THE VISUALIZATION OF THE PHOTOSYNTHETIC COUPLING FACTOR IN EMBEDDED SPINACH CHLOROPLASTS

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ABSTRACT

Spinach chloroplast lamellae were stained with aqueous uranyl acetate immediately after glutaraldehyde-osmium fixation but before dehydration and embedding. Under these conditions, the lamellae are shown in thin sections to have 95-Å × 115-Å coupling factor particles on their surfaces. The particles can be seen only on the matrix side of nonopposed thylakoids, and are shown to occur on both stromal and granal lamellae, regardless of the organization of the lamellae into stacks. It is estimated that, in native, fully coupled chloroplast lamellae, there is on the average one coupling factor for every 500 chlorophyll molecules. The morphological appearance of the particles is not affected by a variety of buffers, by changes in illumination or temperature, or by alterations in the energy state of the membranes during preparation.

The particles can be removed from the membranes with low concentrations of Na_2EDTA, and the photophosphorylating activity of the membranes is concomitantly lost. Both the activity and the appearance of the particles can be restored to the membranes by rebinding EDTA-extracted coupling factors to the uncoupled membranes.

The presence of a 90–100-Å particle on the surface of negatively stained chloroplast lamellae was first reported in 1964 (21) and this observation has been extensively confirmed (13, 14, 29, 30). Because the photosynthetic coupling factor (CF_1) was removed from such lamellae after extraction with *dilute* EDTA[1] solutions (15, 16) with a concomitant loss of the 100-Å particles from the membrane surfaces, and because the isolated CF_1 in negatively stained preparations appeared to be identical to these membrane-bound particles, the 100-Å diameter particles were identified as CF_1 (14). Upon recombining the uncoupled particle-free membranes with the purified CF_1, photophosphorylating activity was restored (14). However, the morphological appearance of such reconstituted membranes was not reported in that study.

Negatively stained and even metal-shadowed preparations of membranes cannot unequivocally show the relationship of the particle with the membrane because of the severe dehydration artifacts inherent in these techniques. Reports from embedded and sectioned lamellae have not yet provided information on this problem, since most of such studies have not even been successful in visualizing the coupling factor (4, 24). Indeed, the systematic failure to visualize the coupling factor as a distinct morphological entity in thin sections

[1] *Abbreviations used in this paper*: CF_1, chloroplast coupling factor; EDTA, ethylenediaminetetraacetic acid; PCA, perchloric acid; STN, buffer solution containing 0.8 M sucrose, 0.01 M Tris-HCl, and 0.01 M NaCl, pH 8.1; Tricine, *N*-Tris(hydroxymethyl)methylglycine; and Tris, *N*-Tris(hydroxymethyl)aminomethane.

was taken—at least by some—to indicate that the approximately 100-Å particle observed in negatively stained preparations of chloroplast and mitochondrial membranes was the result of a modification of the surface of such membranes by the salts employed for negative staining (37). According to such a view, the particulate appearance of those membranes after negative staining was an artifact of denaturation of the membrane surfaces, induced by the continuously increasing concentration of salts as the water was evaporating from the drop of negative stain on the grid. If this interpretation were correct, the surfaces of the above mentioned membranes might very well be smooth while in the native state, which in turn could mean that the coupling factor (ATPase) is either buried within the membrane matrix or is composed of a number of subunits which normally are flattened out on the membrane surface and which under very high salt concentrations (and/or the presence of tungstate) can roll together to form 100-Å spheres.

Since all biological preparations prior to embedding in plastic for thin sectioning are gently cross-linked (fixed) under mild conditions and are *slowly* dehydrated, it is accepted that they suffer very few, if any, morphological distortions that could give rise to detectable structural artifacts. Thus, the shape and size of any structure observable in thin sections would more likely represent the native state of that structure.

It is the intention of this paper to present a method by which a particle of approximately 100-Å diameter can be visualized in embedded material. Reconstitution studies are used to identify the particle as the photosynthetic coupling factor. In addition, it will be shown that the lamellae are *anisotropic* with respect to this molecular moiety, which can thus serve as a morphological marker for membrane sidedness. Finally, the frequency of the coupling factor on the surface of the lamellae is determined, and its possible relationship to the photosynthetic unit is discussed.

MATERIALS AND METHODS

Chloroplast and Chloroplast Membrane Preparation

Chloroplasts were isolated from market spinach as described by Karu and Moudrianakis (16). After a final wash in STN-buffer solution (0.8 M sucrose, 0.01 M Tris-HCl, 0.01 M NaCl, pH 8.1), the chloroplast pellet was resuspended in 20 mM NaCl, and was homogenized in a VirTis homogenizer, model 23 (VirTis Co., Inc., Gardiner, N.Y.), at top speed for 3 min. The homogenate was centrifuged for 5 min at 3000 *g* to remove unbroken chloroplasts. The resulting supernate was then centrifuged for 15 min at 27,000 *g* to sediment the disrupted membranes. All of the above steps were performed at 4°C under dim green light.

EDTA Uncoupling

The membrane pellet from 300 g of spinach was resuspended in 50 ml of 5 mM NaCl and was pelleted at 27,000 *g* for 15 min. The pellet was extracted twice with 10 ml of 1 mM Na_2EDTA solution that had recently been boiled to remove CO_2 and then adjusted to pH 7.0. The uncoupling was performed at 4°C under bright white light with continuous agitation. A third extraction was performed with another 25 ml of the EDTA solution. Each extraction was 15 min in length and the uncoupled membranes after extraction were centrifuged at 27,000 *g* for 15 min. The slightly yellow-green supernates of the first two extractions, without further purification, were pooled and retained as "coupling factor extract."

Reconstitution

Coupling factor extracts from day-old and same-day preparations (from a combined total of 450 g of spinach) were added to thrice EDTA-extracted membranes. The resulting mixture was agitated for 5 min, adjusted to 20 mM NaCl, stirred for 5 min, then adjusted to 3 mM $MgCl_2$, and stirred for an additional 20 min to allow for reconstitution. The membranes were *then* collected by centrifugation at 27,000 *g* for 15 min and the pellet was resuspended in a medium of 20 mM NaCl-50 mM Tricine-NaOH, pH 8.1. The total time elapsed from the initial grinding of the spinach leaves to the final "reconstituted" pellet was 6–7 h.

Two pools of control membranes were prepared: (*a*) by adding a solution of 1 mM Na_2EDTA alone, instead of the coupling factor-EDTA extract, to the thrice-extracted chloroplast membranes ("mock reconstitution"); and (*b*) by adding the coupling factor-EDTA extract to the washed, but not EDTA-extracted, chloroplast membranes ("nonextraction"). In the latter case, STN-washed and broken membranes were suspended in only 0.5 mM NaCl-0.5 mM $MgCl_2$ solution during the extraction step. Then, during reconstitution, a 20-ml aliquot of the day-old coupling factor extract (from a total of 150 g of spinach) was added to the membranes in addition to the NaCl-$MgCl_2$ solution in which they had been suspended. NaCl and $MgCl_2$ were added to final concentrations of 20 mM and 3 mM, respectively, during reconstitution as described above.

Photophosphorylation Assays

Photophosphorylation reactions were performed at pH 8.1 in a medium containing 50 mM Tricine, 20 mM

NaCl, 1 mM ADP, 20 μM pyocyanin, 2 mM Na_2HPO_4 containing 7 μCi/ml $^{32}P_i$, and membranes with the equivalent of 50–65 μg/ml chlorophyll. The reactions were performed under bright saturating white light at 24°C with constant agitation. Reactions were stopped by adding PCA to a final concentration of 10% and the precipitated membranes were removed by centrifugation. The supernate was extracted with butanol-benzene to remove inorganic phosphate, as described by Avron (6) and Adolfsen and Moudrianakis (2). 1 ml of the aqueous fraction was counted in 9 ml water (Čerenkov radiation, see 11) in a Packard scintillation counter, model 2002 (Packard Instrument Co., Inc., Downers Grove, Ill.).

Chlorophyll concentrations were estimated according to the method of Arnon (3).

Electron Microscopy

Membranes were fixed at room temperature for 45 min in 2% glutaraldehyde buffered with 100 mM NaH_2PO_4, pH 7.2, unless otherwise indicated. Samples were then washed in phosphate buffer and postfixed in phosphate-buffered 1% OsO_4 at 4°C for 30 min. After a water wash, the samples were suspended in aqueous 2% uranyl acetate, pH 4.5, at room temperature overnight (to be referred to as *prestaining*). The samples were then sometimes embedded in 2% agar and cut into small blocks for ease of handling during dehydration. Samples were dehydrated in graded ethanol and propylene oxide and were embedded in Araldite-Epon (20).

Silver sections were cut on an LKB or a Sorvall MT-2B ultramicrotome with a glass knife and were picked up on bare 300-mesh copper grids. Sections were poststained at room temperature first with 2% uranyl acetate in 100% ethanol for 15 min, and then with Reynolds' lead citrate (31) for 2 min. Sections were examined with a JEM 100B electron microscope operating at 80 kV with a 50-μm objective aperture and an anticontamination device kept at liquid nitrogen temperature.

RESULTS AND DISCUSSION

Functional Considerations

RECONSTITUTION OF PHOTOPHOSPHORYLATION: It has been demonstrated repeatedly (7, 13–16, 36) that washing chloroplast membrane fragments in dilute EDTA solutions results in loss of photophosphorylation of these membranes. The high-speed supernate of this extraction contains the essential entity needed to restore photophosphorylation to the uncoupled membranes. This entity has been named coupling factor (CF_1) (36) and was purified by sucrose density centrifugation (14). It was found to be a protein which appeared as a 90–100-Å sphere in electron micrographs of negatively stained fractions obtained from the 13S area of the sucrose density gradient profile (13).

Howell and Moudrianakis (14) studied the reconstitution of ATP synthesis after the addition of purified CF_1 to EDTA-uncoupled membranes. Since unbound or loosely bound coupling factor was not removed from the reconstitution reaction medium before the photophosphorylation assay, the possibility existed—although remotely—that not all of the observed reconstituted activity resulted from bound coupling factor *exclusively*. Also, since electron micrographs of recoupled membranes were not included in this earlier study, morphological demonstration of reconstitution was not established. In the present study, to address these questions directly, the reconstituted membranes were removed from the milieu of the reconstitution reaction (thus freed from excess, unbound coupling factor) and were resuspended in fresh reaction buffer before enzymatic analysis and fixation for electron microscopy.

Two controls were used in analyzing the biochemical activity. These included (*a*) "nonextracted" membranes, which were not uncoupled but which were carried through the physical operations of uncoupling and reconstitution, and (*b*) "mock reconstituted" membranes, which were extracted with EDTA but which were not exposed to coupling factor during the reconstitution step.

ATP synthesis was determined by measuring phosphate esterification after 2 min in light, well within the linear phase of photophosphorylation exhibited by these preparations (Fig. 1). The activities of the nonextracted and reconstituted membranes of two representative experiments are listed in Table I. Biochemical activity of the mock reconstituted lamellae was negligible, as was the activity of the supernates containing the unbound coupling factor.[1] Reconstituted lamellae showed about 30% of the activity of the nonextracted controls. This represents a significant level of reconstitution, especially after the extensive washing to which the chloroplast membranes were exposed.

MORPHOLOGICAL ANALYSIS OF RECONSTITUTION: Using techniques described in Materials and Methods to visualize 100-Å particles in embedded and sectioned material, electron micrographs of test and control membranes (Fig. 2) were evaluated to determine whether extraction and reconstitution significantly reduced and re-

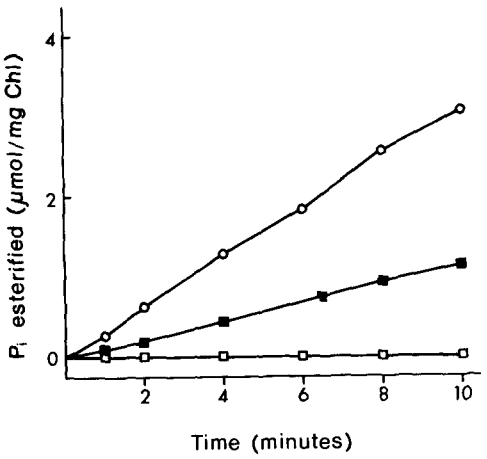

FIGURE 1 Rates of reconstituted photophosphorylating activity (μmol P_i esterified/mg chlorophyll). Nonextracted (O—O), mock reconstituted (□—□), and reconstituted (■—■) membrane activities from experiment 1 are shown. Reaction mixture was 50 mM Tricine-NaOH, pH 8.1, 20 mM NaCl, 1 mM ADP, 20 μM pyocyanin, 2 mM Na_2HPO_4 containing 7 μCi/ml $^{32}P_i$, and chloroplast membranes equivalent to 50–65 μg/ml chlorophyll.

stored the numbers of particles on the membranes, respectively. The frequencies of particles obtained from the three types of membranes were compared, nonextracted with mock reconstituted and mock reconstituted with reconstituted, using the two-tailed *t*-test. In all cases, the probability of chance correlation between the values was less than 1.5%, and in most cases, much less.

The extent of morphological reconstitution for the same experiments for which the previously mentioned biochemical assays were done is given in Table II. Particle frequency was obtained by counting particles per unit length of perpendicularly sectioned membrane and squaring the resultant value. Such linear frequencies are more readily obtainable than areal measurements from tangentially sectioned membranes and therefore were used for comparisons with biochemical data. The last column in Table II lists the percentages of actual reconstitution, representing only those particles which have been *re*associated with the membranes; not included in the values are those particles which were not removed by the EDTA extraction.

It is the values in the last column which should be compared to those of biochemical reconstitution because the particles left on the membranes after extraction and mock reconstitution show no biochemical activity (as indicated in the previous section). The observation that uncoupled membranes still have coupling factor bound to them agrees qualitatively with the finding of McCarty et al. (17, 18) who showed that under their conditions, half of the CF_1 on chloroplast membranes was not active in photophosphorylation.

Morphological Considerations

VISUALIZATION AND DIMENSIONS OF THE PARTICLE: As indicated in the previous section, membrane-bound particles can be seen in micrographs of thin sections from plastic-embedded chloroplast lamellae stained with uranyl acetate *before dehydration*. The particles are visible on both perpendicularly and tangentially sectioned surfaces (Fig. 3). On tangential surfaces, the average particle measures 95 Å × 115 Å (Fig. 4), which closely correlates with the approximately 100-Å diameter of the coupling factor observed on negatively stained preparations (13, 14, 25). The approximately spherical particle observed on the *deeply etched* surfaces of lamellae from freeze-fractured chloroplast preparations (27), and believed by us to correspond to the particle seen on sectioned lamellae, also has a diameter of 100 Å.

In perpendicular sections, the particles extend an average of 120 Å from the outer dense layer of the membrane (Fig. 4). In negatively stained preparations (13, 25), this extension is approximately 100 Å and is obtainable only when the particles are located on the folded edge of a membrane. A height for the particle of about 100 Å was also estimated from metal-shadowed lamellae. The close agreement for the particle extension

TABLE I

Reconstitution of Photophosphorylation to Uncoupled Chloroplast Membranes

	μmol P_i esterified/mg chlorophyll/hour		Percent relative activity
Experiment	NE	R	R/NE
1	18.2	6.6	36
2	7.0	1.7	24

Membranes were washed free of the suspending medium used during reconstitution before being assayed. Mock reconstituted membranes showed negligible activity. NE = nonextracted membranes, R = reconstituted membranes. See text for explanation.

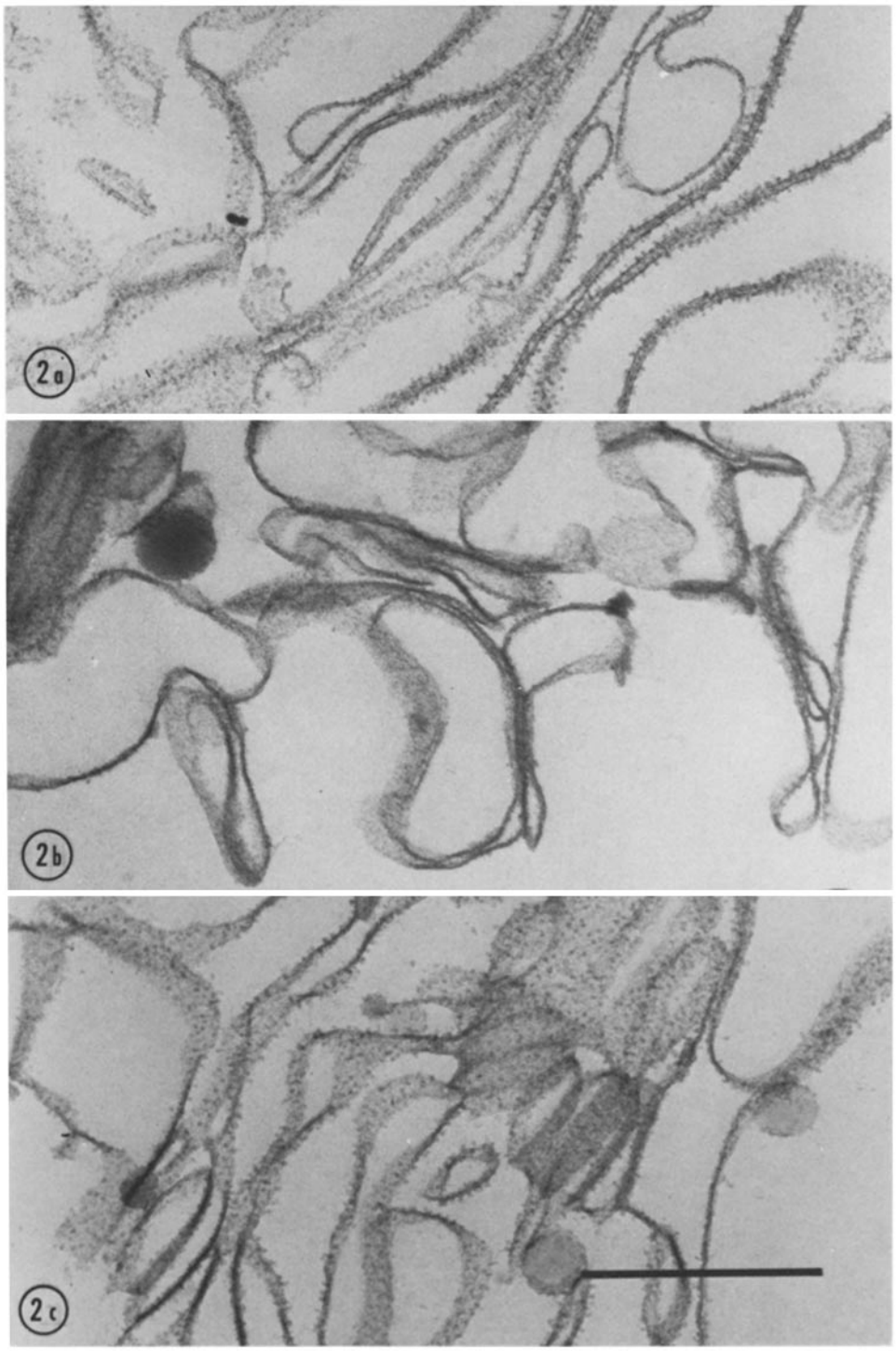

FIGURE 2 Reconstitution of particles to EDTA-uncoupled membranes. In panel *a*, native, nonextracted spinach chloroplast lamellae are shown. As can be seen in panel *b*, these membranes lose a significant number of coupling factor particles after EDTA extraction and mock reconstitution. In panel *c* the particles appear rebound to a significant extent after reconstitution as described in the text. Notice that the membrane-bound particles in panels *a* and *c* are virtually indistinguishable in size and distribution, both in cross sections and in tangential sections of membranes, while very few, if any, are visible in the membranes of panel *b*. For *a* and *c*, bar = 0.5 μm; × 68,000. For *b*, bar = 0.47 μm; × 72,000.

TABLE II

Analysis of Electron Micrographs of Reconstituted Membranes

Experiment	Particles/μm^2				Percent reconstitution		
	NE	MR	R	R-MR	MR/NE	R/NE	(R-MR)/NE
1	1407	312	799	487	22	57	35
2	827	48	534	486	5	64	59

Frequencies were obtained indirectly from linear counts of particles. Procedure is discussed in text. NE = nonextracted membranes, MR = mock reconstituted membranes, R = reconstituted membranes. See text for explanation.

by these three techniques is surprising in view of the variabilities which may occur in each from respective artifacts.

IDENTIFICATION OF THE PARTICLE: The data presented lead to the conclusion that morphological reconstitution of photophosphorylation occurs simultaneously with biochemical reconstitution. Since the results in this study are consistent with what has been established about CF_1 in previous studies, the particles observed on the embedded lamellae can be identified as the photosynthetic coupling factor.

The possibility that the membrane-bound particles are chloroplast ribosomes is eliminated by two considerations. First, chloroplast ribosomes are much larger in diameter (200 Å) when measured in embedded preparations (9). Second, the loss and reconstitution of ATP synthetic activity correlates with the presence of the particles.

EFFECTS OF STAINING: It may be asked why such an abundantly visible structure was not seen earlier. We have twice in recent years shown electron micrographs of sectioned chloroplast fragments with the particle easily visible on the lamellae (22, 23), but until now such pictures have not been very reproducible. After a careful analysis of the preparative techniques, we have concluded that the most important factor in particle visualization is the use of uranyl acetate as a prestain *before dehydration*. Without its use, not only are the particles difficult to observe, but the membranes themselves are less clearly defined (Fig. 5 *a*). In the absence of prestaining, if concentrated methanolic or ethanolic uranyl acetate is used for longer periods of *post*staining (30 min), the 100-Å particle can be observed in thin sections (Fig. 5 *b*), although not as clearly nor as frequently as when the preparations are *pre*stained for even as short a time as 20 min (Fig. 5 *c*).

Particles were also resolved on *Chlamydomonas* chloroplast membranes using uranyl acetate as a prestain (10), and the mitochondrial coupling factor has recently been observed with the use of the same technique (34). Reexamination of micrographs of sectioned chloroplasts published by Sane et al. (32) also indicates the presence of particles on lamellar surfaces that were poststained for 2 h with uranyl acetate, but these authors did not emphasize the significance of that staining step, nor did they comment on the presence of the particles.

It has recently been demonstrated that uranyl acetate used after osmium fixation and before dehydration actually serves as an additional fixative, especially for phospholipids (12). This may account for the greater membrane delineation observed during the course of this study (cf. Figs. 3 and 5 *a*). However, on the basis of the images obtained here after increased poststaining time, it seems that the effectiveness of uranyl acetate as a prestain is due less to its fixative ability than to its staining enhancement. This enhancement must be the result of the *binding* of uranyl ions to the coupling factor *prior* to its *dehydration*. If the aqueous uranyl solution is used *after* the dehydration of the coupling factor, the partition of the uranyl ions into the domain of the dehydrated CF_1 is decreased, yielding lower binding and thus lower contrast in the electron microscope. This difficulty is partially overcome by the use of methanolic uranyl acetate, apparently because this solvent can more effectively partition into the dehydrated staining site and thus can carry the staining species there. Nevertheless, the maximum contrast (i.e., the maximum binding of uranyl ions per CF_1) is obtainable only when the stain is made to interact with the hydrated site, and thus we interpret the contrast to be controlled by the polar properties of the microenvironment of the staining site.

EFFECTS OF THE ENERGY STATE OF THE LAMELLAE: The effect of a number of factors

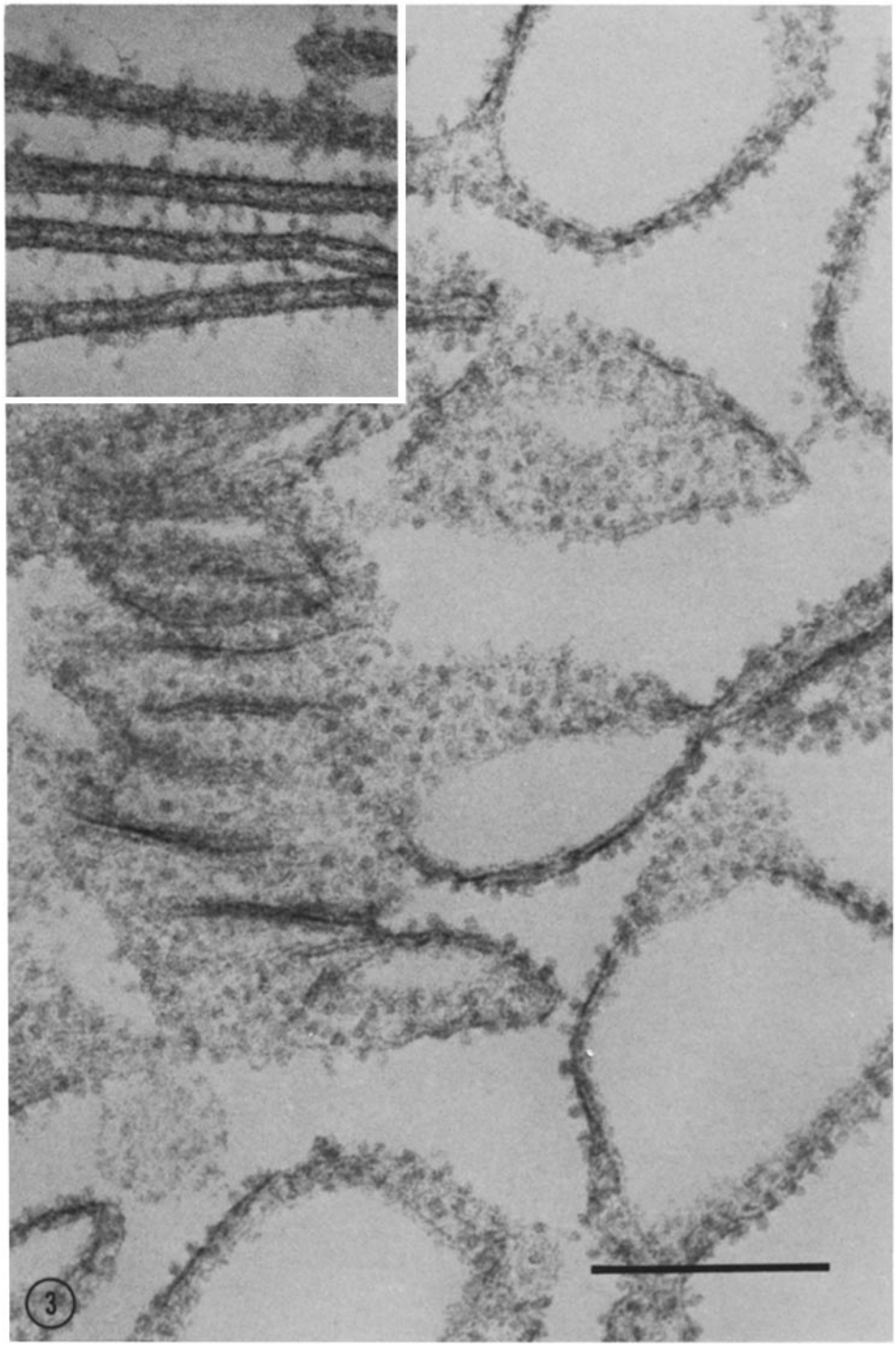

FIGURE 3 Disrupted chloroplast membranes stained with aqueous 2% uranyl acetate before dehydration Particles are visible on both perpendicular and tangential views of the membranes. Bar = 0.2 μm; × 168,000. *Inset*: bar = 0.17 μm; × 192,000.

employed during the isolation of the spinach chloroplast membranes on the appearance of the coupling factor was also tested. The use of a variety of buffers (Na acetate, Veronal acetate, Tris, NH_4Cl) for suspending the membranes, and alterations in the energy state of the chloroplast fragments by the addition of electron transport cofactors or inhibitors had no apparent effect on the coupling factor size and frequency.

DEHYDRATION EFFECTS: Although the evidence presented in this study (and to be discussed later) indicates that the particles are on the outer surface of the thylakoids, *in situ* the coupling factor may be partially embedded within the membrane. During the process of preparation, some membrane component(s) may be extracted and structural relationships may be altered, raising the particles above the remaining structure. There are indications that acetone dehydration removes fewer lipids (1, 5) than dehydration with alcohol-propylene oxide, which was the method routinely used in this study. However, the use of acetone in control experiments did not affect the appearance of the particles or of the lamellae (Fig. 5 *d*).

Furthermore, the fact that the unidentified particle seen on the *outer* surface of thylakoids in deeply freeze-etched chloroplasts appears to be identical to the coupling factor supports a major contention of this work that, *in situ*, the coupling factor on *nonopposed* lamellae is at least partially above the surface of the membrane.

FREQUENCY OF THE PARTICLE: The average frequency of the coupling factor, determined from projected areas of tangentially sectioned lamellae, is 1,600/μm^2 (Fig. 6). This value, however, must be corrected for artifacts due to membrane tilting and undulations within the 650-Å thick section. In a few select micrographs where only tilting was accounted for, the frequency is about 600 particles/μm^2. Using the latter value, which we believe to be closer to the actual frequency, and using a chlorophyll concentration of 310,000/μm^2 (35), which assumes that the chlorophyll is homogeneously distributed and on only one side of the lipoprotein bilayer, we calculate about one coupling factor for every 500 chlorophyll molecules.

The frequency of the spinach coupling factor obtained from negatively stained preparations has previously been reported to be 3,000 particles/μm^2 (25). Park and Biggins (26) observed the same frequency for the "quantosome." The discrepancy between these values and the one obtained in the present study may be attributed to more severe membrane shrinkage during air dehydration and perhaps to precipitation of suspended molecules that settle onto the membrane during drying. Recently Strotmann et al. (33) calculated, from the Ca^{++}-dependent ATPase activity of CF_1, a value of one coupling factor per 850 molecules of chlorophyll.

LOCATION OF THE PARTICLE: Two questions must be answered with respect to the location

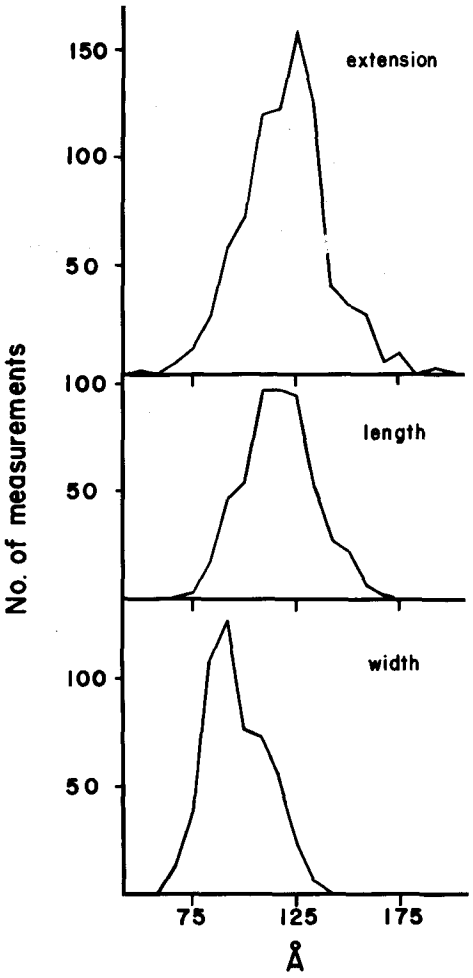

FIGURE 4 Distribution of particle dimensions. Extension (height) was measured from sections cut perpendicularly to the lamellar surface, while length and width were measured from sections cut tangentially to the lamellar surface. Average extension = 120 Å, average length = 115 Å, and average width = 95 Å. Standard deviation for all measurements is 15%.

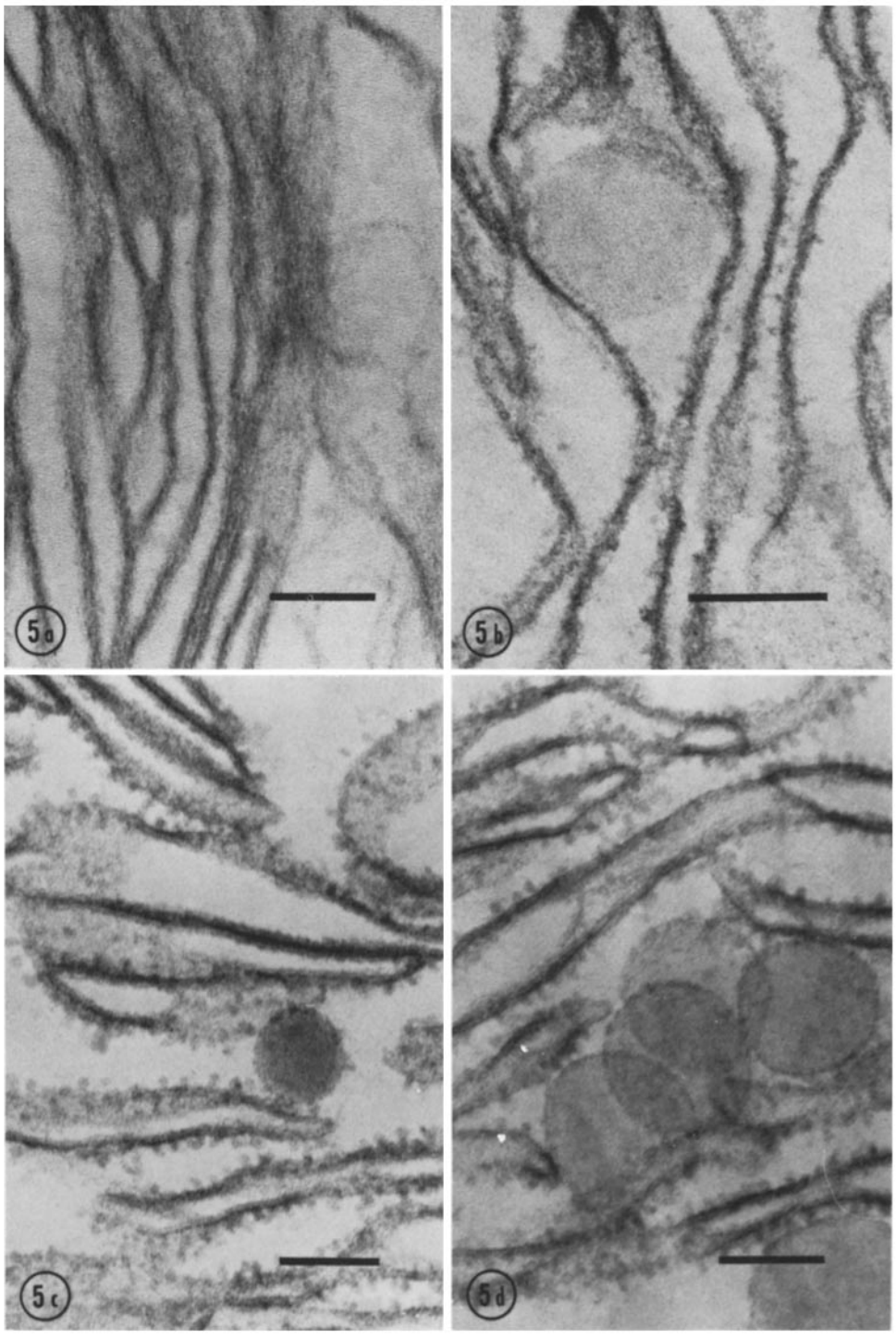

FIGURE 5 (*a*) Membranes prepared without prestaining, but poststained as usual. Particles are not visible and membranes are not clearly delineated. × 144,000. (*b*) Membranes prepared with a 30-min poststain in 5% uranyl acetate in 100% ethanol (no prestaining used). Particles and membranes are not as clear as with prestain (cf. Fig. 1). × 176,000. (*c*) Short 20-min prestain with uranyl acetate. Enzyme particles are clearly visible. × 140,000. (*d*) Membranes dehydrated in acetone. There is no apparent difference between these membranes and membranes dehydrated in ethanol-propylene oxide. × 144,000. All bars = 0.1 μm.

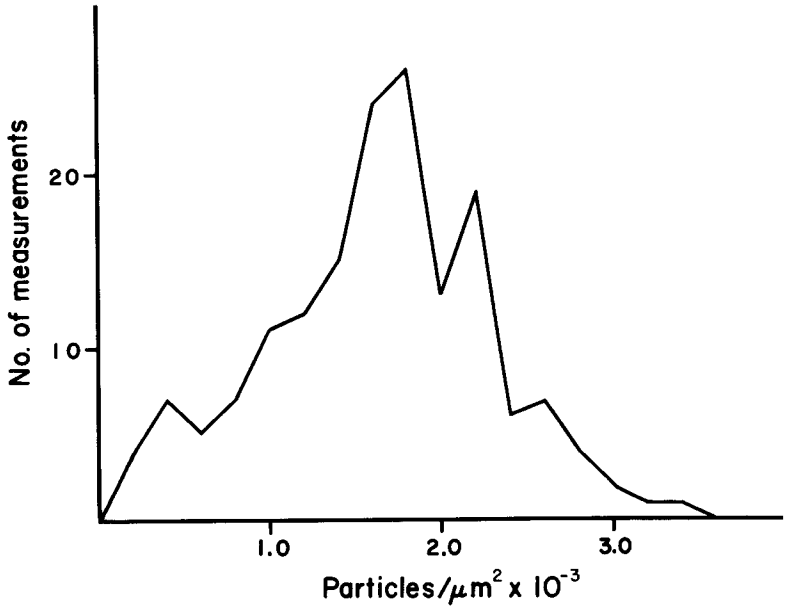

FIGURE 6 Distribution of particle frequency based on projected areas (uncorrected). The average frequency is 1,600 particles/μm^2 with a standard deviation of 50%. Based on areas corrected for membrane tilts within the thickness of the section, the average frequency is 600 particles/μm^2.

of the coupling factor: (*a*) is it found on all chloroplast lamellae, and (*b*) what is its relationship to the membranes? In this section, data will be presented to show that the coupling factor exists *on all lamellae* and, when found on nonopposed membranes, it is located only on the membrane surface *facing the stromal matrix*.

In sections of Class II chloroplasts, which have lost their limiting outer membranes, coupling factor particles can be observed on the stromal lamellae extending into the matrix (Fig. 7 *a*). On these sections, similarly oriented coupling factors can also be seen occasionally on granal lamellae whenever the fused membranes appear slightly separated (Fig. 7 *a insets*).

Although they are not visible between the membrane stacks in Class II chloroplasts because of the close membrane opposition, the fact that particles occur on granal lamellae is strongly corroborated by the results obtained from osmotically disrupted chloroplasts. These preparations contain mainly single disks (Fig. 7 *b*), with the enzyme occurring on practically all external surfaces. Since spinach chloroplast membranes *in situ* are predominantly arranged in grana, and since there is no reason to suspect an enrichment of stromal lamellae during membrane preparation, these disk preparations should also contain a preponderance of granal lamellae. Thus it may be that the coupling factor particles are located on the granal as well as on the stromal lamellae. If coupling factors redistributed from stromal to granal areas during the isolation of the thylakoids, particle frequency would be found to be less on single disks than on stromal areas of Class II chloroplasts. However, the frequencies are very similar and therefore, redistribution seems unlikely.

Because of the lack of cross-sectional information, it is difficult to interpret the orientation of the coupling factor on the surfaces of negatively stained or metal-shadowed lamellae. The possible presence of altered membranes and folded, everted, and/or collapsed thylakoids obscures the determination of the "sidedness" of the coupling factor. Nevertheless, attempts have been made on metal-shadowed lamellae, with contradictory interpretations (26, 29). The interpretive problems encountered in these techniques are not found with embedded and sectioned chloroplast lamellae. Indeed, the solitary disk profiles on Fig. 7 *b* clearly exhibit coupling factor particles that are located only on the thylakoid surface which would face the stroma *in situ*. The possibility of thylakoid eversion during preparation, which would reverse the

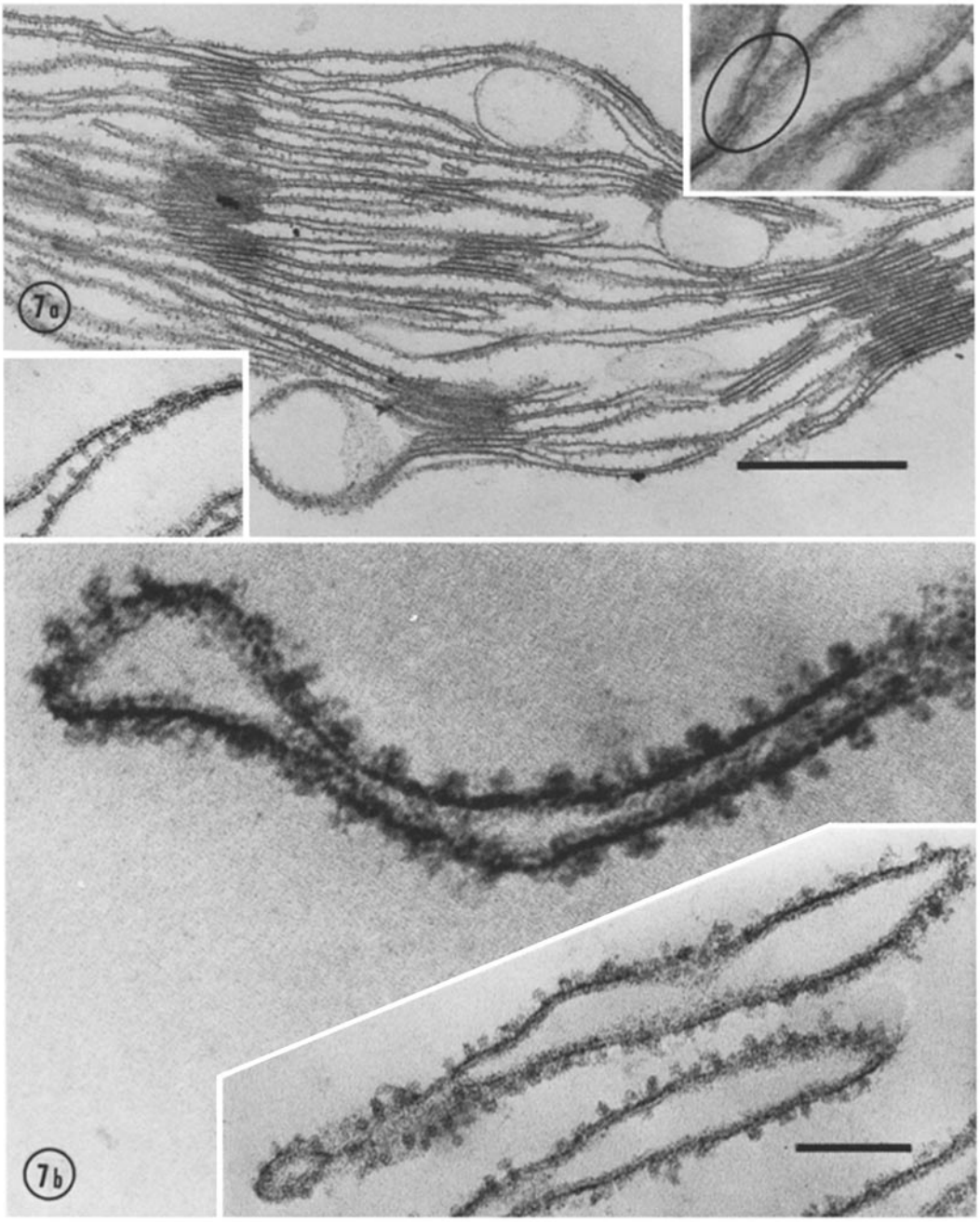

FIGURE 7 (*a*) Class II chloroplast. Coupling factor particles are seen on all stromal surfaces of non-opposed membranes and between granal membranes when stacks separate. *Insets* show areas where stacked membranes are separating; the ellipse emphasizes one such area. The position of the particles identifies the "outside" surfaces of separating thylakoids. Bar = 0.5 μm; × 47,500. Upper righthand *inset*: bar = 0.14 μm; × 176,000. Lower lefthand *inset*: bar = 0.16 μm; × 153,000. (*b*) Preparations of isolated disks. Particles are seen on only those surfaces which would face the stromal matrix *in situ*. Bar = 0.05 μm; × 320,000. *Inset*: bar = 0.1 μm; × 160,000.

apparent orientation of the particles, appears unlikely since Class II chloroplasts, having been exposed to minimal mechanical forces, also exhibit particles oriented toward the stroma (Fig. 7 *a*).

In intact grana, the adjacent disks are so closely opposed that there may not be enough room to accomodate fully extended 100-Å structures between the membranes. Under these conditions, the coupling factors may partially or totally reside in the membrane matrix.

If the particles are indeed located on the outer surface of *nonopposed* thylakoids, as indicated in this study, then the particles should also be visible on deeply etched freeze-fractured chloroplast membranes where sublimation has exposed external surfaces. With the use of such methods, Park and Pheifhofer (27, 28) have, in fact, observed particles whose dimensions are 100–120 Å in diameter and which can be removed with EDTA solutions.

Freeze-fracture studies on closely *opposed* thylakoids should indicate to what extent the coupling factors are embedded in the membrane. Branton and Park (8) observed structures which have a diameter of 110 Å and a height of 90 Å in freeze-fractured, intact grana. Based on the location of the fracture plane, they interpreted the location of the particles as being in the outer (with respect to the disk) half of the membrane. Whether these subunits are identical to the coupling factor is not known. The nonremoval of these particles during EDTA extractions could simply indicate that EDTA cannot penetrate the membrane matrix, rather than that these particles are not morphological counterparts of the coupling factor.

The asymmetric orientation of the coupling factor on the lamellae reflects an inherent asymmetry in the membrane structure itself. Although vectorial activity of the enzyme cannot be established by this morphological observation, the asymmetry, as also occurs with the mitochondrial coupling factor (34), is consistent with one of the requirements of Mitchell's chemiosmotic hypothesis (19).

In closing we should like to point out that by the use of the technique described here and without any addition of external components, a morphological marker has become available (the coupling factor particle) for developmental studies of the origin and the direction of folding of the internal membranes of the chloroplast and mitochondrial organelles.

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