. 2). Each of the two size classes of particles is associated with one of the two complementary faces exposed by freeze-fracturing (Branton and Park, 1967). The B face of the 6045 chloroplast reveals the larger particle (175 Å, average size) while the C face reveals a smaller particle (120 Å, average size). The distribution of these particles and the relationship of the B and C faces in control chloroplasts is similar to that observed in spinach chloroplasts by Branton and Park (1967) and Arnzen et al. (1969). The same image is a consistent feature of virtually all freeze-fracture studies of chloroplasts in higher plants.

Chloroplast Structure in Cell Line STV

Thin sections of chloroplasts in cell line STV reveal an altered arrangement of membranes. Compared to chloroplasts of control cells (Fig. 1), the chloroplasts of the STV cell line contain one to four supergrana often containing 30 or more thylakoids (Fig. 3). These supergrana are usually as long as the chloroplast itself (1-3 µm) and are not interrupted by interconnecting stroma lamellae. The partitions of the supergrana are the same width (120 Å, average) as those in the control chloroplasts. There do not appear to be any interconnections between the supergrana, and the ends of the thylakoids terminate at the end of the granum or project as short single lamellae or inflated vesicles (Fig. 3).

Replicas of freeze-fractured chloroplasts from cell line STV differ markedly from those of control chloroplasts (Figs. 2, 4-6). One membrane face contains widely spaced particles with an average diameter of 80 Å (Figs. 4-6). The other membrane face seen in STV chloroplasts is essentially smooth, lacking particles on its surface (Figs. 4, 6). Since both of these faces differ from those of control chloroplasts, particle size alone cannot be used to identify the membrane faces that are exposed during freeze-fracturing of STV chloroplasts. By assuming that the shadow length of a face is proportionate to its height (Branton and Park, 1967) above the next face, it is possible to analyze the arrangement of faces in the supergrana of STV plastids. Assuming that these membranes fractured preferentially through their hydrophobic interiors (Branton and Park, 1967), the two membrane faces seen in the STV replicas can best be interpreted as a smooth, particle-free C face and a B face containing 80-Å particles. Thus, the short shadow frequently seen between a smooth surface and a particle-containing surface (Fig. 5) represents the short transition from a C to a B face. The transition from particle-containing B face to a smooth C face is always marked by a long shadow which is the result of the fracture crossing the thylakoid interior (see Figs. 7, 8).

Comparison of Photosynthetic Activities of Control and STV Cell Lines

Cells of the STV line contain an equal amount of chlorophyll per gram fresh weight as the control cells (0.1 g chlorophyll/g fresh weight) but exhibit a markedly decreased light dependent O2 evolution and CO2 fixation ability. The ability of whole cells from the two cell lines to fix 14CO2 is compared in Fig. 9. Control cells, 6045, exhibit a rate of light-dependent 14CO2 fixation equal to 7.5 µM/h/mg Chl. Although this rate is far below that reported for spinach (Jensen and Bassham, 1966) or Chlamydomonas (Goode-nough et al., 1969), it is consistent with the rates observed in other plant tissue cultures (Laetsch and Stetler, 1965; Laetsch and Kortschak, 1972; Neuman and Raafat, 1973). The compact nature of plant cell cultures appears to inhibit CO2 incorporation, especially when compared with leaves that are anatomically specialized to facilitate gaseous exchange. The low, but consistent, light-stimulated 14CO2 fixation seen in control
Figure 5 Freeze-fractured replica of a supergranum. Two faces are revealed. The B face contains 80Å particles and the C face is essentially smooth. Bar equals 0.1 µm. × 150,000.

Figure 6 Freeze-fractured replica of a supergranum showing relationship between B and C faces. Bar equals 0.1 µm. × 150,000.

Figure 7 Artist's interpretation of chloroplast structure in cell line 6045 (control) showing the B and C faces exposed by freeze-fracturing. Note that the exterior surface of the thylakoid (A) and the interior surface of the thylakoid (D) are not revealed by freeze-fracturing. The thylakoid membrane contains the complementary B and C faces which are revealed when the membrane splits down the center. The 175Å particles are revealed on the B surface while the 190Å particles are revealed on the C surface.

Figure 8 Artist's interpretation of a supergranum from cell line STV showing faces exposed by freeze-fracturing and the 80Å particles on the B surface.
2.5
0.5
0

TIME (min)

FIOtIRE 10

Tracings from oxygraph showing oxygen evolution of whole cells from cell line 6045 (control) and cell line STV.

Cells of control line 6045 exhibit a light-dependent, DCMU-sensitive oxygen evolution. No light-dependent oxygen evolution or change in respiratory oxygen utilization is found in cell line STV.

DISCUSSION

On the basis of the evidence presented here, we conclude that in the supergranal chloroplasts from the STV cell line, extensive membrane aggregation occurs without the formation of 175-Å particles on the B face. This is in contrast to the suggestion by Goodenough and Staehelin (1971) that the large particles seen in freeze-fracture may be related to the aggregation of thylakoid membranes into stacks. Their evidence suggests that, at least in Chlamydomonas, the large (160 ± 10 Å) B face particles are more densely distributed in stacked regions (B₈) than in unstacked regions (B₉) of the plastid membranes.

The lack of correlation between the 175-Å particle distribution and membrane aggregation seen in the STV supergrana is also seen in the freeze-fracture studies of another green alga, Oocystis, carried out by Pendland and Aldrich (1973). The B faces of stacked regions in Oocystis reveal particle distributions similar to the B₈ faces of unstacked regions in Chlamydomonas chloroplasts. No B₈ faces were found in Oocystis even though extensive stacking is present in the chloroplast membranes. Unstacked regions of Oocystis chloroplasts exhibit two freeze-fractured faces. One is a C face (type 4) with tightly packed 90-Å particles, and the other is a B face (type 1).

Pendland and Aldrich's (1973) elegant applica-
demonstrated a four unit substructure in the control chloroplasts. Park and Pfeifhofer (1969) normal 175-A particles found on the B face of the STV B face may represent a subunit of the 90-A particles of Oocystis. Our evidence suggests, however, that these particles occur on a B face in the supergranum and are less densely distributed than the 90-A particles of Oocystis. These 80-A particles on the STV B face may represent a subunit of the normal 175-A particles found on the B face of control chloroplasts. Park and Pfeifhofer (1969) demonstrated a four unit substructure in the 175-A particles found on B faces of deep-etched spinach chloroplasts. Pendland and Aldrich (1973) also noted the possibility that their 90-Å particle may be a subunit of a larger particle.

The lack of the 175-Å particle on the B face of the supergranal plastids in the STV cell line may be unrelated to the aggregation of the membranes in supergranula, but rather may be related to their inability to fix CO₂ or evolve O₂. This conclusion would be supported by the work of Arnzen et al. (1969) which showed that freeze-fracturing of membrane fragments enriched in either PS I or PS II activity revealed the 175-Å particles on membrane faces enriched in PS II activity and the 110-Å particles on the membrane faces enriched in PS I activity. Sané and Park (1971) and Goodchild and Park (1971) have indicated that the larger (175 Å) particle is restricted to the areas of the thylakoid membranes that aggregate to form partitions in a granum, while the stroma lamellae, according to these authors, lack the 175-Å particle and also lack PS II activity. The smaller (110 Å) particle is found in both partition regions and stroma lamellae and they believe that PS I occurs in both regions of the thylakoids.

In the present study, chloroplasts with supergrana are found in tissue cultures that lack the ability to differentiate vascular tissues or organs and contain a presumptive spherical virus. Chloroplasts with large numbers of thylakoids arranged in supergranum have also been seen by Arnott et al. in leaf cells of tomato that were infected with tobacco mosaic virus (1969). These leaf cells had mixed populations of normal and supergrana chloroplasts. The tissue culture system used here allows heterotrophic growth of plant cells, permitting the study of photosynthetically deficient cells that would not survive under autotrophic conditions.

Our preliminary evidence indicates that the STV cell line is deficient in photosynthetic CO₂ fixation and oxygen evolution. In addition, the 110-Å particles characteristic of higher plant C faces are absent, and an 80-Å particle rather than the 175-Å particle is present on the B face. Further investigations of PS I and PS II activity, as well as electrophoresis of membrane proteins are currently in progress to test the hypothesis that the lack of these normal particles is associated with the photosynthetic deficiency of the STV cell line.

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