Structural Localization of the O₂-Evolving Apparatus to Multimeric (Tetrameric) Particles on the Lumenal Surface of Freeze-etched Photosynthetic Membranes

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Abstract. Isolated appressed chloroplast membranes, highly enriched in photosystem II (PSII) activity, were examined by freeze-etch electron microscopy. The exposed surfaces of these Triton X-100 solubilized membrane fragments correspond to the lumenal or ESs surface of intact stacked thylakoid membrane regions (Dunahay, T. G., L. A. Staehelin, M. Seibert, P. D. Ogilvie, and S. P. Berg. 1984. *Biochim. Biophys. Acta.* 764:179–193). The sequential removal from this sample of three extrinsic proteins (17, 23, and 33 kD) associated with the O₂-evolving apparatus and the concomitant loss of O₂ evolution, was related to subtle changes in the height and substructure of characteristic multimeric (often tetrameric) particles that protrude from the ESs membrane surface. After removal of these proteins, the multimeric particles disappeared and dimeric particles of similar diameter but of lesser height (6.1 vs. 8.2 nm in the controls) were observed. Reconstitution of the depleted membrane fragments with the extrinsic proteins led to rebinding of the three proteins, to a 63% recovery of the control rates of O₂ evolution, and to the reappearance of the larger multimeric particles. Analysis of the structural changes associated with the loss and rebinding of the extrinsic proteins is consistent with a stoichiometry of one PSII complex for either one or two copies of the 17-, 23-, and 33-kD proteins, and these are symmetrically arranged on the lumenal surface of the complex. These results demonstrate that the multimeric ESs particles correspond to part of the intact O₂-evolving apparatus of PSII, thus confirming previous indirect studies relating these particles to PSII. The dimeric particles probably contain the rest of the O₂-evolving complex.

PHOTOSYNTHETIC electron transport in O₂-evolving organisms is driven by two photochemical reaction centers associated with two photosystems, photosystem II (PSII)[1] and photosystem I (PSI), that operate in series. PSII produces the oxidizing potential required to oxidize water, thereby extracting electrons used in the electron transport chain; PSI generates the reducing potential needed to convert NADP⁺ to NADPH for CO₂ fixation. Oxygen, of course, is a waste product of the water-splitting reaction. The proteins involved in this process are known collectively as the O₂-evolving complex and are tightly coupled to PSII on a functional basis (Andersson, 1986; Babcock, 1987). Topological studies have shown that PSII is a transmembrane protein complex, whereas the extrinsic proteins associated with the O₂-evolving complex reside on the lumenal surface of the thylakoid membrane (Åkerlund and Jansson, 1981).

Until recently, the lumenal location of the O₂-evolving complex was a major obstacle to its biochemical characterization. However, the introduction of new methods to differentially solubilize thylakoids with mild detergents (Yamamoto et al., 1981; Berthold et al., 1981; Kuwabara and Murata, 1982) and thereby produce PSII-enriched membrane fragments with the O₂-evolving complex exposed to the medium has dramatically changed the situation. The resistance of O₂-evolving PSII membrane fragments to detergent fractionation treatments is related to the lateral differentiation of higher plant and green algal thylakoid membranes into PSII-enriched, appressed (grana) and PSI-enriched, non-appressed (stroma) regions. When exposed to a detergent, the mechanically unsupported stroma thylakoids are preferentially solubilized, leaving behind fragments of appressed membranes. The exposed surfaces of these membrane fragments correspond to the lumenal surfaces of intact, grana thylakoid membranes (Dunahay et al., 1984). From a functional point of view, the mechanism of O₂ evolution in these PSII membranes is not altered by the isolation procedure (Seibert and Lavorel, 1983).

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1. *Abbreviations used in this paper:* Chl, chlorophyll; DCBQ, 2,6-dichlorobenzoquinone; DMBQ, 2,6-dimethylbenzoquinone; EF particles, freeze-fracture particles associated with the exoplasmic (lumenal) membrane leaflet; ESs surface, exoplasmic (lumenal) surface in a stacked membrane region; ESu surface, exoplasmic (lumenal) surface in an unstacked membrane region; LDS, lithium dodecyl sulfate; PSI, photosystem I; PSII, photosystem II.

Biochemical studies of these membrane fragments have revealed that the O_2-evolving complex includes three extrinsic membrane proteins of molecular masses 17, 23, and 33 kD (Yamamoto et al., 1981) as well as Mn^{2+}, Ca^{2+}, and Cl^- ions (Babcock, 1987). Because of the "inside-out" orientation of the appressed membrane fragments, it is possible to sequentially remove these proteins by various treatments and to concomitantly monitor the loss of O_2-evolving capability (Kuwabara and Murata, 1983). It is also possible to reconstitute lost functional activity by adding the proteins back to the depleted PSII membranes.

Freeze-fracture electron micrographic studies of thylakoid membranes have demonstrated that proteins associated with PSII give rise to membrane-spanning freeze-fracture particles associated with the exoplasmic (lumenal) membrane leaflet (EF particles). These particles protrude into the lumenal space and can be visualized as particles associated with the exoplasmic (lumenal) surface in either a stacked membrane region (ESs) or an unstacked membrane region (Esu) by freeze-etch techniques (Miller, 1976; Staehelin, 1976; Machold et al., 1977). The ESs particles exhibit a distinct multimeric (often tetrameric) substructure, but to date it has not been possible to correlate the multimeric particles with specific molecular constituents of PSII.

The goal of this study has been to correlate the loss and rebinding of the three extrinsic proteins associated with the O_2-evolving complex to changes in both functional capacity and the structural appearance of the (lumenal) surface of PSII membrane fragments. Our results indicate that the three extrinsic proteins are constituents of the multimeric ESs particles previously identified generally with PSII. A preliminary communication of this work was presented at the VII International Congress on Photosynthesis (Staehelin et al., 1986).

Materials and Methods

Experimental Samples

O_2-evolving PSII appressed membrane fragments were made by the Triton X-100 extraction procedure (Kuwabara and Murata, 1982) from market spinach, and frozen until use. The extrinsic proteins were removed from the membranes by salt, alkaline-Tris, or $CaCl_2$ treatment. The 17-kD, and the 17- and 23-kD proteins were extracted by exposure to 250–300 mM NaCl or 1 M NaCl, respectively, in a 25 mM 2-(N-morpholino)ethane sulfonic acid (MES) (pH 6.5), 300 mM sucrose buffer (Kuwabara and Murata, 1983). PSII membranes were salt-treated (chlorophyll [Chl] concentration, 0.5 mg/ml) for 30 min in the light at 4°C, washed once by centrifugation for 10 min at 25,000 g in 20 mM MES (pH 6.5), 15 mM NaCl, 5 mM $MgCl_2$, and 400 mM sucrose (buffer A), resuspended in the same buffer, and frozen until use. All three extrinsic proteins were removed either by alkaline-Tris (Kuwabara and Murata, 1983) or $CaCl_2$ (Ono and Inoue, 1983) treatment. In this case, PSII membranes (Chl, 0.5 mg/ml) were exposed to 0.8 M Tris-HCl (pH 8.2) and 300 mM sucrose for 30 min in the light at 4°C, washed and resuspended in buffer A as described above, and frozen until use. $CaCl_2$ treatment was accomplished by exposing PSII membranes (Chl, 1.0 mg/ml), which were washed twice in 40 mM MES (pH 6.5), 10 mM NaCl, and 300 mM sorbitol (buffer B), to 0.9 M $CaCl_2$ in buffer B for 30 min in the dark at 4°C. Ca-treated membranes to be used for lithium dodecyl sulfate (LDS)-PAGE were washed three times in buffer A and stored frozen until use.

The three extrinsic proteins were isolated as a set by dialyzing and concentrating the supernatant fraction from 1 M-$CaCl_2$-treated PSII membranes. Dialysis was against two changes of 10 mM MES (pH 6.5) for a total of 4 h followed by a 30,000 g spin for 15 min to pellet any residual membrane. The material was then concentrated using an ultrafiltration cell (Amicon Corp., Danvers, MA) with a PM-10 filter. Reconstitution of the three

extrinsic proteins onto $CaCl_2$-treated PSII membrane fragments was accomplished by an adaption of a procedure (Ono and Inoue, 1984a) used for reconstituting 33-kD protein onto $CaCl_2$-treated PSII membranes. The extrinsic protein concentrate described above was used instead of isolated 33-kD protein. Freshly prepared Ca-treated PSII membranes (see above) were washed once in buffer B and then diluted to 0.25 mg/ml Chl in a final concentration of 40 mM MES (pH 6.5), 10 mM NaCl, 300 mM sorbitol, and 10 mM $CaCl_2$. Included in this suspension was a saturating amount of the extrinsic protein concentrate as determined by preliminary titration experiments using reconstituted O_2 evolution capacity as an assay. The material was incubated for 1 h in the dark at 4°C, assayed for O_2 evolution, washed twice in buffer A to remove unbound protein, and prepared immediately for electron microscopy. Reconstituted samples used for LDS-PAGE were frozen until use.

Assays

Steady-state O_2 evolution rates were measured with a Clark electrode (Yellow Springs Instrument Co., Yellow Springs, OH) in 20 mM MES (pH 6.0), 15 mM NaCl, and 300 mM sucrose buffer (unless otherwise indicated). Oxygen rates of control PSII membrane fragments were 250–300 μmol $O_2 \cdot$mg $Chl^{-1} \cdot h^{-1}$ using 0.4 mM 2,6-dimethylbenzoquinone (DMBQ) as an acceptor and 500–650 μmol $O_2 \cdot$mg $Chl^{-1} \cdot h^{-1}$ using 0.4 mM 2,6-dichlorobenzoquinone (DCBQ) as an acceptor. Manganese was assayed using an atomic absorption spectrometer (model 2380; Perkin-Elmer Corp., Norwalk, CT) equipped with a graphite furnace. Samples for LDS-PAGE were brought to 1% LDS and 50 mM dithiothreitol, and the proteins were separated on 10–15% polyacrylamide gels (Metz et al., 1986). Molecular mass standards were obtained from Bio-Rad Laboratories (Richmond, CA), and the gels were stained with Coomassie Brilliant Blue.

Electron Microscopy

The different PSII samples were fixed with 1% glutaraldehyde in 50 mM phosphate buffer (pH 6.8) for 20 min, washed three times in distilled water, and resuspended in water at a Chl concentration of 1 mg/ml. Freshly cleaved mica mounted on a regular copper freeze-etch specimen support was first glow discharged at 4 kV under a 100–200 mTorr vacuum for 5 min, then treated with a drop of 5 mM Alcian Blue 8GX (Sigma Chemical Co., St. Louis, MO) for 5–10 s, rinsed well with water, and dried with N_2 gas. A drop of the PSII sample was applied to the mica surface for 10 s, and nonbound membrane fragments were washed away with a stream of distilled water. The specimen was placed face down onto slightly damp filter paper to absorb excess water, and then immediately frozen in liquid N_2-cooled Freon 12 and stored in liquid N_2. Freeze-drying (1 h at −75°C in a Balzers [Hudson, NH] freeze-etch apparatus) was used to remove the frozen water from around the bound membrane fragments to expose the ES membrane surface, and a Pt/C replica was produced after cooling to −105°C. The thickness of the evaporated Pt/C (2 nm) and of the C (15 nm) layers was controlled by a quartz-crystal film thickness monitor. The replicas were cleaned on bleach and examined in the electron microscope. The height of the ES particles was determined by measuring, on micrographs enlarged 200,000×, the length of the particle shadows produced by the Pt/C applied at an angle of 45°. Between 150 and 260 particles were measured for each sample (two to three micrographs from two different replicas), and the results were standardized to 150 particles for the histograms. Particle widths were determined as described by Staehelin (1976).

Results

Fig. 1 is an LDS-PAGE protein profile of control PSII membranes and membranes from the same sample treated with either 0.25 M NaCl, 1 M NaCl, or 0.8 M Tris-HCl. A comparison of the different profiles shows that the treatments removed the 17-, the 17- and 23-, and the 17-, 23-, and 33-kD extrinsic proteins, respectively. All other polypeptides are essentially unaffected by the treatments. However, the Tris-HCl treatment also removed $\sim$3.5 of the 4 Mn per reaction center, making this type of sample unsuitable for reconstitution experiments. O_2 evolution rates for the four samples were 502, 406, 117, and $\sim$0 μmol $O_2 \cdot$mg $Chl^{-1} \cdot h^{-1}$, respectively, using DCBQ as an acceptor.
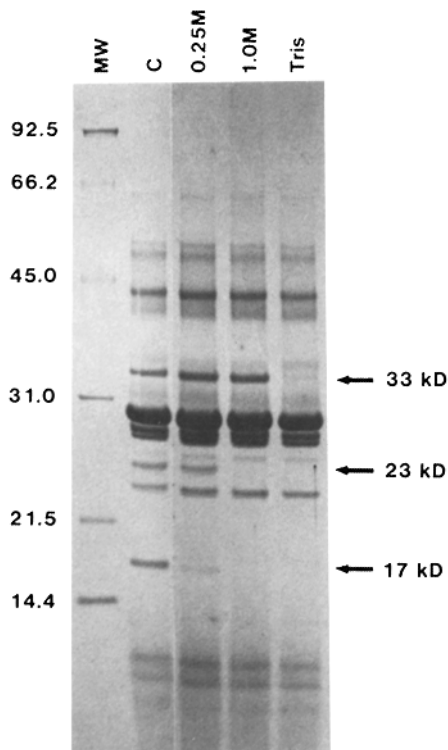
Figure 1. LDS-PAGE of PSII control (*C*) membranes; membranes washed with 0.25 M NaCl to remove the 17-kD protein (*0.25 M*); with 1 M NaCl to remove the 17- and 23-kD proteins (*1.0 M*), and with 0.8 M Tris-HCl (pH 8.2) to remove the 17-, 23-, and 33-kD proteins (*Tris*). The locations of the three extrinsic proteins is indicated by arrows on the right. The first column (*MW*) contains molecular standards of indicated molecular mass.

Fig. 2 *a* shows the lumenal surface of an intact spinach thylakoid exposed by deep-etching. The PSII-enriched stacked membrane region (ESs) in the center of the micrograph is clearly differentiated from the surrounding unstacked areas (ESu) by the large and distinct, multimeric (often tetrameric) particles, which protrude from a very smooth background. As illustrated in Fig. 2 *a*, the ESs particles can be randomly organized or arranged in arrays exhibiting different degrees of order and possessing lattice repeats of ~17.5 and 20.4 nm, respectively. Arrayed particles are only occasionally encountered in isolated spinach thylakoids. The origin of the arrays is unknown, but since they are most frequently encountered in spinach samples purchased during winter months at supermarkets, they may result from certain cold storage/transport conditions. In the context of this study, the arrayed particles are of interest for two reasons. Since they are preserved during mounting of the membrane fragments on mica (see below) and their lattice parameters do not change, they confirm that mica binding does not affect the general lateral organization of the ESs particles. In addition, the arrayed ESs particles invariably display a tetrameric substructure, whereas the randomly distributed ESs particles appear mostly multimeric and only sometimes tetrameric (compare Figs. 2, *a* and *c* and 3 *a*). The reason for this difference in appearance is unknown and unfortunately difficult to test experimentally due to the unpredictability of appearance of these arrays.

As seen in Fig. 2 *b*, PSII membrane fragments, freeze-etched and replicated in the same manner as the intact membrane shown in Fig. 2 *a*, reveal a morphology that closely resembles the ESs regions of thylakoid membranes. However, because of the irregular curvature of the relatively small membrane fragments, it is both impossible to visualize more than a few optimally shadowed particles in any one area, which is essential for evaluating the uniformity of a response to a given treatment, and to obtain precise measurements of particle height on such membranes. The ability to precisely measure particle height is a prerequisite for correlating changes in composition with changes in size of the protruding components of PSII complexes on ESs surfaces. To circumvent this problem, we bound PSII membrane fragments to freshly cleaved mica sheets prior to freezing, etching, and Pt/C shadowing at an angle of 45°. Figs. 2 *c*, 3, *a–d*, and 5, *a* and *b* show different PSII membrane samples processed in this manner. The changes in appearance and size of the multimeric ESs particles caused by the sequential removal of the three extrinsic proteins (Figs. 3, *a–d*) tend to be subtle, but were consistently seen in all of our samples. As is to be expected, the greatest difference is evident between control membranes and membranes from which all three proteins have been removed (compare Fig. 3 *a* with Figs. 3 *d* and 5 *a*). After the loss of the 17-, 23-, and 33-kD proteins, the remaining ESs particles no longer appear multimeric, but are mostly dimeric, irrespective of the type of treatment they received. Typical of these dimeric particles is a tendency to aggregate into short rows (Figs. 3 *d* and 5 *a*). Particle size measurements of the different samples reveal that while the average diameter of the multimeric particles (~16 nm) changes only slightly with the loss of the peripheral proteins, their average height decreases significantly (discussed below).

To substantiate the correlation between removal of peripheral proteins of the O_2-evolving enzyme complex and changes in ESs particle size and morphology, we have also reconstituted depleted PSII membranes by the readdition of the three extrinsic proteins. Fig. 4 shows an LDS-PAGE profile of control PSII membranes, 0.9 M $CaCl_2$-treated membranes, and $CaCl_2$-treated membranes reconstituted with the three extrinsic proteins. $CaCl_2$ treatment removed all of the 17- and 23-kD proteins and ≥90% of the 33-kD protein. The reconstituted sample bound control levels of 33-kD protein, most of the 23-kD protein observed in control membranes, and some 17-kD protein. Table I reveals that $CaCl_2$-treated samples in Fig. 4 have lost virtually all O_2 evolution capacity while reconstituted samples achieved 63% recovery of the control level of O_2 production. This experiment demonstrates that in the reconstituted sample (Fig. 4) the majority of the proteins had rebound to their native sites. The reason for choosing 0.9 M as the $CaCl_2$ treatment concentration was a trade-off between the desire to remove as much 33-kD protein as possible without removing the Mn ions essential for O_2 evolution (see Table II) or destroying the capability of the membrane to rebind the three extrinsic proteins in a functionally active manner. In our hands, this concentration of $CaCl_2$ produced the maximum ratio of O_2 evolution measured in the presence and absence of 50 mM $CaCl_2$ (B/A in Table II; see Ono and Inoue, 1984*b*, for an explanation of the mechanism of Ca-enhancement of O_2 evolution in Ca-treated PSII membranes), and the ratio was
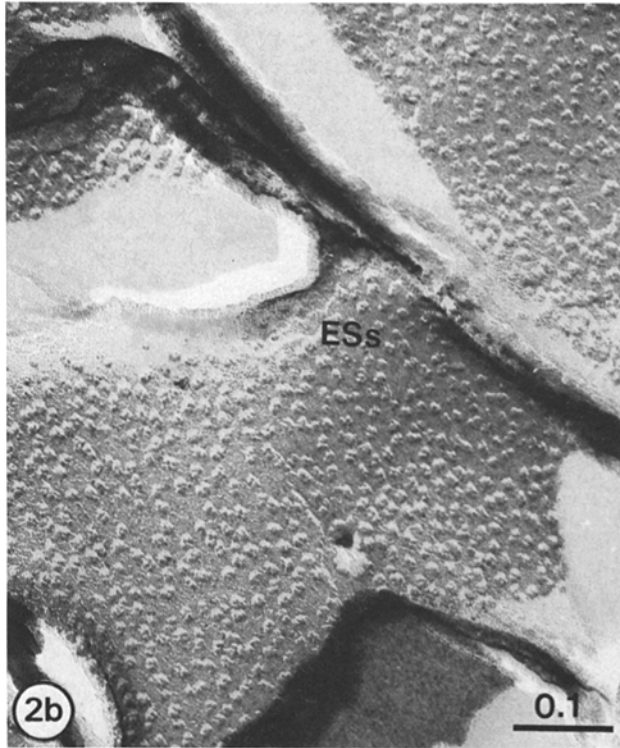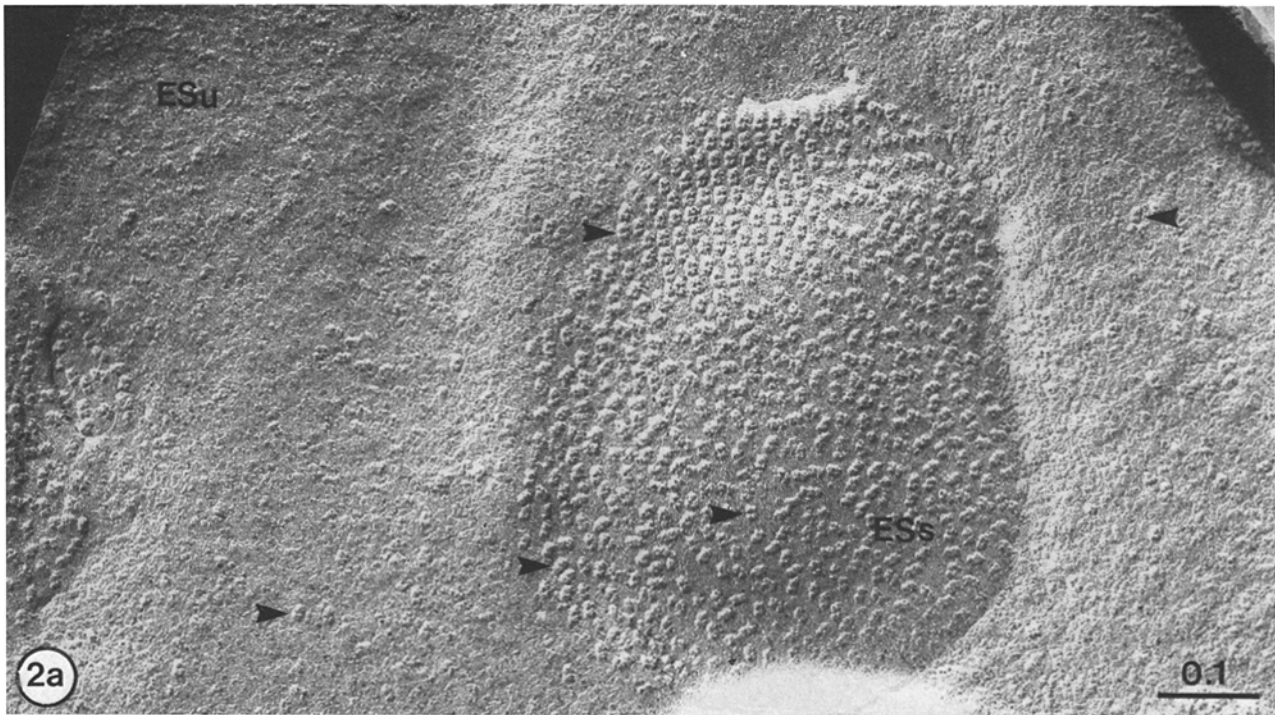
Figure 2(a). Lumenal surface of a spinach thylakoid as revealed by freeze-etch electron microscopy. The stacked membrane region (*ESs*) in the center of the micrograph is clearly differentiated from the surrounding unstacked area (*ESu*) by the large, distinct multimeric and tetrameric particles (*arrowheads*) that protrude from the smooth background. A small number of similar particles is also seen in the ESu regions. Note that the majority of the randomly distributed ESs particles exhibit a multimeric substructure, whereas the arrayed ones show a tetrameric substructure. Bar, 0.1 µm. (*b*) Freeze-etch electron micrograph of isolated, appressed thylakoid membrane fragments derived from Triton X-100 solubilized thylakoids. These membrane fragments are highly enriched in PSII activity, including O_2 evolution capacity. A comparison with *a* shows that the exposed surface of these fragments corresponds to the lumenal surface of stacked (*ESs*) membrane regions. As discussed in the text, the curvature of these membrane fragments in conventionally processed freeze-etch samples makes quantitative analysis of the particle heights and comparisons of particle morphologies difficult. Bar, 0.1 µm. (*c*) Freeze-etch micrograph of mica-mounted PSII membrane fragments derived from stacked regions of thylakoid membranes. The exposed surface of these membrane fragments corresponds to the lumenal ESs surface of thylakoid membranes (compare with *a*). This micrograph demonstrates that mounting of the membrane fragments on mica does not affect the general lateral organization of the particles, and that, as in intact thylakoids, arrayed particles appear as tetramers. The multimeric and tetrameric substructure of the randomly distributed particles is more clearly visible than in *a* and *b*. Bar, 0.1 µm.
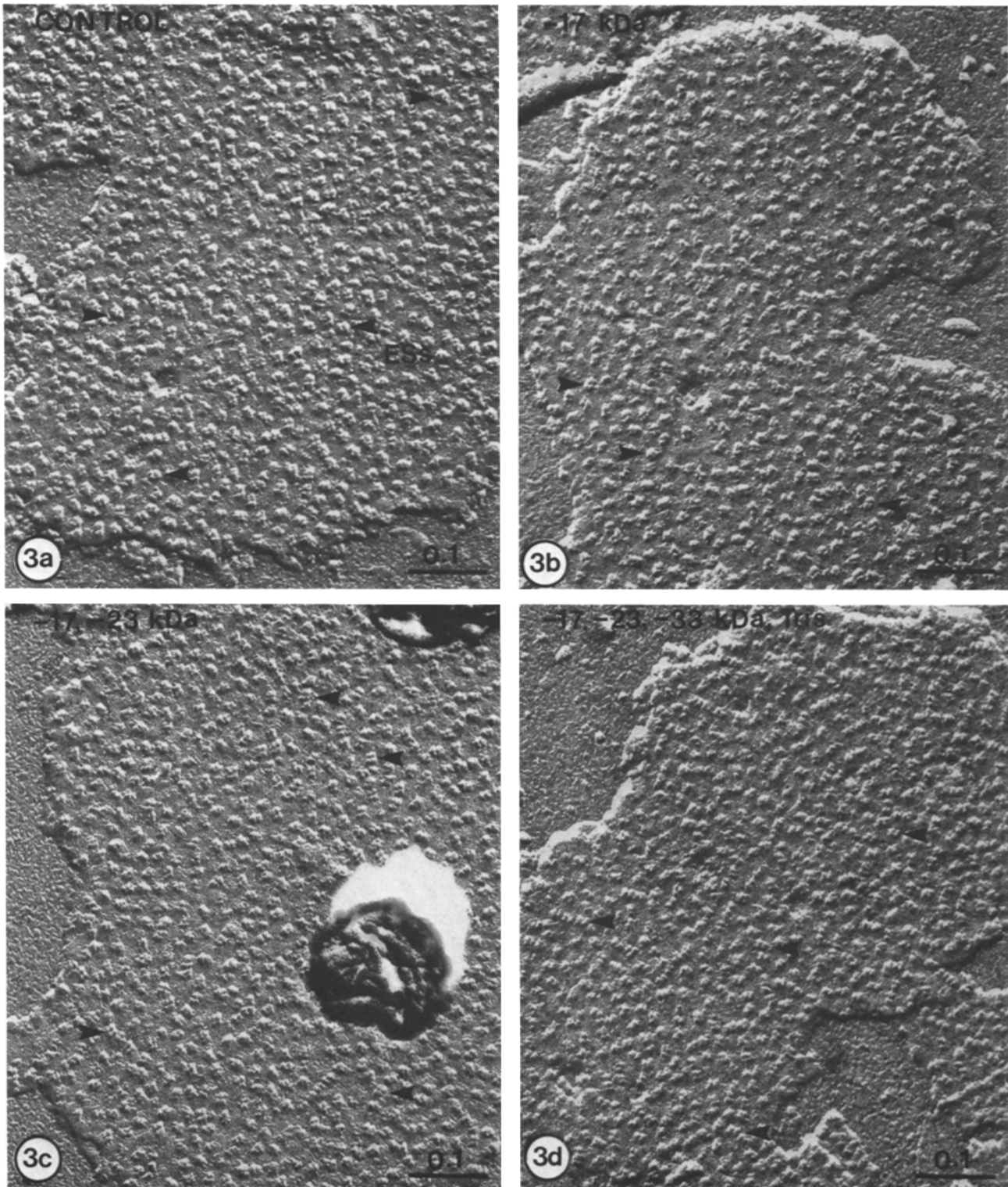
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Figure 3(a). Typical PSII control membrane samples mounted and freeze-etched on mica. The individual ESs particles can be clearly recognized against the relatively smooth background. Most of the particles possess a multimeric substructure while some appear as tetramers. Bar, 0.1 μm. (*b*) PSII membrane fragment after removal of the 17-kD extrinsic protein with 0.25 M NaCl (see LDS-PAGE, Fig. 1). The particles have a similar appearance to those seen on control membranes, but their surface relief is slightly lower (see histogram, Fig. 6). Bar, 0.1 μm. (*c*) Removal of the 17- and 23-kD extrinsic proteins from PSII membranes with 1.0 M NaCl leads to a further lowering of the ESs particle profiles (see histogram, Fig. 6). In this sample, many of the particles exhibit either a tetrameric or dimeric substructure. Bar, 0.1 μm. (*d*) PSII membrane fragments from which all three (17, 23, and 33 kD) extrinsic proteins have been removed by a wash with 0.8 M Tris at pH 8.2. The profile of the particles is further reduced (see histogram, Fig. 6), and upon closer examination most of the particles reveal a distinctly dimeric substructure. The loss of particle height is most evident when compared with the control sample (*a*). Bar, 0.1 μm.
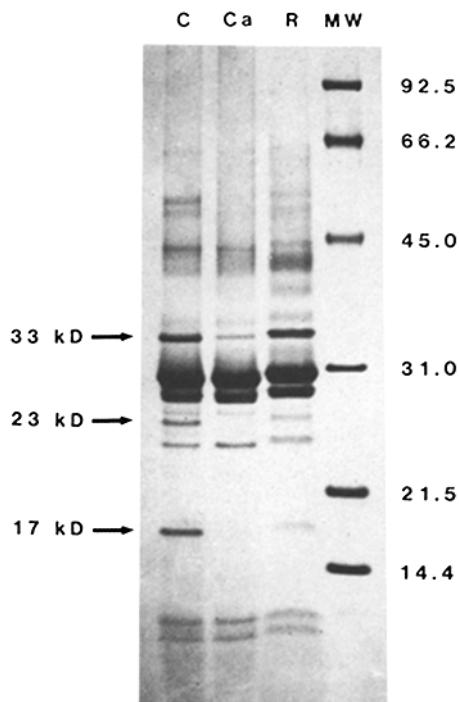
C   Ca   R   MW

92.5

66.2

45.0

33 kD →

31.0

23 kD →

21.5

17 kD →

14.4

Figure 4. LDS-PAGE of control PSII membranes (*C*), CaCl₂-washed PSII membranes (*Ca*), and CaCl₂-washed PSII membranes reconstituted with the three extrinsic proteins (*R*).

used as an easily measurable assay for successful reconstitution potential.

Fig. 5, *a* and *b* depicts PSII membrane fragments treated with 0.9 M CaCl₂ and those reconstituted with the three peripheral proteins, respectively. The CaCl₂-washed sample appears nearly identical to the alkaline Tris-washed preparation shown in Fig. 3 *d*. On the other hand, the reconstituted sample closely resembles the control membrane seen in Fig. 3 *a*.

To prove in a more definitive manner that the removal and reconstitution of the extrinsic proteins of the O₂-evolving

Table I. Steady-State O₂-Evolution Rates of PSII Membranes

Sample	O₂ Evolution rate*	Percentage of control rate
	μmol O₂·mg Chl⁻¹·h⁻¹	
Control PSII membranes	293	100
0.9 M CaCl₂-treated	8	3
CaCl₂-treated PSII membranes reconstituted with the three extrinsic proteins associated with O₂ evolution	184	63

* O₂ evolution rates were obtained using 2 mM DMBQ as an acceptor because residual rates of O₂ production observed when the 33-kD protein is removed (in the absence of added CaCl₂) is much less than that observed when DCBQ was used as an acceptor. This anomaly predominates when the rate of O₂ evolution due to loss of proteins drops to <15% of the control rate. Above this rate, the percentage of control O₂ evolution exhibited by a sample does not depend on which acceptor (DMBQ or DCBQ) is used. The control O₂ yield with 0.4 mM DCBQ was 597 μmol O₂·mg Chl⁻¹·h⁻¹. The assay buffer contained 20 mM MES (pH 6.0), 15 mM NaCl₂, and 300 mM sucrose in addition to the acceptor.

complex involves changes in ESs particle height, we have quantitated our findings as shown in the histograms of Fig. 6. Removal of all of the extrinsic proteins decreases the height of the particles protruding from the membrane surface from 8.2 to 6.1 nm. The largest individual decrease is associated with the loss of the 33-kD protein. Concomitant with the reattachment of the proteins to depleted PSII membranes in the reconstituted sample and the change in morphology (compare Figs. 5 *a* and 5 *b*), the height of the particles is seen to increase to ~7.5 nm (Fig. 6). No other major changes in the membrane surface architecture of the CaCl₂-treated and protein-reconstituted membranes were noted. The slightly greater height of the CaCl₂-treated samples versus the Tris-treated samples is most likely related to the fact that the CaCl₂-treated membranes still contained some bound 33-kD protein (Fig. 4), whereas all 33-kD protein was removed from the Tris-treated specimens (Fig. 1). The rather broad particle height distribution of the reconstituted samples suggests that the 63% recovery of the control rates of O₂ evolution is due to incomplete reassembly of the three enzymes in a fraction of the O₂-evolving complexes.

Discussion

Large tetrameric (multimeric) particles protruding into the lumenal space of thylakoid membranes were first observed in electron micrographs of air-dried, heavy metal–shadowed spinach thylakoids, and were postulated to correspond to "quantasomes," minimal photosynthetic units (Park and Biggins, 1964). Subsequent freeze-fracture and freeze-etch studies (reviewed by Staehelin et al., 1977) revealed that these ES particles corresponded to protruding elements of freeze-fracture EF particles, whose distribution between grana and stroma thylakoids matched the partitioning of PSII activities in biochemical fractionation experiments. This initial correlation between EF (and ES) particles of thylakoid membranes and PSII complexes has since been confirmed in numerous structural/biochemical studies of PSII mutants (Simpson et al., 1977; Miller and Cushman, 1979; Wollman et al., 1981; Leto et al., 1982; Lacambra et al., 1984) of greening chloroplasts (Armond et al., 1977) and of plants grown under Mn-deficient conditions (Simpson and Robinson, 1984). The goal of this study, then, was to determine more precisely the composition of the lumenally exposed components of PSII complexes that give rise to the typical multimeric ESs particles on freeze-etched thylakoid membranes. Since the O₂-evolving complex is located on the lumenal side of thylakoid membranes and is known to be coupled functionally to PSII, we have focused our attention on the spatial organization of the extrinsic protein components of the O₂-evolving complex with respect to the ESs surface particles.

Numerous reports have identified and characterized three extrinsic proteins (17, 23, and 33 kD) associated with the photosynthetic water oxidation process (reviewed by Andersson, 1986) that can be removed from exposed surfaces of PSII membrane fragments. Nearest neighbor profiles of these proteins have been obtained by immunological means (Ljungberg et al., 1984), but detailed corroborative structural studies at the electron microscopic level have not yet been reported. In this study we show clearly that extraction of each of the three extrinsic proteins, schematically illus-

Table II. Effects of CaCl₂ Treatment of PSII Membranes on O₂ Evolution and Mn Content

Treatment	A* O₂ Evolution	B* O₂ Evolution with + 50 mM CaCl₂	Ratio B A	Mn per PSII reac- tion center
	$\mu mol \; O_2 \cdot mg \; Chl^{-1} \cdot h^{-1}$	$\mu mol \; O_2 \cdot mg \; Chl^{-1} \cdot h^{-1}$		
Control	293	—	—	3.72
0.7 M CaCl₂	31	88	2.8	—
0.8 M CaCl₂	17	60	3.5	—
0.9 M CaCl₂	8	43	5.4	3.87
1.0 M CaCl₂	11	34	3.1	—
1.5 M CaCl₂	∼0	∼0	—	0.92

* O₂ evolution rates were measured as in Table I. In B the assay medium contained 50 mM CaCl₂ in addition to the normal assay buffer and DMBQ.

trated in Fig. 7, affects the characteristic multimeric surface particles located on the ESs surface of appressed grana membranes (Fig. 3, a–d). A dimeric substructure remains in the place of the multimeric particles when all three proteins are removed from the membrane surface by either the Tris or CaCl₂ wash methods (Figs. 3 d and 5 a). These dimeric particles are about the same diameter as the polymeric particles, but are about 2 nm lower in height (Fig. 6).

To confirm that the multimeric particles are in fact related to both the three extrinsic proteins and water-oxidation capacity, we reconstituted the proteins to depleted membranes prepared by the CaCl₂ wash procedure. This procedure removes the three proteins but not the Mn required for O₂

evolution. Reconstitution (Fig. 4) leads to the reappearance of the multimeric particles (Fig. 5 b), an increase in particle height (Fig. 6), and recovery of 63% of the control O₂ evolution capacity (Table I).

The stoichiometry of the three extrinsic proteins per PSII reaction center is still controversial. Murata et al. (1984) determined that each reaction center is coupled to one copy of each extrinsic protein, while the data of Andersson et al. (1984) suggest two copies of each protein per reaction center. Depending on the identity of the dimeric surface particles in the absence of extrinsic proteins, our data might be explained by either stoichiometry. For example, one of the striking features to come from the x-ray crystallographic studies of the
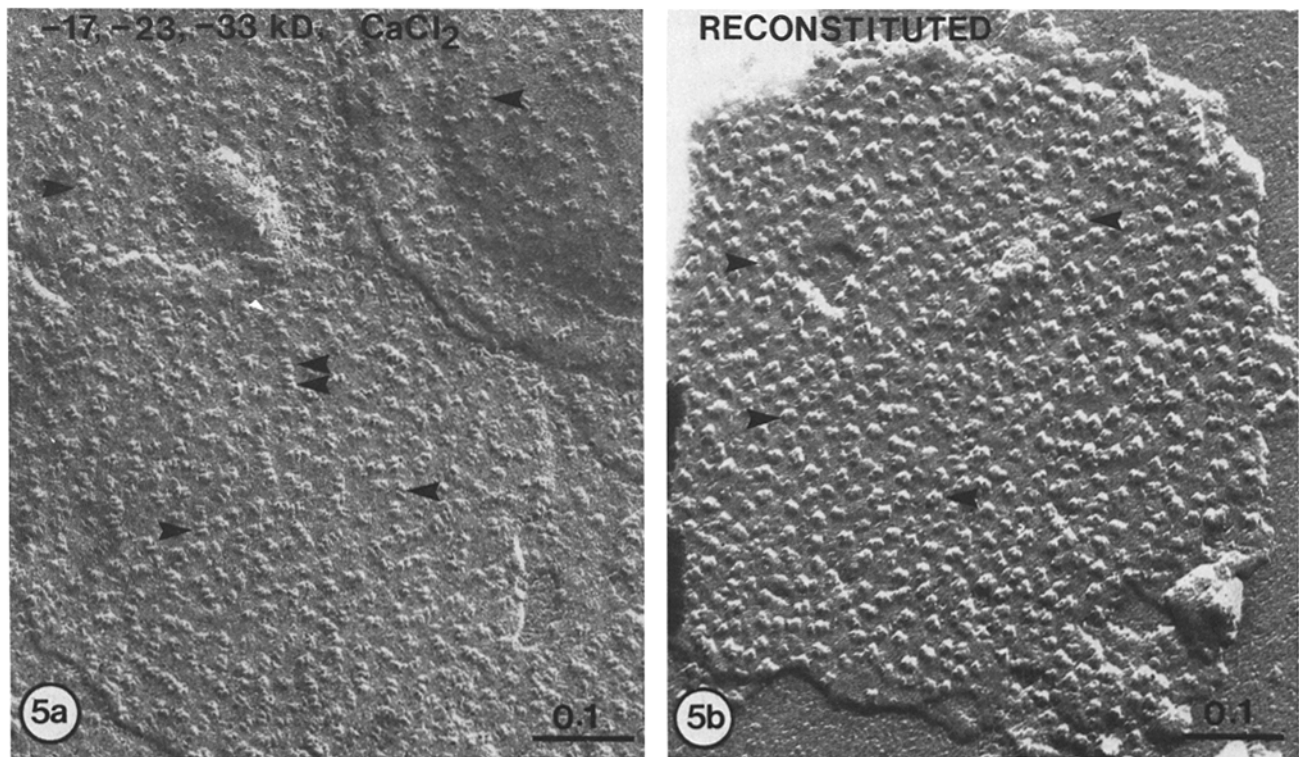

Figure 5(a). PSII membrane fragments treated with 0.9 M CaCl₂ to remove the 17-, 23-, and 33-kD extrinsic proteins while maintaining near normal amounts of bound Mn (Table II). As in Fig. 3 d, most of the low profile particles exhibit a distinct dimeric substructure. Bar, 0.1 µm. (b) Reconstituted PSII membrane fragments washed in 0.9 M CaCl₂ and then incubated in a medium containing the 17-, 23-, and 33-kD extrinsic proteins in the presence of 10 mM CaCl₂. In this reconstitution experiment, a 63% recovery of the control rates of O₂ evolution was achieved (Table I). Note the general increase in size of the ESs particles compared with the CaCl₂-washed sample (a). However, there is also a greater spread in particle size than in the control membranes (see histogram, Fig. 6), suggesting that not all complexes reconstituted equally well. Bar, 0.1 µm.
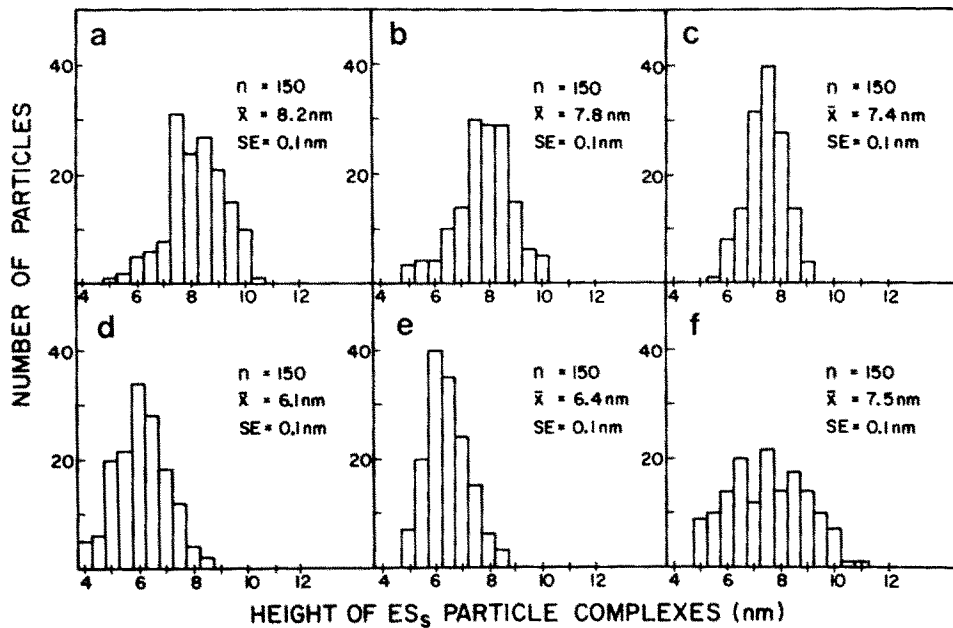
Figure 6. Histograms showing the distribution of particle height in PSII membrane fragments. (*a*) Control as in Fig. 3 *a*; (*b*) minus the 17-kD protein as in Fig. 3 *b*; (*c*) minus the 17- and 23-kD proteins as in Fig. 3 *c*; (*d*) minus all three extrinsic proteins as in Fig. 3 *d* (Tris-treated); (*e*) minus all three extrinsic proteins as in Fig. 5 *a* (CaCl₂-treated); (*f*) reconstituted membranes as in Fig. 5 *b*.

reaction center of the purple bacterium, *Rhodopseudomonas viridis* (the postulated evolutionary progenitor of the PSII reaction center) is the basic, twofold symmetry of the reaction center complex. Of particular interest is the symmetry of the L and M subunits, the equivalents of which are the D1 and D2 polypeptides of PSII (Deisenhofer et al., 1985; Michel and Deisenhofer, 1986; Renger, 1986). In addition to the D1 and D2 proteins, each PSII core complex contains two major chlorophyll/protein complexes (CP43 and CP47) and one to two cytochrome b_{599} molecules (Ort, 1986; Renger, 1986; Nanba and Satoh, 1987). A roughly symmetrical arrangement of these components together with the bound light-harvesting center II antenna CP29 (Greene, B. A., D. R. Allred, D. Morshige, and L. A. Staehelin, manuscript submitted for publication) could explain the dimeric appearance of the protruding elements of the PSII complexes after removal of the extrinsic proteins of the O₂-evolving apparatus (Figs. 3 *d* and 5 *a*). Since the same complexes often ex-

hibit a pronounced twofold symmetry even with bound 17-, 23-, and 33-kD proteins (Figs. 2 *a* and 3 *a*), and removal of these proteins leads to symmetrical changes in appearance, it appears likely that each PSII complex (in this case equivalent to the entire dimeric ESs particle seen after removal of the extrinsic proteins) contains two copies of each of the three extrinsic proteins as suggested by the Andersson et al. (1984) data. Alternatively, if each of the dimeric ES particles contains two PSII reaction centers, a 1:1 photochemically active reaction center complex to extrinsic protein ratio is possible, consistent with the Murata et al. (1984) studies. From the point of view of size, two PSII reaction centers (D1/D2 complexes) could fit into a 16 by 16-nm surface area judging from the known size of the bacterial reaction center (Diesenhofer et al., 1985). There is also evidence that under some conditions, a particular O₂-evolving complex can donate electrons to more than one reaction center (Packham and Barber, 1983). Unfortunately, the limited resolution of our
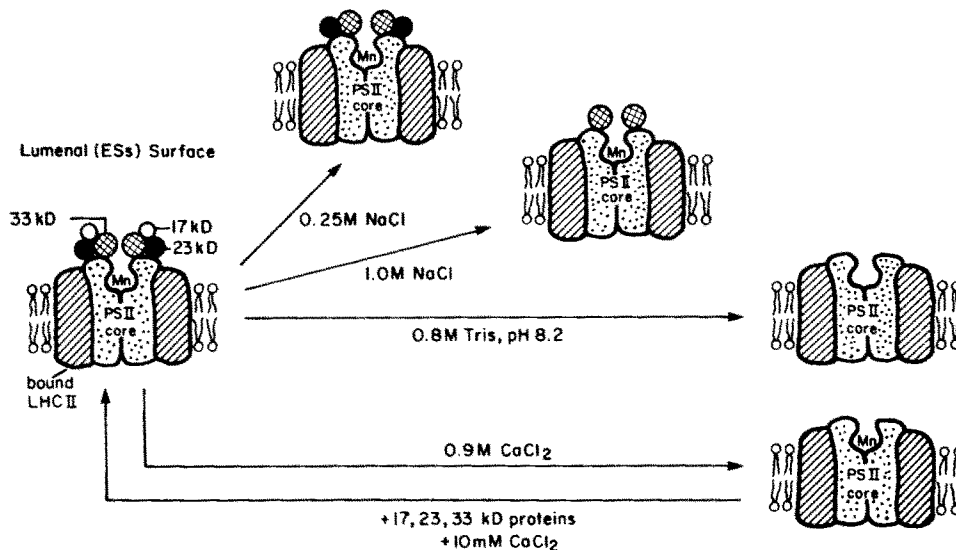

Figure 7. Schematic diagram summarizing the experiments and findings of the current study to localize the extrinsic proteins associated with the O₂-evolving complex on freeze-etched thylakoid membranes. Step-by-step removal of the different proteins leads to a gradual decrease in height of the multimeric (tetrameric) ESs particles. Reconstitution produces an increase in ESs particle size. After removal of all three extrinsic proteins, a dimeric core complex with a lower profile is observed.

electron micrographs does not allow us to determine how the individual proteins are arranged in the multimeric particle complexes.

During the final drafting of this report, we learned that Simpson and Andersson (1986) have also correlated the multimeric (tetrameric) ESs particles with the extrinsic proteins of the O_2-evolving complex. However, in that study, inside-out thylakoid vesicles were used, and removal of the extrinsic proteins produced only a general loss in definition of the multimeric surface particles. No dimeric particles were revealed. After reconstitution (28% O_2 recovery vs. 63% in the current study), the particles became slightly more distinctive again. Furthermore, no quantitative analysis of surface particle heights was attempted.

We conclude that the 17-, 23-, and 33-kD water-soluble extrinsic proteins of the O_2-evolving complex give rise to the multimeric particles observed on the ESs surface of appressed grana thylakoids. The dimeric particles observed after extraction of these proteins probably contain the rest of the water-oxidation apparatus and other components of the PSII complex, including the D1 and D2 proteins and the chlorophyll-protein complexes CP47, CP43, and possibly CP29 (Metz et al., 1986; Nanba and Satoh, 1987; Ort, 1986; Staehelin, 1986).

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