Reconstitution of Cytochrome f/b_6 and CF_0-CF_1 ATP Synthetase Complexes into Phospholipid and Galactolipid Liposomes

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ABSTRACT Cytochrome f/b_6 and ATP synthetase (CF_0-CF_1) complexes from spinach chloroplasts have been reconstituted into liposomes prepared from soybean phospholipids and purified spinach galactolipids. Freeze-fracture analysis revealed homogeneous populations of particles spanning the lipid bilayers with their elongated axes perpendicular to the membrane plane. The lipid composition of the liposomes had no effect on the size of the reconstituted complexes, the average diameter of cytochrome f/b_6 complex measuring 8.5 nm, and of the CF_0 base piece of the ATP synthetase, 9.5 nm. When reconstituted cytochrome f/b_6 complexes were cross-linked by means of antibodies prepared against the whole complex, the thus aggregated particles formed either hexagonal or square arrays. In both instances the center-to-center spacing of the particles was 8.3 nm, thereby suggesting that this value could be closer to the real diameter of the complexes than the one obtained from measuring individual particles. Assuming an ellipsoidal shape for these particles, and using a measured height of 11 nm, a molecular weight of ~280,000 could be calculated for the reconstituted cytochrome f/b_6 complex, consistent with a dimeric configuration. In many instances the crystalline sheets of antibody-aggregated cytochrome f/b_6 complexes were found to be free in the buffer solution; apparently the antibody-induced strains caused the sheet-like aggregates to pop out of the liposomal membranes. Agglutination studies of inside-out and right-side-out thylakoid vesicles revealed the antigenic determinants of the cytochrome *f* and cytochrome b_6 polypeptides to be exposed on the inner thylakoid surface and to be present in stacked and unstacked membrane regions. The molecular weight calculated from the size of freeze-fractured CF_0 base pieces was over twice the value determined by x-ray scattering data. This discrepancy may be caused by significant lipid domains within the base piece, or by an unusual fracturing behavior of the base piece in reconstituted liposomes.

During the last decade significant progress has been made in the isolation of functional complexes from chloroplast membranes. Efforts are underway to correlate those functional units with structural membrane components (reviewed in 22). The first enzyme and pigment-protein complexes of photosynthetic membranes to be localized and positively identified by means of immunological and electron microscopical techniques were the CF_1-ATP synthetase, the ribulose 1,5 diphosphate carboxylase-oxygenase, and the phycobilisomes of red algae and cyanobacteria (9, 15, 34, 35). While positive identification of integral membrane protein complexes has yet to be achieved, several lines of indirect evidence obtained from freeze-fracture studies of greening chloroplasts, chloroplast mutants, and reconstituted membrane systems have led to the following tentative structure-function correlations: photosystem II reaction center-bound light-harvesting complexes and 10–17-nm EF particles (5, 33, 53, 58), photosystem I-light-harvesting complexes and 10–11-nm PF particles (32, 36), CF_0-CF_1 ATP synthetase complexes and 9–10-nm particles (37), and "free" chlorophyll *a/b* light-harvesting complexes and 8–9-nm PF particles (31, 40, 49, 54). No size estimate for the cytochrome f/b_6 particle has been reported yet.

The need for positive identification of chloroplast mem-

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brane complexes in intact membranes is highlighted by two recent developments. Biochemical fractionation techniques have revealed that all but possibly the cytochrome f/b_6 complexes are nonrandomly distributed between stacked grana and unstacked stroma membrane regions (2, 4, 12). Furthermore, this distribution can be experimentally altered by changes in the ionic environment (6, 53) and by illumination of chloroplasts (24). The latter changes have been shown to be part of a phosphorylation-dependent mechanism for regulating chloroplast activity (24).

We hope to be able to positively identify the integral protein complexes of thylakoid membranes by using antibodies to form aggregates of specific complexes in lipid-enriched membranes. The methodology for increasing the lipid-to-protein ratio of membranes in vitro was pioneered by Schneider et al. (45, 46), who used phospholipid vesicles and inner mitochondrial membranes. A similar enrichment of chloroplast membranes with phospholipids has been reported by Siegel et al. (48). However, in this latter study, significant alterations in membrane structure, other than the expected dilution of particles, were observed. These included loss of grana stacks, vesiculation of membranes, changes in freeze-fracture particle sizes, and crystallization of integral protein complexes. Since the majority (~77%) of the unpigmented chloroplast membrane lipids are uncharged mono- and digalactolipids, the structural changes could be caused by the flooding of the membranes with charged phospholipid molecules, which constitute only 7% of the normal photosynthetic membranes (38).

Based on the above-listed findings, the goals of the current study were: (*a*) to determine the size of reconstituted cytochrome f/b_6 and CF_0-CF_1 complexes in freeze-fractured liposomes of different lipid composition and thereby gain information on the composition and possible size of these enzymes in thylakoid membranes, (*b*) to determine if antibodies against the cytochrome f/b_6 complex can aggregate the reconstituted particles within the plane of the bilayer membrane, and (*c*) to reevaluate the topographical location of the cytochrome f/b_6 molecules within the thylakoid membrane.

MATERIALS AND METHODS

Isolation of the CF_0-CF_1 ATP Synthetase and the Coupling Factor CF_1: The CF_0-CF_1 ATP synthetase complex was isolated according to the method of Pick and Racker (42). Commercial spinach leaves were washed and destemmed prior to homogenization in a Waring blender in cold buffer (0.1 M NaCl, 200 mM sucrose, 50 mM Tris-HCl, pH 7.8). The homogenate was filtered through cheesecloth and centrifuged at 300 *g* for 2 min at 4°C to remove unbroken cells and large debris. Crude thylakoids were collected from the 300 *g* supernatant by centrifugation at 1000 *g* for 10 min (4°C), washed twice in 0.15 M NaCl, 10 mM Na-Tricine (pH 8.0), and incubated with 15 mM octylglucoside (Calbiochem-Behring Corp., San Diego, CA) and 0.1% Na-cholate (15 min, 0°C, 2 mg chlorophyll/ml). After centrifugation (225,000 *g*, 35 min, 60 Ti rotor, Beckman Instruments, Inc., Spinco Div., Palo Alto, CA) to remove the unsolubilized membranes, the supernatant was precipitated by ammonium sulfate and the 38–50% fraction separated on a 7–30% sucrose density gradient in 30 mM Tris-succinate (pH 6.5), 1 mM EDTA, 0.1 mM ATP, and 0.1% phospholipids (Sigma IVS soybean phosphatidylcholine; Sigma Chemical Co., St. Louis, MO; 15 h, 150,000 *g*, 4°C). The ATP synthetase complex was identified by the $^{32}P_i$-ATP exchange assay according to Carmeli and Racker (11). The CF_0-CF_1-ATP synthetase fractions were rapidly frozen in liquid nitrogen and stored at −70°C.

The chloroplast coupling factor CF_1 was isolated by the procedure of Binder et al. (10). Complete separation of CF_1 from ribulose 1,5 diphosphate-carboxylase-oxygenase was achieved by two density gradient centrifugation steps (10–30% sucrose density gradient in 20 mM Tris HCl, 2 mM EDTA, pH 7.8). Prior to immunological use, sucrose was removed from the protein solution by elution on Sephadex G-50 in the appropriate buffer and the protein subsequently concentrated by ultrafiltration (Diaflo membranes PM 30, XM 100).

Isolation of the Cytochrome f/b_6 Complex: The cytochrome complex was isolated according to the method of Hurt and Hauska (20). The isolated thylakoids were washed twice in 0.15 M NaCl and 10 mM Na-Tricine (pH 8.0) followed by a washing in 2 M NaBr to remove peripheral membrane proteins. The cytochrome f/b_6 complex was then solubilized with 30 mM octylglucoside and 0.5% Na-cholate (4°C, 30 min, 1.5 mg chlorophyll/ml) and the suspension centrifuged at 300,000 *g* (1 h). The supernatant was precipitated with ammonium sulfate; the fraction with the highest cytochrome content (45–55% ammonium sulfate) was dialyzed against 0.3% Triton X-100 or 30 mM octylglucoside in Tris-succinate buffer and separated on a 7–25% sucrose density gradient in the presence of 0.3% Triton X-100 or 30 mM octylglucoside and 0.1% phospholipids (24 h, 280,000 *g*, Beckman Instruments, Inc. SW 41 rotor). The deep brown cytochrome fraction was recovered, concentrated by centrifugation, rapidly frozen in liquid nitrogen, and stored at −70°C.

Reconstitution of the CF_0-CF_1-ATP Synthetase Complex and the Cytochrome f/b_6 Complex into Liposomes: The cytochrome f/b_6 and the CF_0-CF_1-ATP synthetase complexes were reconstituted into galactolipid and phospholipid liposomes. Digalactolipids isolated from spinach chloroplasts (51) were kindly provided by Dr. S. Sprague (Univ. of Colorado). Digalactolipid liposomes were formed in 30 mM Tris-succinate buffer, pH 8.0, by heating to 65°C and sonicating briefly (52). The indicated membrane proteins were reconstituted using a modification of the procedure of McDonnel and Staehelin (30). The protein solutions (5–10 mg/ml) were mixed with an equal volume of galactolipid liposomes (10–20 mg/ml), followed by three cycles of freeze-thawing. The preparation was flushed with nitrogen, incubated for 1 h, and then treated with Bio-Beads SM2 (Bio-Rad Laboratories, Richmond, CA) for 2 h at room temperature to remove the detergent. Incorporation of the membrane complexes into phospholipid liposomes was accomplished as described above except that the heating step was omitted. The liposomes were concentrated by centrifugation (100,000 *g*, 30 min, Beckman Instruments, Inc. type 65 rotor), resuspended in 30% glycerol, and processed for freeze-fracture electron microscopy (30). Particle sizes were measured according to the method of Staehelin (53).

Preparation of Inside-Out Vesicles: Inside-out thylakoid membrane vesicles were prepared from pea chloroplasts by two-phase partition according to the method of Andersson and Akerlund (3). The isolated chloroplasts were broken by French-press treatment (1,500 psi). The concentration of polyethyleneglycol 4,000 and dextran 500 (Pharmacia Fine Chemicals Div., Uppsala, Sweden) were 6.4 and 6.0% (wt/vol), respectively. For antibody agglutination experiments, the thylakoid fractions B3 and T2 were diluted to 0.1 mg chlorophyll/ml and scored after short (1 h) and long (16 h) incubations (8).

Immunological Procedures: Antibodies against CF_1-ATP synthetase and the cytochrome f/b_6 complex were raised by injecting rabbits subcutaneously in the back. The rabbits were initially injected at multiple sites with a total of 1 and 1.5 mg proteins, respectively, and received booster injections after 6 weeks. The antisera were purified according to Mayer and Walker (29).

Antibody specificity against the native membrane complexes was tested by using the Ouchterlony double diffusion test (41), while the specificity against the constituent polypeptides of the isolated protein complexes was tested by labeling with ^{125}I-protein A (14). Transfer blots (56) from SDS gels of cytochrome f/b_6 were incubated with the appropriate antibodies, and labeled with ^{125}I-protein A (10^6 cpm) (1). Autoradiograms were exposed at −70°C for 5–12 h with intensifying screens. Antiserum against cytochrome *f* was prepared by preadsorption of cytochrome f/b_6 antiserum with cytochrome b_6 polypeptides that were obtained by elution of gel slices after SDS PAGE.

Aggregation Experiments: Reconstituted cytochrome f/b_6 particles were aggregated directly using their respective antibodies alone, or indirectly by additional application of a goat-anti-rabbit antibody. The antibody titer necessary for precipitation was determined empirically by dilution experiments; the reaction times for the antisera were 30–60 min. After incubation, the samples were centrifuged (20,000 *g*, Beckman Instruments, Inc. 75 Ti rotor), glycerinated, and frozen for freeze-fracture electron microscopy.

Analytic Procedures: Gel electrophoresis of the native membrane complexes was performed according to Davis (13), and SDS gel electrophoresis of the proteins according to the procedure of Laemmli (25) in 0.7- and 3-mm thick, 12% acrylamide gels. Cytochrome contents were estimated according to Hurt and Hauska (20), and chlorophyll and protein according to Kirk (23) and Bensadoun and Weinstein (7), respectively.

RESULTS

Isolation: Polypeptide Pattern

The cytochrome f/b_6 complex was isolated according to the method of Hurt and Hauska (20). A final centrifugation step used to concentrate the cytochrome complexes for incorpo-

ration into lipids resulted in a further enrichment of cytochrome relative to chlorophyll. The absorption and difference spectra of the purified complex are shown in Fig. 1. SDS gel electrophoresis (Fig. 2) consistently resolved five polypeptides with apparent molecular weights of 34,000, 33,000, 19,500, 17,500 and 16,000. In some preparations one or two polypeptides with molecular weights less than 14,000 and a 40,000-mol-wt polypeptide were also present. All attempts to remove this latter polypeptide by a second density gradient purification or by gel filtration of the complex in the presence of detergent (Triton X-100 or octylglucoside) and phospholipids failed. Instead, these additional purification steps resulted in a partial breakdown of the complex (Fig. 2). In particular, the 17,500-mol-wt polypeptide, shown to be the Rieske iron center by Hurt et al. (21), was selectively removed from the complex, and the ratio of cytochrome *f* to cytochrome b_6 dropped from 2 to 1.5. Thus the lability of the complex prevented a quantitative determination of its polypeptide composition. Specific staining of the cytochromes with diaminobenzidine (31) showed that the polypeptides of 34,000, 33,000, and 19,500 were heme-containing (Fig. 2); the 34,000 and 33,000 bands probably correspond to cytochrome *f* polypeptides, and the 19,500 band to cytochrome b_6. SDS gel electrophoresis of CF_0-CF_1 ATP synthetase gave a banding pattern nearly identical to the one reported by Mullet et al. (37).

Electron Microscopy: General Characteristics

The cytochrome *f*/b_6 and the CF_0-CF_1 ATP synthetase complexes were reconstituted into phospholipid or galactolipid liposomes. Reconstitution was achieved by mixing preformed liposomes with the purified, detergent-solubilized protein complexes, and by subsequent removal of the detergent by adsorption to polystyrene beads (Bio-Beads). In this manner denaturation or dissociation of the labile proteins by heat, ultrasonic treatment, or prolonged dialysis was avoided. Figs. 3 and 4 show the concave and convex fracture faces of the two types of proteoliposomes. Most liposomes were of the unilamellar type; the rare multilamellar liposomes contained protein particles in both outer and inner layers. The size of

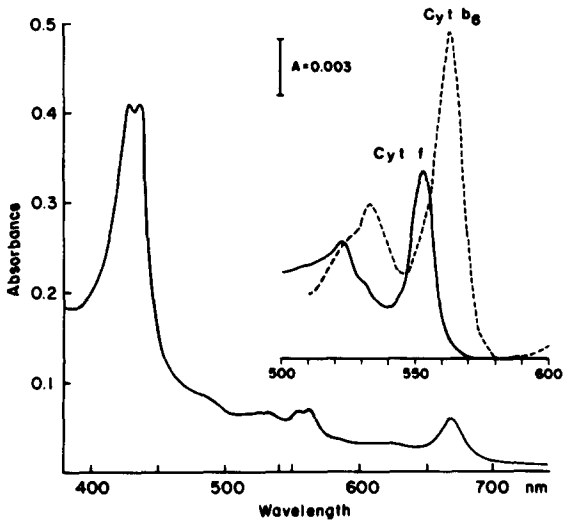

FIGURE 1 Absorption spectrum of the reduced cytochrome *f*/b_6 complex in 30 mM Tris-succinate, pH 6.5. *Inset:* redox difference spectra indicating cytochrome b_6 (– – –, redox reagents: dithionite/ascorbate) and cytochrome *f* (——, ascorbate/ferricyanide).

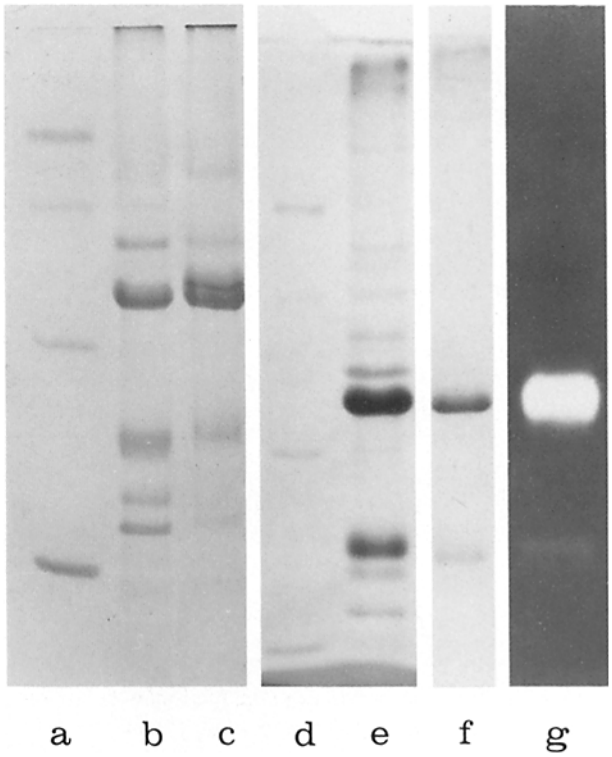

FIGURE 2 SDS gel electrophoresis of the cytochrome *f*/b_6 complex before (*b*) and after (c) a second density gradient centrifugation. Analysis of the heme-containing polypeptides by staining with diaminobenzidine (*f*), compared with a Coomassie Blue poststained preparation (e). Autoradiogram of a transfer blot of the cytochrome *f*/b_6 complex labeled with antibody-Protein A-^{125}I (*g*). Reference proteins (*a* and *d*) from the top: bovine serum albumin (68,000), ovalbumin (45,000), chymotrypsinogen A (25,000), and lysozyme (14,300).

single liposomes varied from 0.05 to 1.0 μm. The particle density depended on the ratio of lipid to protein applied in each experiment. However, the extent of incorporation varied considerably from liposome to liposome (in one experiment 100–800 particles/μm^2).

To further confirm the incorporation of the purified complexes into the liposomes, the reconstituted vesicles were pelleted and the distribution of the complexes between pellet and supernatant determined. Using the $^{32}P_i$ATP exchange assay (11) for the CF_0-CF_1 ATP synthetase, and a spectrophotometric assay for the cytochrome *f*/b_6 system (20, 21), we found that close to 100% of the complexes became incorporated into the liposomes. Absorption and difference spectra of the cytochrome *f*/b_6 proteoliposomes were identical to those for the isolated complexes in Triton X-100 micelles. Furthermore, SDS gel electrophoresis revealed that all polypeptides present in both types of isolated complexes also became associated with the reconstituted liposomes. Based on these observations we conclude that the particles seen on the freeze-fractured, reconstituted liposomes correspond to CF_0-CF_1 ATP synthetase and cytochrome *f*/b_6 complexes. Control liposomes lacking protein exhibited smooth fracture faces.

Appearance of Cytochrome *f*/b_6 and ATPase Particles

Replicas of freeze-fractured cytochrome *f*/b_6 and the CF_0-CF_1 ATP synthetase proteoliposomes revealed randomly dis-

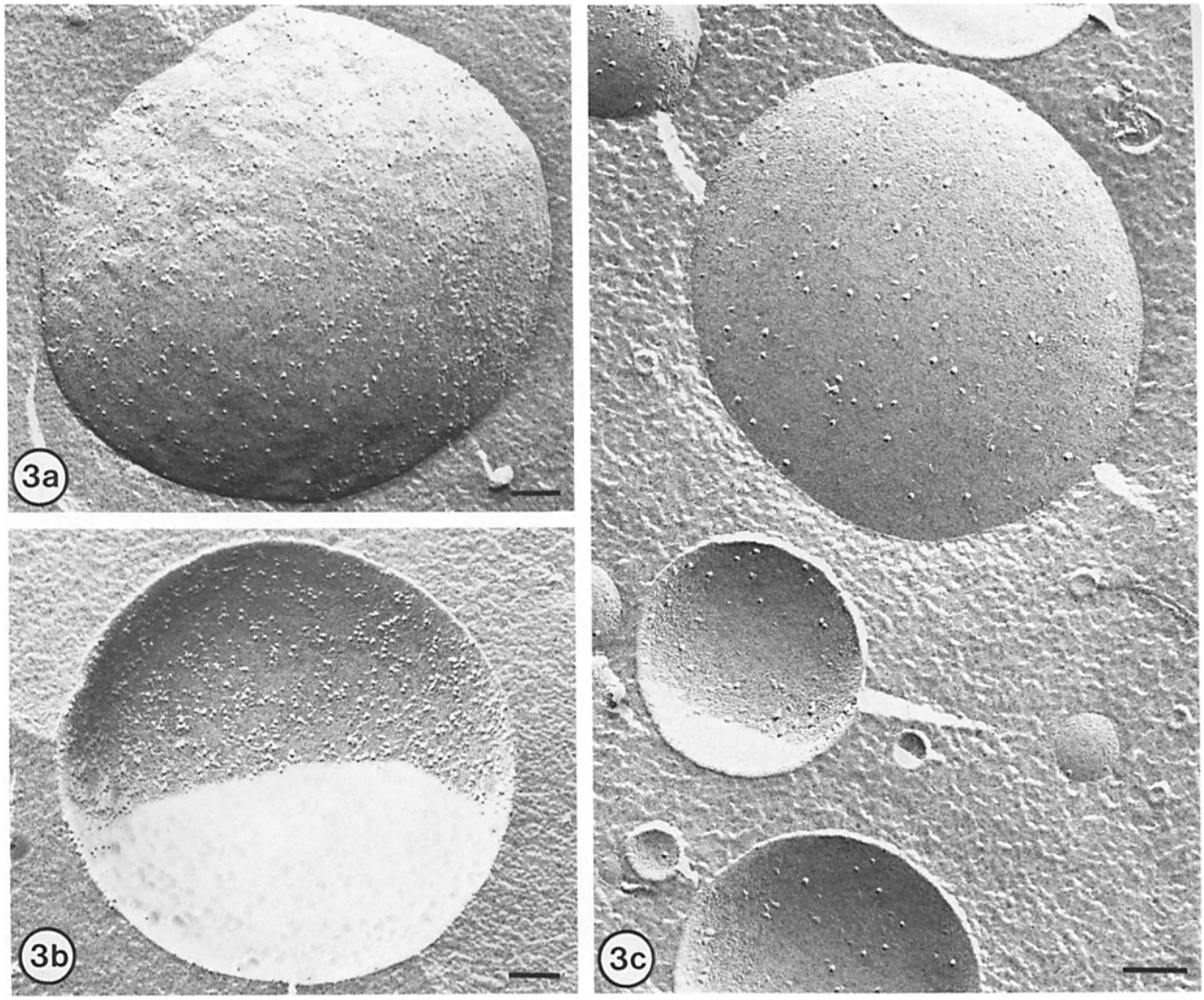

FIGURE 3 Freeze-fractured liposomes with incorporated cytochrome f/b_6 particles. Phospholipid liposomes (*a* and *b*) and galactolipid liposomes (*c*). Bars, 100 nm. *a* and *b*, × 70,000 and *c*, × 90,000.

persed particles on both concave and convex fracture faces (Figs. 3 and 4), suggesting a random insertion of the complexes into the lipid bilayers. Clustering of the particles on the liposomes that would interfere with the antibody aggregation studies was never observed under the conditions used (Figs. 3, *a–c* and 4, *a* and *b*). The average diameter of the CF_0-ATP synthetase particles was 9.7 nm in phospholipid and 9.5 nm in galactolipid liposomes (Fig. 5*a*). The cytochrome f/b_6 particles were smaller, measuring 8.7 and 8.6 nm in the same lipids (Fig. 5*b*). No size differences were observed on the concave and convex fracture faces. The height of the freeze-fracture particles was calculated from the length of the shadows in the center of the liposomes, where the shadowing angle was close to 45°. The values obtained were ~6.0 nm for the cytochrome f/b_6 particles and 5.3 nm for the CF_0-ATP synthetase particles. If one assumes that the particles are roughly symmetrical in shape with respect to the central plane of the lipid bilayer, the overall height of the cytochrome and the CF_0-ATP synthetase complexes would be ~12.0 and 10.6 nm. Measurements of the height of cytochrome f/b_6 particles in cross-fractured, reconstituted liposomes (Fig. 6*f*) gave a value of 11.0 nm, which is close to the 12.0 nm calculated from the shadow length measurements of individual particles. The greater difference in height of CF_0 particles determined by the same two methods, 7–8 nm in cross-fractured liposomes and 10.6 nm based on shadow length, may be attributed to a greater amount of plastic deformation of the particles, or by the fracturing of the CF_0-CF_1 complex in such a manner that the stalk peptides partition with the CF_0 base piece. These measurements clearly support the notion that the height of both complexes is greater than the width of an average bilayer (5–6 nm), i.e., that they are transmembrane complexes and protrude from the bilayer into the aqueous phase. The cytochrome f/b_6 particles rarely exhibit any substructure. In contrast, images of some of the fragmented freeze-fractured CF_0-ATP synthetase particles suggest the presence of subunits (Fig. 4*d*).

When CF_0-CF_1 ATP synthetase liposomes are fractured perpendicular to their membranes, the whole ATP synthetase complex is visible, as shown in Fig. 4*c*. The hydrophobic CF_0-subunit is embedded in the membrane as a transmembrane particle and connected through a small, ~3.5 nm-long stalk to the CF_1 heads (Fig. 4*c*, arrowhead). The presence of CF_1 heads on both sides of the bilayer membrane is consistent with the notion that the particles are randomly oriented within the membrane.

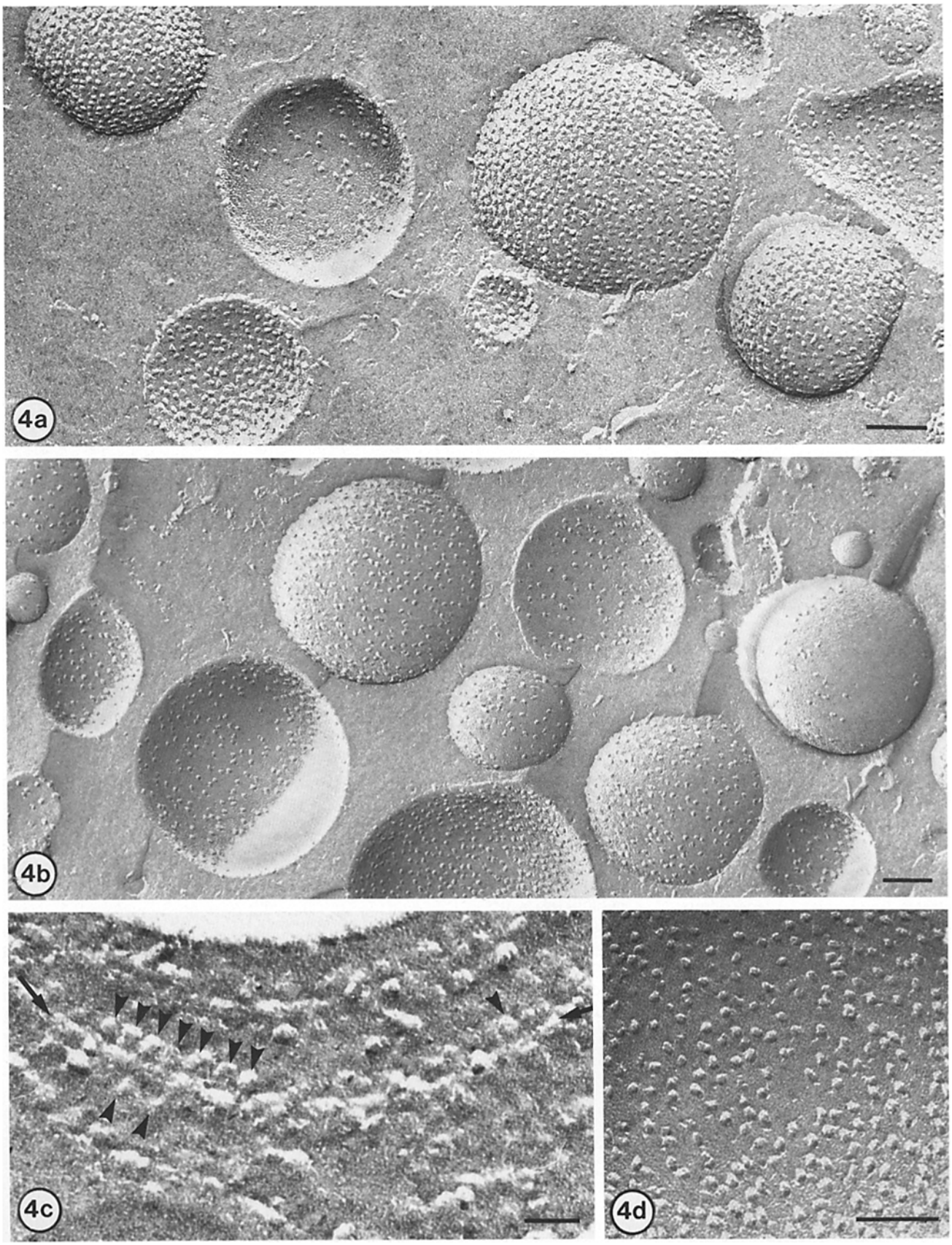

FIGURE 4 Freeze-fractured phospholipid liposomes (*a* and *c*) and galactolipid liposomes (*b* and *d*) with incorporated CF_0-CF_1-ATP-synthetase particles. In *c* the CF_0-CF_1 proteoliposome has been fractured perpendicular to the plane of the membrane. Rows of CF_1-ATP synthetase particles (arrowheads) are seen on either side of the membrane bilayer (arrows). In several instances a space between the membrane surface and the CF_1 units was seen as well as stalk-like connections between the two structures. (*d*) Detail from a freeze-fractured galactolipid CF_0-CF_1 liposome. Some of the CF_0 particles show evidence of subunits. Bars (*a* and *b*), 100 nm; (*c* and *d*), 50 nm. *a*, × 110,000; *b*, × 90,000; *c*, × 300,000; *d*, × 200,000.

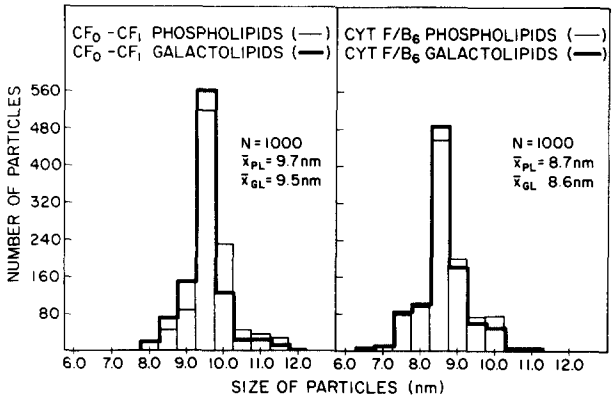

FIGURE 5 Histograms of particle sizes. *Left:* CF_0-CF_1-ATP-synthetase particles incorporated in phospholipid and galactolipid liposomes. *Right:* Cytochrome f/b_6 freeze-fracture particles in phospholipid and galactolipid liposomes.

Aggregation Studies

To evaluate the possibility of using antibodies to aggregate and thereby positively identify cytochrome complexes in intact thylakoid membranes, liposomes containing purified cytochrome f/b_6 complexes were exposed to appropriate antibody preparations. The antibodies were raised against the whole complex because we did not know which polypeptides were surface exposed. However, Western transfer blots showed that our antiserum contained antibodies that reacted primarily to cytochrome *f* and only very slightly to cytochrome b_6 (Fig. 2*g*). Antibodies against cytochrome *f* alone were obtained by preadsorption with electrophoretically separated cytochrome b_6 from the whole complex. Aggregation reactions were carried out either directly with the corresponding antibodies alone, or indirectly (to enhance aggregation) by the application of a second, goat-anti-rabbit antibody. Both the direct and indirect aggregation experiments gave rise to aggregates of freeze-fracture particles, as shown in Fig. 6, *a–g*. However, not all reconstituted vesicles displayed particle aggregates as clearly as shown in Fig. 6, *c–g*. In general, the clusters were larger and more dominant in the samples treated by indirect immunoaggregation, but on the average no more than ~50% of the particles were in the aggregated configuration, consistent with a random insertion of the complex into the bilayer membrane and the presence of the major antigenic sites on one side of the complex only. As shown in Fig. 6, *c*, *d*, *e*, and *g*, the clustered particles formed two-dimensional crystals in hexagonal or square patterns, suggesting that the particles were forced into the densest packing configuration by antibody treatment. Crystalline aggregates of cytochrome f/b_6 particles tended to form rather flat, sheet-like structures which did not readily conform to the curvature of smaller liposomes (Fig. 6, *c* and *d*). As a result, these aggregates seemed to have a high probability of popping out of the liposomes and floating around as planar particle arrays, as is shown in Fig. 6, *e* and *f* and diagrammed in Fig. 7. The center-to-center spacing of the hexagonal and square particle arrays was 8.3 nm. This value was independent of the amount of shadowing material deposited and might correspond more closely to the true diameter of the particles than to the diameter of 8.5 nm measured on the randomly distributed complexes. However, both values are in good agreement.

Agglutination Studies

To learn more about the localization of the cytochrome *f* and b_6 molecules within the thylakoid membranes, right-side out and inside-out thylakoid vesicles were prepared according to the procedure of Andersson and Åkerlund (3). Thylakoids and right-side out vesicles showed no agglutination with cytochrome f/b_6- and cytochrome *f*-specific antibodies. However, they agglutinated with antibodies against CF_1-ATP synthetase as expected from the stroma-oriented localization of CF_1 (9, 34). In contrast, inside-out thylakoid vesicles were readily agglutinated with antibodies against the cytochrome complex and with purified antibodies against cytochrome *f* at low titers (1:32–1:256), while the antibodies against the CF_1-ATP synthetase had no effect. These experiments proved that cytochrome *f*-specific (and possibly b_6-specific) determinants are exposed on the inner thylakoid surface. Our findings also support the idea that cytochrome f/b_6 is present in stacked membrane regions and not only in stroma regions. When anti-cytochrome f/b_6 antibodies were mixed with pure cytochrome f/b_6 complexes prior to incubation, no agglutination of the thylakoid vesicles was observed.

DISCUSSION

As previously mentioned, the functional identification of EF particles of freeze-fractured thylakoid membranes is more advanced than that of PF particles (22). The relatively slow progress that has been made in the characterization of PF particles can be traced to their uniformity in size, their diversity in function, and their density of packing within the plane of the membrane. It is now generally assumed that many of the functional complexes prepared from detergent-solubilized thylakoid membranes could closely resemble the complexes present in intact membranes. Thus one of the indirect methods suitable for studying structural parameters of purported PF particles is to isolate the functional complexes, reconstitute them into liposomes, examine their appearance in freeze-fracture replicas, and compare the particles formed in this manner with the "in vivo" particles of thylakoid membranes. It is clear that the physiological conditions of membrane assembly are very different from those employed in reconstitution studies, and that these differences have to be considered in the interpretation of the data. However, the fact that the reconstituted ATP synthetase complex can phosphorylate (42) and that the cytochrome f/b_6 complex exhibits a significant plastoquinol-plastocyanin oxidoreductase activity (20) suggests that the two complexes examined in this study could closely resemble their "in vivo" forms.

Effect of Different Lipids on Complex Size

Siegel et al. (48) have reported that enrichment of isolated thylakoid membranes with phospholipids leads to significant alterations in thylakoid membrane structure and to a decrease in the mean size of the particles. Fluorescence emission studies indicated that some of the changes were brought about by the dissociation of light-harvesting complexes from photosystem II reaction centers. In the context of the present study it seemed therefore important to determine whether the composition of the liposomes had any effect on the morphology of the reconstituted cytochrome f/b_6 and CF_0-CF_1 ATP synthetase complexes. Chloroplast membranes contain nearly

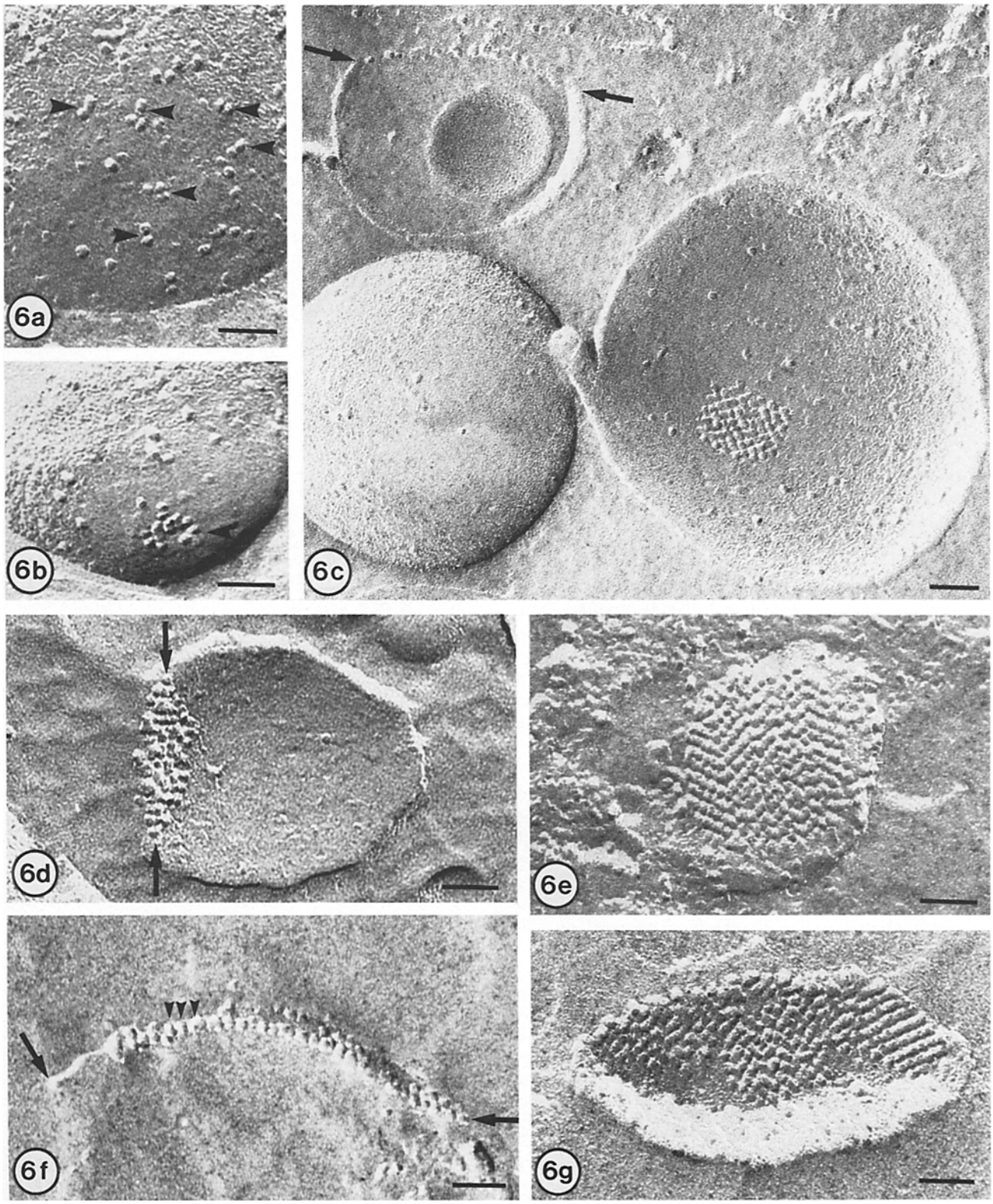

FIGURE 6 Liposomes containing cytochrome f/b_6 particles aggregated by antibodies prepared against the cytochrome complex. *a* and *b*, treated with anti-cytochrome f/b_6 IgG only, depict dimeric and trimeric particle aggregates (*a*, arrowheads), and a small multimeric particle patch (*b*, arrowhead). Samples shown in *c–g* were treated with rabbit-anti-cytochrome f/b_6 IgG followed by goat-anti-rabbit antibodies and show crystalline arrays of antibody-aggregated particles. The center-to-center spacing of the particles in these lattices is 8.3 nm. Note also how the flat arrays can deform the liposomes (*c* and *d*, arrows), leading in some instances to the separation of particle arrays from the liposomes (*e* and *f*). In *f* the free ends of a membrane fragment containing aggregated cytochrome f/b_6 particles are seen (arrows). The arrowheads point to individual complexes seen edge-on, and which have a height of ~11 nm. Bars, 50 nm. *a, b, d, e, g,* × 200,000; *c,* × 165,000; *f,* × 185,000.

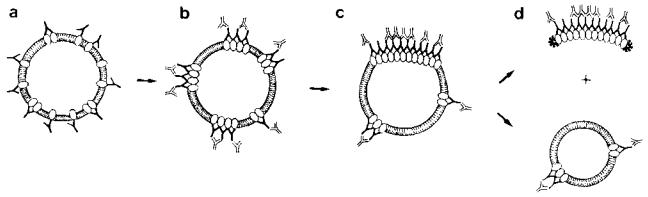

FIGURE 7 Diagram showing steps associated with the aggregation of liposomal particles by indirect antibody treatment. (a) Binding of the corresponding antibody. (*b* and *c*) Enhancement of aggregation by application of the second antibody. (*d*) Formation of sheet-like particle patches which can pop out of the liposomal membrane.

80% uncharged mono- and digalactolipids and only 7% phospholipids (38). The changes in membrane structure observed by Siegel et al. (48) may have been caused by the incorporation of large quantities of zwitterionic lipid molecules into the membranes. For our reconstitution studies we chose a commercial preparation of soybean phospholipids and purified digalactolipids from spinach thylakoids. As judged by the lack of any changes in size of the reconstituted particles, the lipid composition of the liposomes used in our studies had no effect on the general morphology of the isolated cytochrome f/b_6 and CF_0-CF_1 ATP synthetase complexes.

The average size of the CF_0 segment of the ATP synthetase complex (~9.5 nm) and of the cytochrome f/b_6 complex (~8.5 nm when dispersed, ~8.3 nm in crystalline arrays) falls well within the range of PF_u particles (7–12 nm) of control thylakoid membranes. Taken together with estimates of particle height (~7.5 nm for the CF_0 subunit and 11 nm for the cytochrome complex), these values allow us to calculate (ellipsoid particle shape, partial specific volume of 0.73 for protein) the upper limits for the molecular weight of these reconstituted complexes as being ~260,000 for the CF_0 segment of the ATP synthetase and ~280,000 for the cytochrome f/b_6 complex. These calculations are valid for soluble proteins with a compact, globular configuration, but may not always be appropriate for complexes of integral membrane proteins visualized by freeze-fracture electron microscopy: Aside from the possibility of plastic deformation occurring during the fracturing process (50), the latter complexes may contain sizable lipid domains, as is the case for bacteriorhodopsin (19). Alternatively, a complex may possess a dumbbell-like configuration, which could lead to the formation of freeze-fracture particles containing a significant amount of boundary lipids. This may be the case for glycoprotein particles such as those produced by glycophorin (16, 47). On the other hand, Lepault et al. (28) have reported that the size of mitochondrial cytochrome *c* reductase particles in freeze-fractured reconstituted membranes is essentially the same as determined by three-dimensional image analysis of two-dimensional crystals of this complex.

Calculation of the Molecular Weights of the Isolated Complexes

Using x-ray scattering data, Pick and Racker (42) have calculated a molecular weight of 435,000 for the whole CF_0-CF_1 ATP synthetase complex, while Suss and Schmidt (55) report a value of 490,000. The difference has been attributed to variations in subunit stoichiometries ($\alpha_2\beta_2\gamma$ vs. $\alpha_3\beta_3\gamma$). These molecular-weight estimates would allow ~110,000 for the CF_1-base piece. Our size measurement of the CF_0 base piece of the ATPase (9.5 nm diam) is essentially identical to the value (9.6 nm) reported by Mullet et al. (37) but considerably smaller than the size of the reconstituted yeast mitochondrial ATPase particles (26). Mullett et al. (36) also examined liposomes reconstituted with pure DCCD-binding proteolipid from CF_0 base pieces and observed particles averaging 8.3 nm diam, which they interpreted as self-associated aggregates of those molecules. The CF_0 base piece of the ATP synthetase contains at least three additional proteins besides the DCCD-binding protein (39), which would be expected to increase the diameter of the particle if arranged around the periphery of the channel-forming proteolipid molecules as shown in the model of Nelson et al. (39). However, without additional data, the discrepancy in the molecular-weight estimates for the CF_0 subunit as calculated from x-ray scattering data (42) and from our size measurements on freeze-fractured particles cannot be resolved unambiguously. The most plausible explanation would seem to be that the CF_0 particles, as visualized by freeze-fracture electron microscopy, contain significant amounts of lipid molecules in addition to the proteins. This might also explain why, to the best of our knowledge, no research group has yet succeeded in isolating a stable and pure CF_0 particle.

The subunits of the cytochrome f/b_6 complex add up to 128,000 mol wt when a 1:1 stoichiometry is assumed; using the cytochrome *f*-to-protein ratio, Hurt and Hauska (20) calculated a particle weight of 148,000. The estimated molecular weight of ~280,000 for the particles seen in our reconstituted vesicles suggests that the reconstituted and the "in vivo" cytochrome f/b_6 complexes could have a dimeric structure. Hurt and Hauska (20), with caution, proposed a monomeric configuration. On the other hand, dimeric cytochrome complexes are not uncommon and have been postulated for cytochrome *c* reductase of *Neurospora* mitochondria (27) and cytochrome *c* oxidase (18) based upon electron microscopy of crystalline membrane structures. The dimeric cytochrome *c* reductase of mitochondria has an aggregate weight of ~550,000 and dimensions of 9.0 × 7.0 nm in projection and 15.0 nm in height. When inserted into a 5-nm thick bilayer, it protrudes 7.0 nm on one side and 3.0 nm on the other (27, 57). Based on these data, it seems reasonable to propose a dimeric configuration for the 8.3 × 8.3 × 11 nm cytochrome f/b_6 complex of chloroplast membranes.

Sidedness of the Cytochrome f/b_6 Complex

Using well-defined thylakoid fractions, right-side-out and inside-out thylakoid vesicles prepared by the phase partitioning method of Andersson and Åkerlund (3), we have localized the main antigenic determinants of the cytochrome f/b_6 complex, and of cytochrome *f* in particular, on the inner thylakoid surface. This result is in agreement with the studies of Hauska (17) and Racker et al. (43), who localized cytochrome *f* by immunoagglutination studies of sonicated thylakoids on the inner thylakoid surface. In contrast, Schmid et al. (44) found that their antibodies prepared against cytochrome *f* bound to components on the outer thylakoid surface. The different findings of the latter authors could be caused by either a transmembrane disposition of cytochrome *f* or by the presence of antibodies against a minor contaminant in the cytochrome *f* preparation. It should also be mentioned that clean separation of right-side-out from inside-out thylakoid membrane preparations has only become possible since the introduction

to chloroplast research of the aqueous dextran-polyethylene glycol two-phase partitioning system (3).

Antibody-induced Aggregation of Cytochrome f/b_6 Complexes

The use of antibodies to cross-link and aggregate the cytochrome *f*/b_6 complexes within the plane of the reconstituted liposomes indicates that it should be possible to employ this approach to positively identify these complexes within thylakoid membranes, providing that methods can be developed to lipid enrich these membranes without the side effects observed by Siegel et al. (48). That our antibodies were only able to aggregate ~50% of the intramembrane particles of the reconstituted liposomes provides further support for the hypothesis that the cytochrome *f*/b_6 complexes become randomly inserted into the liposomal membranes and that our antibodies are against antigenic determinants localized on one side (the inner thylakoid surface side) of the complex. The intriguing observation that sheets of antibody-aggregated cytochrome *f*/b_6 complexes can apparently pop out of the liposomal membranes is of interest from a membrane-dynamics point of view. More importantly, it suggests that under certain conditions antibodies against membrane components may produce functional inactivation of such components by physically extracting them from the membranes.

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