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Particle Movements in Chloroplast Membranes: Quantitative Measurements of Membrane Fluidity by the Freeze-Fracture Technique

(*Chlamydomonas reinhardtii*/membrane interactions/chloroplast membrane structure)

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ABSTRACT **Stacked chloroplast membranes isolated from *Chlamydomonas reinhardtii* have differentiated particle arrays when examined by freeze-fracture electron microscopy. When the membranes are isolated unstacked, these particle arrays are lost and the fracture faces have a homogeneous appearance. The changes in appearance are due to a rearrangement of existing membrane components by lateral particle movements in the plane of the fluid chloroplast membranes, since quantitative measurements demonstrate almost complete conservation of numbers and sizes of membrane particles during experimentally controlled stacking and unstacking.**

The freeze-fracture technique, which preferentially splits frozen membranes down a unique internal plane (1), has been used to study the movement of components intercalated into the membrane. Lateral movements of freeze-fracture particles, presumed to be protein, have been observed in response to environmental pH changes in erythrocyte ghosts (2), during phase transitions in membrane lipids (3), in membrane receptors during secretion in *Tetrahymena* (4), in lymphocytes (5), possibly in membranes undergoing pinocytosis (6), and elsewhere. However, quantitative measurements on the translocation of freeze-fracture particles are lacking. In this report, utilizing the chloroplast membranes of the green alga *Chlamydomonas reinhardtii*, we present such information for the first time. We will demonstrate that there is nearly complete conservation of membrane particles on both fracture faces during experimentally induced particle redistributions. These results allow reinterpretation of the earlier data of Goodenough and Staehelin (7), who first showed that different concentrations of intramembranous particles exist in stacked and unstacked *Chlamydomonas* chloroplast membranes, to provide strong support for a fluid membrane model (8) of contact formation in these membranes.

MATERIALS AND METHODS

Cell Culture. Wild-type (137 C) and mutant strains (*ac-5*, *ac-31*) of *Chlamydomonas reinhardtii*, kindly provided by Dr. R. P. Levine, Harvard University, were grown in 4-liter bottles containing 2 liters of liquid minimal media supplemented with 0.3 g of sodium acetate per 100 ml (9). Continuous illumination was provided by cool white fluorescent lights at an intensity of 4000 lux. *Ac-5* mutants were phototropically grown by culturing the cells in minimal media.

Chloroplast Membrane Isolation. Logarithmic phase cultures were harvested at 1000 × *g*, washed twice with either 1 mM Tris·HCl, pH 7.8 (Tris), or Tris containing 20 mM $MgCl_2$ (Tris-Mg buffer). This and all subsequent operations were done at 4°. The cells were then resuspended in either Tris or Tris-Mg buffer containing 0.3 M sucrose and broken in a French pressure cell, usually at 6000 lbs./inch^2.

Chloroplast membranes were isolated as described (10) except that either Tris or Tris-Mg buffer was substituted as the buffer for the sucrose gradient. Only the membrane fraction collected at the 1.5–2.0 M sucrose interface was used.

Unstacking and Stacking Experiments. These experiments compare two preparations derived simultaneously from one cell population. The stacked membrane configuration is maintained when Tris-Mg buffer is used in the isolation and freezing procedures. Unstacked membranes are isolated in an identical manner when Tris is substituted for Tris-Mg buffer. After removal from the sucrose gradient, the isolated chloroplast membranes were washed twice with either Tris or Tris-Mg buffer. Duplicate samples of stacked and unstacked membranes for all strains were prepared for both thin section and freeze-fracture electron microscopy.

Electron Microscopy. For sectioning, the membranes were fixed in 2% glutaraldehyde–7 mM potassium phosphate buffer, pH 7.0, for 30 min, washed in the buffer, and then postfixed in 1% O_sO_4–potassium phosphate for 60 min. The material was then dehydrated in a graded ethanol series and embedded in Epon 812. Thin sections were cut, mounted on formvar-coated grids, stained with uranyl acetate and lead citrate, and examined in a Siemens Elmiskop 1 electron microscope. For freeze-fracturing, membranes were resuspended without fixation in either Tris or Tris-Mg buffer containing 20% glycerol as a cryoprotectant. Droplets were pipetted onto 3-mm cardboard discs, rapidly frozen in liquid Freon 22, and placed in a Balzer's apparatus at −115° for fracturing and replication.

Quantitative Measurements. For particle density measurements, micrographs of stacked and unstacked membrane preparations were taken. Contacted, noncontacted, and unstacked membrane regions of both fracture faces were selected. The defined areas were measured with a compensating polar planimeter and the freeze-fracture particles counted to give particle density. At least 1 μm^2 of each membrane region was analyzed. The percentage of membrane contact for stacked membranes was determined by measuring four to six micrographs of sections taken at random with a map reader.

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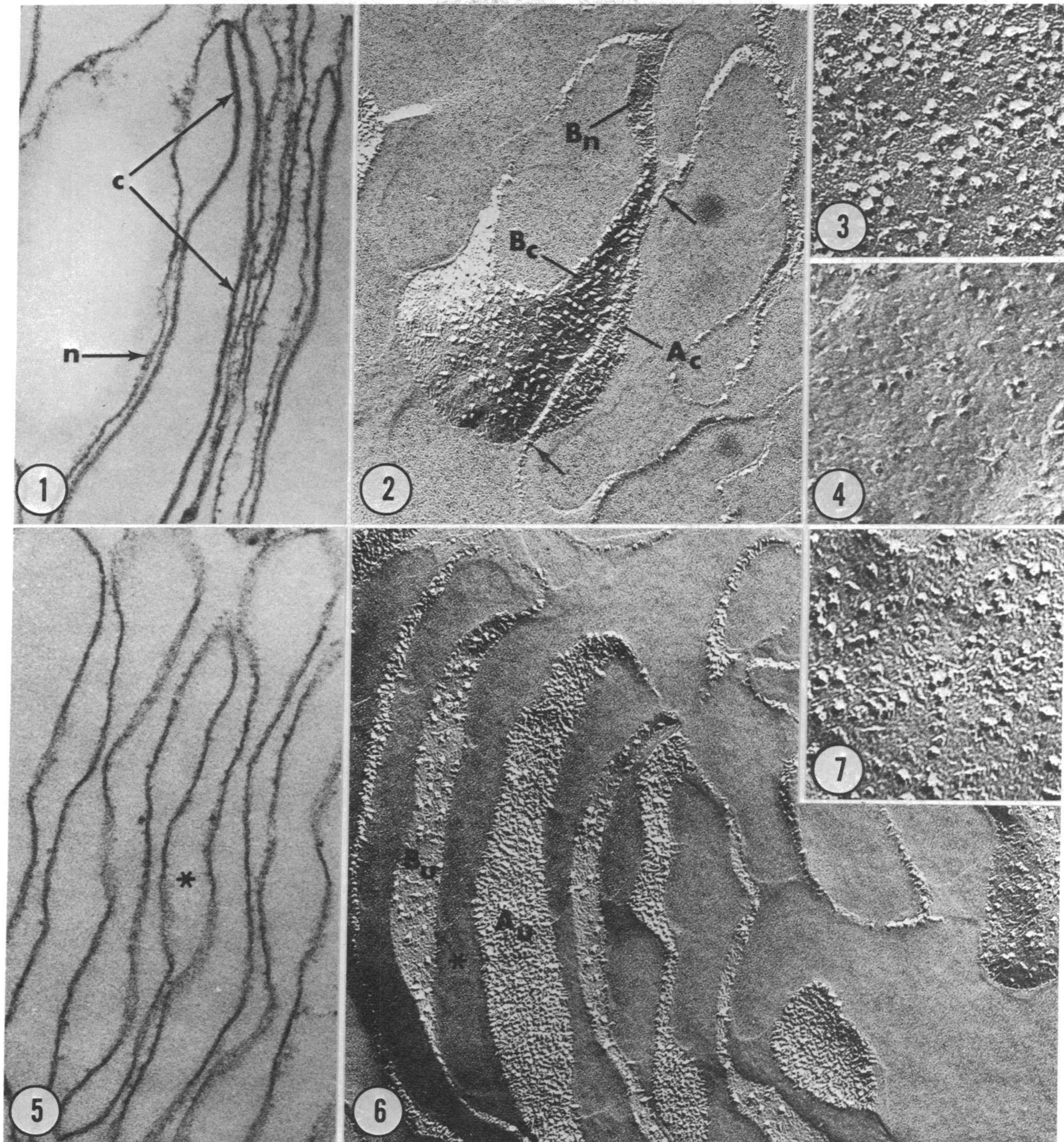

FIGS. 1–7. All micrographs are for the wild-type strain; shadow direction of freeze-fracture replicas is *bottom* to *top*. (Figs. 1–4) Chloroplast membranes: isolated, stacked. (*1*) Thin section. A membrane stack with the contacted (*c*) and noncontacted (*n*) regions identified; 100,000×. (*2*) Freeze-fracture. In the region of membrane contact (between the *arrows*) are the A_c and B_c fracture faces. Note that the B_n face is coplanar with B_c; 72,000×. (*3*) B_c face; 144,000×. (*4*) B_n face; 144,000×. (Figs. 5–7) Isolated unstacked membranes with the newly formed interthylakoid space (*). (*5*) Thin section showing the separate, individual membrane discs; 100,000×. (*6*) Freeze-fracture. Alternating A_u and B_u faces are present. The particles on the B_u face are homogeneously distributed; 72,000×. (*7*) B_u face; 144,000×.

To determine the diameter of the particles present on each type of fracture face, measurements were made on enlarged micrographs (144,000X) with a 7X magnifier equipped with a calibrated micrometer grating. Due to irregularities in shadowing, minimum particle diameters were taken whenever particle boundaries appeared unclear or fuzzy. A total of 800 particles, 400 each from duplicate experiments, was measured for each histogram.

Terminology. By current convention, for most membrane systems studied with the freeze-fracture technique, the cytoplasmic membrane half is termed A while the complementary half is B. We have adopted this convention for the chloroplast membrane. The highly particulate A fracture face borders the stroma while the B face borders the intrathylakoid space (Figs. 2 and 6). This differs from the terminology previously used for chloroplast membranes in which the particulate face is named C, instead of A (7, 11).

RESULTS

Isolated Wild-Type Chloroplast Membranes. Chloroplast membranes of wild-type *Chlamydomonas* isolated in Tris-Mg buffer (Fig. 1) maintain the stacked configuration found

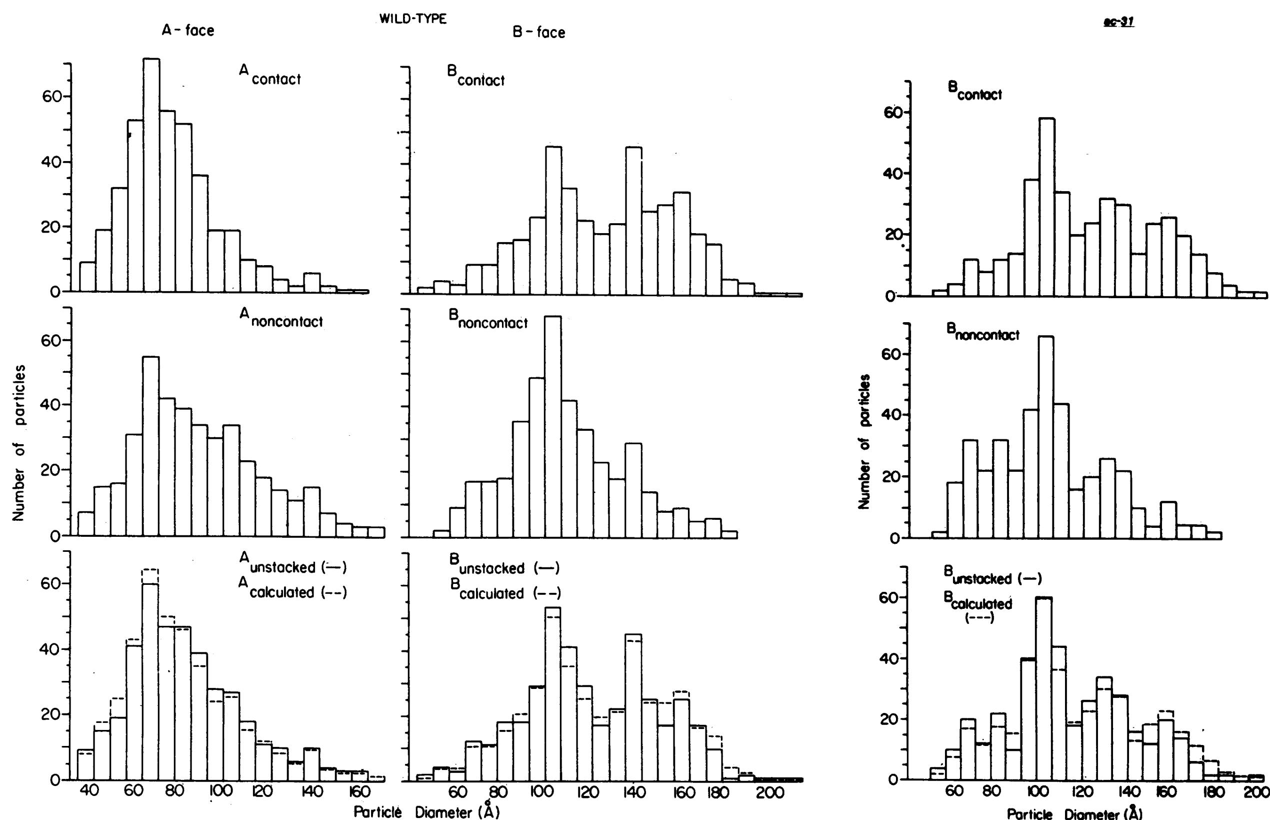

FIG. 8. Histograms demonstrating the particle size distribution for wild-type and *ac-31* strains. The *bottom figure* for each fracture face compares the measured unstacked (*solid line*) to the calculated unstacked (*broken line*) distribution.

in vivo. Although the thylakoids appear slightly swollen, the regions of membrane contact appear unaltered. Regions of contact (*c*) are defined as two adjacent membranes approaching close enough to produce an electron-opaque fusion line. All membranes at the end of a stack and those that do not approach close enough to produce the electron-opaque line are considered to be noncontacted (*n*). About 57% of an average isolated, wild-type membrane stack is in a contacted configuration (Table 1). This percentage of membrane contact compares favorably to the 58.5% that we calculate from *in vivo* stacking data of Goodenough and Levine (12).

Freeze-Fracture of Stacked Membranes. When stacked chloroplast membranes are freeze-fractured, contacted and noncontacted regions occur in areas along a single membrane. The freeze-fracture appearance of the contacted region is unique and distinctly different from that of the noncontacted region (Figs. 2–4). This differentiation is found on both fracture faces, but is most obvious on the *B* face, which contains numerous large particles in the contacted region (B_c face, see Figs. 2 and 3), while the coplanar noncontacted region (B_n face) has few particles (Figs. 2 and 4). A sharp boundary exists between the B_c and B_n faces at the point of contact between the two membranes. In Fig. 2, the adjacent *A* face identifies the contacted region unequivocally.

The B_c face has a particle density of about 1900 particles per μm^2 (Table 1), which is comparable to that of the B_c face in *Euglena* (13) and in spinach (14). A histogram of the particle size distribution on the B_c face shows distinct peaks at 105, 140, and 160 Å (Fig. 8). The A_c face contains 3500 particles per μm^2 (Table 1), the majority of which are about 70 Å in diameter (Fig. 8). Because all measurements of particle size group around these four peaks (70, 105, 140, and 160 Å) with what appear to be normal distributions, all particles in the membrane have been assigned to one of these four size classes.

The B_n fracture face also has a striking appearance due to the relative paucity of particles (Figs. 2 and 4). The particle density is only about 600 particles per μm^2 (Table 1), but all

TABLE 1. *Particle conservation on the A and B fracture faces*

		Stacked regions*			Unstacked*		
Strain	Face	Contacted	Noncontacted	g	Measured	Calculated†	Ratio‡
wt	*A*	3510 ± 71	3665 ± 247	57.6 ± 1.3	3440 ± 57	3565 ± 140	96.6 ± 4.9
wt	*B*	1943 ± 37	606 ± 22	57.1 ± 2.2	1337 ± 64	1368 ± 63	97.7 ± 4.0
ac-5	*B*	2080 ± 245	762 ± 28	53.1 ± 2.0	1405 ± 184	1464 ± 244	96.2 ± 2.5
ac-31	*B*	2514 ± 190	888 ± 235	42.8 ± 4.1	1600 ± 144	1605 ± 308	99.5 ± 2.2

* Particles per μm^2 ± standard error.

† Calculated from Eqs. 2 and 3; see *text*.

‡ Measured/calculated × 100.

four classes are represented (Fig. 8). The A_n face has a particle density similar to that of the coplanar A_c face (Table 1). The freeze-fracture appearance of stacked chloroplast membranes is identical to that previously reported for *Chlamydomonas* (7) and, while there are small differences in the particle size distributions, these results are confirmatory.

Morphology of Unstacked Chloroplast Membranes. Chlamydomonas chloroplast membranes isolated in Tris appear as a series of separate, flattened discs (Fig. 5). No membrane contact is ever observed when the membranes are isolated in Tris or other low-ionic-strength solutions (7). When the unstacked chloroplast membranes are freeze-fractured, alternating A and B faces are still seen but they are now separated by not only the intrathylakoid, but also, by the newly-formed interthylakoid space (Fig. 6). The characteristic low ridge (Fig. 2) separating the A_c and B_c fracture faces of stacked membranes is no longer present. The unstacked B fracture face (B_n) lacks the highly differentiated particle regions. Instead, it appears homogeneous with a random distribution of membrane particles. The unstacked A fracture face (A_u) appears also homogeneous with no evidence of differentiated membrane regions.

Quantitation of Membrane Particle Movements. Two alternative hypotheses that would account for the observed morphological differences in stacked compared to unstacked chloroplast membranes are: (*i*) a redistribution of existing membrane components by lateral diffusion in the fluid matrix or (*ii*) conformational changes resulting in altered expression of these components. Demonstration of total particle conservation during stacking or unstacking would support hypothesis *i*, while changes in particle size and number would be considered as evidence for hypothesis *ii*.

The following equations express the conditions for particle conservation:

$$A_u + B_u = g\,(A_c + B_c) + (1 - g)(A_n + B_n) \quad [1]$$

$$A_u = g\,A_c + (1 - g)\,A_n \quad [2]$$

$$B_u = g\,B_c + (1 - g)\,B_n \quad [3]$$

where A_u, A_c, A_n, B_u, B_c, and B_n are the particle densities for the A and B fracture faces designated by the subscript; g is the proportion of contacted membrane, and $(1 - g)$ is the proportion of noncontacted membrane.

Eq. 1 describes the entire membrane and states that for conservation the total number of particles in the unstacked membranes (measured value) must be equal to the weighted contributions of the contacted and noncontacted portions of the stacked membranes (calculated value). Eqs. 2 and 3 are more stringent and describe each membrane half individually. The two membrane halves can be treated separately and are additive.

Comparisons of measured with calculated particle densities for both fracture faces are summarized in Table 1. The calculated density for the wild-type A fracture face is 3565 particles per μm^2, similar to the 3440 particles per μm^2 measured directly on unstacked membranes. For the wild-type B fracture face, the calculated density of 1368 particles per μm^2 agrees closely with the measured density of 1337 particles per μm^2. Similar results are presented for the *ac-5* strain (Table 1). Statistical analysis by Student's *t*-test shows these slight deviations to be insignificant ($P = 0.4$) for both fracture faces.

TABLE 2. *Particle size conservation after unstacking of wild-type chloroplast membranes*

Diameter (Å)	Measured	Calculated*	Ratio†
70	2236‡	2364‡	95.0
105	1445	1484	97.4
140	702	676	103.8
160	285	336	84.8
Total	4668	4860	96.0

* Calculated from Eq. 1; see *text*.
† Measured/calculated × 100.
‡ Unstacked particle densities; particles/μm^2.

The particle size distribution expected when the chloroplast membranes are unstacked can also be calculated, and compared to the measured unstacked distribution. Little change can be observed in either the percent of particles in a given size range (Fig. 8) or in the density of particles of a given size in the total membrane (Table 2) except in the 160 Å class, where the density decreases by 15% upon unstacking. Since the 160 Å class only comprises about 7% of total membrane particles, the 15% decrease has less than a 2% effect on total particle density. Within these limits, we conclude that there is total conservation of number in each particle size class.

Particle Movements upon Membrane Stacking in the ac-31 Strain. In the *ac-31* strain of *Chlamydomonas*, chloroplast membranes are unstacked *in vivo* and after isolation in Tris. Membrane isolation in Tris-Mg buffer will produce membrane stacks (7). Thin-section and freeze-fracture morphology of both unstacked and stacked membrane fractions is qualitatively identical to that of the wild type. Conversation of both particle size and numbers while going from the unstacked to the stacked configuration is demonstrable on the B fracture face (Table 1; Fig. 8). The small differences between calculated and measured particle densities are not statistically significant ($P = 0.8$). There appears to be a 20% increase in 160 Å particles when the *ac-31* membranes are stacked.

DISCUSSION

Quantitative measurements of particle number and diameter have demonstrated almost complete conservation of membrane particles when going from the stacked to the unstacked membrane configuration, and vice versa (Table 1; Fig. 8). We interpret these results to indicate that a redistribution of existing particles occurs within the chloroplast membrane by lateral movement when the configurations are experimentally manipulated. Particle movements for both fracture faces are summarized in Fig. 9. Although redistribution occurs on both fracture faces, it is more clearly defined on the B face, to which the majority of the larger particles adhere.

This is the first time that the precise quantitative distribution of all freeze-fracture particles within a single class of membranes has been determined. Partial data for one fracture face, such as are available for erythrocyte membranes, are insufficient in this regard since, of course, fracture faces do not exist in the intact membrane. At least some classes of particles may be able to adhere to either membrane half on fracturing. The measure of relative adherence to the A compared to the B face has been defined as the "particle partition coefficient" (Kp) (15). For the chloroplast membrane, the overall Kp for the stacked membrane for any particle class is

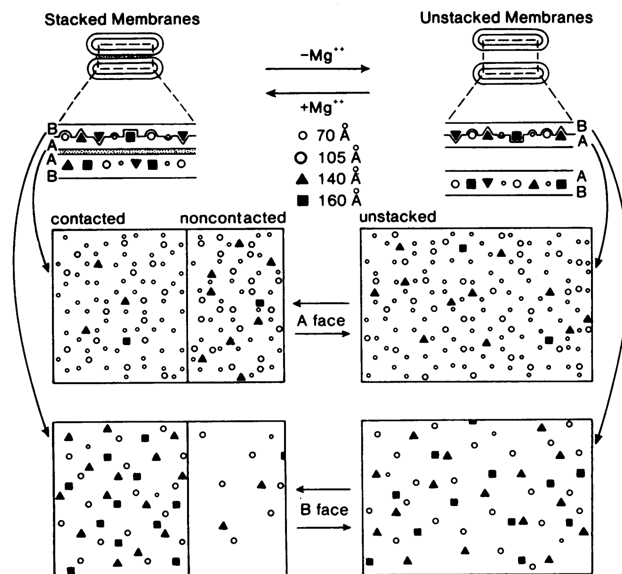

FIG. 9. Summary diagram illustrating lateral particle movements during unstacking (wild type) or stacking (*ac-31*). The *boxes* represent 1 μm^2, and the particles are drawn according to their relative proportions for the wild-type membranes.

identical to the overall *Kp* for the unstacked membrane; that is, regardless of stacking, the relative strengths of association of particles with opposite halves of the membrane remain unchanged. This accounts for the particle conservation that occurs during stacking or unstacking for the *A* and *B* faces independently.

It has been proposed that in spinach chloroplast membranes (16) the large freeze-fracture particles are associated with photosystem 2. One obstacle to identifying the 160 Å diameter particles in *Chlamydomonas* chloroplast membranes with photosystem 2 has been the fact that, upon unstacking, photosystem 2 remains in these membranes, but it has previously been assumed that the great majority of large (160 Å) particles disappear by a conformational change (7). This obstacle is now removed by our work, since we find that all but 15–20% of the 160 Å particles are conserved. Nevertheless, the conformational change hypothesis may be important for the small fraction of the 160 Å particles that are not conserved. These could be a unique functional component of the membrane, since, at this time, we are unable to separate diverse biochemical components that are represented by a single particle class. Although we define only four classes of particles by size, 15 to 20 proteins have been identified in *Chlamydomonas* chloroplast membranes (17).

Our data do not support the Pendland and Aldrich model of the contacted region (18), where a large particle is shared by two contacted membranes. According to this model, during unstacking, the shared particle would remain with only one of the two membranes. Since a shared particle could only be counted once, we should find a corresponding 50% decrease in large particle density during unstacking. This is much greater than the decrease found here.

Chloroplast membranes should be fluid since they contain a high degree of unsaturated lipid and no cholesterol (19, 20), two parameters that would allow a high degree of molecular motion (21). In membrane systems that exhibit gel-to-liquid crystalline phase transitions, particle aggregations occur when the membranes are quenched from temperatures below the critical phase transition temperature (3). Although all particle movements described here took place at 4°, we think it highly unlikely that they are produced by phase transitions in the membranes before freezing, since unstacked chloroplast membranes quenched from 4° have randomly dispersed particles.

Our results provide clear confirmation of the fluid mosaic membrane model (8). We envision the mosaicism to be at a supramolecular level, involving the formation of special contacted zones where freeze-fracture particles, representing specific membrane proteins, are concentrated by cooperative interactions. The particles could move about freely in the fluid lipid matrix until they entered the developing contacted zone, at which time they would be entrapped. Divalent cations apparently are important in maintaining this interaction. When the membranes are isolated unstacked in Tris, the constraints holding the particles in position are released. This allows particle dispersal and, as a result, the intermediate A_u and B_u fracture faces are produced. In the mature chloroplast membrane, this process involves rearrangement of particles with little or no change in size and numbers of existing components. The stacking and unstacking of isolated chloroplast membranes provides a good model system for studying control of particle movements in membranes, and may have wide applicability to cell junction biogenesis, cap formation, and other processes.

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