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STRUCTURAL, BIOCHEMICAL AND BIOPHYSICAL CHARACTERIZATION OF FOUR OXYGEN-EVOLVING PHOTOSYSTEM II PREPARATIONS FROM SPINACH

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Four procedures utilizing different detergent and salt conditions were used to isolate oxygen-evolving Photosystem II (PS II) preparations from spinach thylakoid membranes. These PS II preparations have been characterized by freeze-fracture electron microscopy, SDS-polyacrylamide gel electrophoresis, steady-state and pulsed oxygen evolution, 77 K fluorescence, and room-temperature electron paramagnetic resonance. All of the O_2-evolving PS II samples were found to be highly purified grana membrane fractions composed of paired, appressed membrane fragments. The lumenal surfaces of the membranes and thus the O_2-evolving enzyme complex, are directly exposed to the external environment. Biochemical and biophysical analyses indicated that all four preparations are enriched in the chlorophyll *a/b*-light-harvesting complex and Photosystem II, and depleted to varying degrees in the stroma-associated components, Photosystem I and the CF_1-ATPase. The four PS II samples also varied in their cytochrome *f* content. All preparations showed enhanced stability of oxygen production and oxygen-rate electrode activity compared to control thylakoids, apparently promoted by low concentrations of residual detergent in the PS II preparations. A model is presented which summarizes the effects of the salt and detergent treatments on thylakoid structure and, consequently, on the configuration and composition of the oxygen-evolving PS II samples.

Introduction

Detergent fractionation of thylakoid membranes has been widely employed in an effort to separate grana physically from stroma membranes and, consequently, Photosystem I (PS I) activity from Photosystem II (PS II) activity [1–3]. Early PS II preparations suffered from detergent-induced inhibition of oxygen evolution, although they retained PS II-dependent photochemistry [4–7]. Nevertheless, there were some reports [8–10] of O_2-evolving PS II preparations which attracted little attention.

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Abbreviations: Chl, chlorophyll; Chl *a/b*-LHC, chlorophyll *a/b*-light harvesting pigment-protein complex; DCIP, 2,6-dichlorophenol indophenol; EPR, electron paramagnetic resonance; Mes, 4-morpholineethanesulfonic acid; octyl glucoside, *n*-octyl-β-D-glucopyranoside; P-430, a secondary PS I electron acceptor; P-700, the PS I reaction center; Pipes, 1,4-piperazinediethanesulfonic acid; PS I, Photosystem I; PS II, Photosystem II; SDS, sodium dodecyl sulfate; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; BBY, membrane preparation described by Berthold, D.A. Babcock, G.T. and Yocum, C.F. (Ref. 17); K&M, idem by Kuwabara, T. and Murata, N. (Ref. 19); YUSN, idem by Yamamoto, Y., Ueda, T., Shinka, H. and Nishimura, M. (Ref. 27); LHC, light-harvesting complex; MDT, magnesium-digitonin-Triton.

Since 1978, several new methods for producing active, O_2-evolving PS II preparations have been published [11–21]. The first to be described in detail was the method for isolating so-called 'inside-out' thylakoid vesicles by means of phase partitioning of mechanically disrupted thylakoids [11,12]. These inside-out vesicles are thought to originate from appressed regions of the grana [13] and contain a significantly higher PS II to PS I ratio than intact thylakoids. All other procedures for obtaining oxygen-evolving PS II preparations rely on detergent solubilization of the membranes. Using this approach, O_2-evolving PS II preparations have been reported for cyanobacteria [14–16], spinach [17–19], pea [20], maize [21] and red algae [22]. Some of these studies have included polypeptide analysis by means of SDS-gel electrophoresis [14,18,23], but the lack of uniformity of the protocols has precluded any detailed comparative analysis. Structural analysis by electron microscopic techniques has been performed on some of the older PS II preparations incapable of O_2 evolution [24,25]. Of the O_2-evolving preparations, however, only one of the early preparations [8,26] and the grana-derived inside-out thylakoid vesicles [11] have been studied in this manner. Several other physical and biochemical parameters of different O_2-evolving preparations have also been described in the literature. For instance, major differences can be found in the reported rates of PS II activity (as O_2 evolution), the lowest [20] value being about 30 and the highest [14] 1300 μmol $O_2 \cdot$ mg $Chl^{-1} \cdot h^{-1}$. Of the spinach preparations, Berthold et al. [17] and Kuwabara and Murata [19] have noted almost complete depletion of P-700 in their preparations, while others purportedly contained significant levels of P-700 [18] *.

The various O_2-evolving PS II preparations produced by detergent fractionation of thylakoids employ different salts, salt concentrations, detergents, and detergent concentrations. This makes it difficult to correlate the functional and compositional properties of the PS II preparations with specific differences in sample preparation. To assess these differences, we have compared several spinach oxygen-evolving PS II preparations by means of electron microscopy, gel electrophoresis, steady-state and pulsed oxygen detection, low-temperature fluorescence, and room-temperature EPR. The main purpose of this work was to answer the question; what is an O_2-evolving PS II preparation? We have found that all preparations consist of highly enriched, non-vesicular appressed grana membranes, which exhibit many similarities as well as some surprising differences. The mode of action of different detergents is also discussed, together with an evaluation of detergent versus mechanically prepared PS II preparations.

* From a historical perspective, we should note that both the Berthold et al. [17] and Kuwabara and Murata [19] preparations, and the Yamamoto et al. [18,27] preparation are quite similar to the Briantais [8] and Huzisige et al. [9] preparations, respectively. However, Briantais used high buffer rather than high salt concentrations to maintain stacked membranes, while Huzisige apparently used neither.

Materials and Methods

Oxygen-evolving Photosystem II preparations

Four different O_2-evolving PS II membrane preparations were examined in this study. The first three were described by others, and we shall refer to them as BBY [17], K&M [19] and YUSN [27] preparations. All were prepared as directed in the original references, except (1) the second detergent extraction step was eliminated in the BBY procedure because this step produced abnormal flash patterns as measured on an O_2 rate electrode [28], (2) *N*-morpholineethanesulfonic acid (Mes) was substituted for $Na_2P_2O_7$ in the YUSN preparation because the modification gave samples with higher O_2-evolution rates [29], and (3) the ratio of Triton X-100 to Chl concentration in the K&M preparation was reduced from 25 : 1 to 15 : 1 in order to obtain an O_2-evolving PS II pellet. Procedures for these three membrane preparations are listed in Table I. A fourth procedure (MDT) is also presented in the table. It was developed by one of us (MS) and incorporates the high magnesium and salt conditions of the BBY preparation and the gentle digitonin/Triton extraction conditions of the YUSN procedure. All preparations used market spinach, obtained during November to January.

Freeze-fracture electron microscopy

The different oxygen-evolving PS II samples were infiltrated at 4°C with 35% glycerol and

rapidly frozen in Freon 22, held at approx. −150°C. The frozen samples were fractured and replicated in a Balzers BA360M freeze-etch apparatus and examined in a Hitachi 600 electron microscope. The fracture faces of the thylakoid membranes are labeled as described by Staehelin [30].

Polyacrylamide gel electrophoresis

Chlorophyll-protein complexes were visualized as green bands on unstained SDS-polyacrylamide gels. Spinach thylakoid membranes were isolated as in Ref. 31 with the addition of 5 mM aminocaproic acid and 1 mM benzamidine-HCl to all buffers. Thylakoids or PS II membrane preparations were solubilized in 0.88% octyl glucoside with or without 0.22% SDS (octyl glucoside/Chl = 20 : 1; see Fig. 1 for details) and run at 4°C on gels prepared according to Ref. 32.

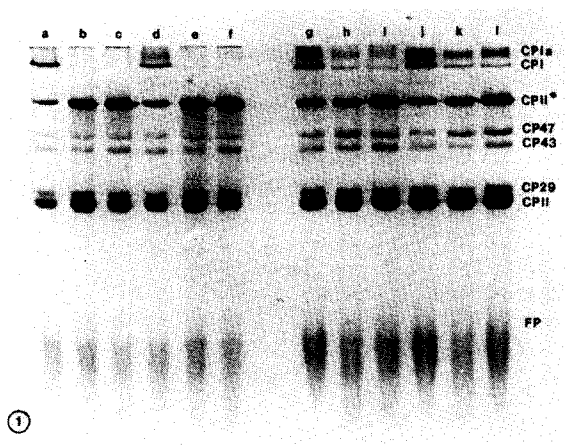


Fig. 1. Unstained 10% polyacrylamide gel of spinach thylakoids (lanes a, d, g and j), and two PS II preparations (BBY – lanes b, e, h and k; YUSN – lanes c, f, i and l) solubilized under different detergent and salt conditions. The solubilization buffers used were: (a–c): 0.88% octyl glucoside in 2 mM Tris-maleate (pH 7.0); (d–f): same as (a–c) with the addition of 0.22% SDS; (g–i): 0.88% octyl glucoside in 20 mM Pipes-NaOH (pH 6.6)/15 mM NaCl/5 mM $MgCl_2$/100 mM sucrose; (j–l): same as (g–i) with the addition of 0.22% SDS. All samples were washed twice in the appropriate buffer, then stirred in detergent for 5 min on ice. N.B. The nomenclature used for chlorophyll-protein bands on gels is currently non-uniform in the literature. We have used terminology here that we found to be common and useful in discussions with other investigators. This nomenclature is a mixture derived from that of Refs. 35, 36 and 37.

The peptide composition of the PS II preparations was determined using the Laemmli gel system [33]. The samples were solubilized in 1% SDS and 1% β-mercaptoethanol (SDS/Chl = 40 : 1) for 10 min at room temperature, then heated to 100°C for 1–2 min. The gels were run at room temperature in a water-cooled electrophoresis apparatus (Hoefer Scientific Instruments), stained with Coomassie blue, and destained by standard procedures.

Cytochrome *f* contained in the PS II preparations was identified on SDS gels using the diaminobenzidine staining procedure of McDonnell and Staehelin [34], with the following modification in the membrane solubilization procedure. The membrane samples were suspended in buffer containing octyl glucoside and SDS (octyl glucoside/SDS/Chl = 20 : 5 : 1), then stirred for 5 min at room temperature and run on Laemmli gels [33]. We found that this mild solubilization procedure resulted in more intense staining of the cytochrome *f* band. Following diaminobenzidine staining, the cytochrome *f* band was marked with India ink, and the gels stained with Coomassie blue in order to identify the cytochrome *f* band within the total peptide pattern.

O_2 measurements

Steady-state O_2 evolution ($H_2O \rightarrow$ 2,6-dimethyl-*p*-benzoquinone) and O_2 uptake or Mehler (DCPIP → methyl viologen) reactions were measured at 25°C under saturating water-filtered tungsten light using a Clark-type O_2 electrode [29]. Oxygen yields, elicited by saturating 3-μs xenon flashes spaced 1.5 s apart, were recorded at 22°C with a Joliot-type O_2 rate electrode. Samples were dark-adapted for 30 min at 4°C and then equilibrated 5–10 min in the dark on the electrode. Exogenous electron acceptors were not added to the flow buffer [28,29].

Spectroscopy

Absorbance spectra were run on a Cary 17D Spectrophotometer (Varian, Palo Alto, CA). Room temperature EPR spectra were recorded with a Varian E-109 Spectrometer. Low-temperature (77 K) fluorescence emission spectra ($\lambda_{ex} = 430$ nm) were obtained with a Spex Fluorolog (Methuchen, NJ) using dilute samples ($A_{680} < 0.1$). Experimen-

TABLE I

SUMMARY OF PROCEDURES USED TO MAKE FOUR OXYGEN-EVOLVING PS II PREPARATIONS

All procedures are performed rapidly at 4°C in dim light.

BBY (Ref. 17) Solutions		Procedure [a]
B-1:	0.4 M NaCl 2 mM $MgCl_2$ 0.2% BSA 20 mM Tricine (pH 8.0)	(1) Grind 100 g deveined spinach in 250 ml B-1 in Waring Blender (2) Filter through four layers cheesecloth; centrifuge out debris (300 × *g*, 1 min) (3) Centrifuge supernatant from (2) to pellet broken chloroplasts (4000 × *g*, 10 min) (4) Wash pellet once in B-2 (5) Suspend pellet in B-3 plus Triton X-100; incubate 25 min at 4°C; Triton/Chl = 25 : 1; final Chl concn. = 2 mg/ml
B-2:	0.15 M NaCl 5 mM $MgCl_2$ 0.2% BSA 20 mM Tricine (pH 8.0)	(6) Centrifuge (3500 × *g*, 5 min); discard white pellet (7) Centrifuge supernatant from (6), (40000 × *g*, 30 min) (8) Suspend pellet in B-4; store at −80°C
B-3:	15 mM NaCl 5 mM $MgCl_2$ 20 mM Hepes (pH 7.5)	
B-4:	B-3 plus 0.4 M sucrose	

K&M (Ref. 19) Solutions		Procedure
K-1:	0.1 M sucrose 0.2 M NaCl 50 mM Na/K phosphate (pH 7.4)	(1) Grind 150 g deveined spinach in 300 ml K-1 in Waring Blender (2) Filter through 16 layers cheesecloth; centrifuge filtrate (3000 × *g*, 5 min) (3) Suspend pellet in K-1; centrifuge (500 × *g*, 30 s), discard pellet (4) Centrifuge supernatant from (3), (3000 × *g*, 5 min)
K-2:	0.3 M sucrose 50 mM NaCl 50 mM Na/K phosphate (pH 6.9)	(5) Suspend pellet in K-2; adjust Chl concn. to 2–3 mg/ml (6) Add 20% w/v Triton X-100 while stirring so that final Triton/Chl ratio = 15 : 1 [b] (7) Centrifuge (1000 × *g*, 2 min); discard pellet (8) Centrifuge supernatant from (7), (35000 × *g*, 10 min)
K-3:	40 mM Na/K phosphate (pH 6.9)	(9) Suspend pellet in K-3; centrifuge (1000 × *g*, 2 min); discard pellet (10) Centrifuge supernatant from (9), (35000 × *g*, 10 min)
K-4:	0.4 M sucrose 15 mM NaCl 5 mM $MgCl_2$ 20 mM Mes (pH 6.5)	(11) Suspend pellet in K-4; store at −80°C

[a] We found that the second Triton X-100 treatment in Ref. 17 decreases the steady-state O_2 evolution, results in abnormal O_2 sequences on the O_2 rate electrode, and significantly reduces the EPR Signal I (oxidized P-700). We also found that a wash step inserted between (7) and (8) dramatically increases O_2-evolution stability at room temperature.

[b] We used a Triton/Chl ratio of 15 : 1 at this step because a ratio of 25 : 1 (as in Ref. 19) gave no pellet in step (8).

tal conditions are given in the text where appropriate.

Results

Polyacrylamide gel electrophoresis

When subjected to SDS-gel electrophoresis under mildly denaturing conditions, all four PS II membrane preparations were found to be depleted in the PS I-related complexes (CP I and CP Ia) and enriched in the complexes associated with light-harvesting (CP II, CP II* and CP 29) and with the PS II reaction center (CP 43 and CP 47). However, the apparent pigment-protein composition of each sample (Fig. 1) varied qualitatively and quantitatively depending on the conditions

YUSN (Ref. 27)

Solutions	Procedure
Y-1: 0.33 M sorbitol 4 mM $MgCl_2$ 10 mM Mes (pH 6.5) 2 mM ascorbate	(1) Grind 60 g deveined spinach in 120 ml Y-1 (2–3 s) in Waring Blender (2) Filter through four layers gauze, then through eight layers gauze containing two layers cotton wool (3) Centrifuge (8000× *g*, 50 s) (4) Suspend pellet in Y-2; let stand 1 min; centrifuge (8000× *g*, 5 min)
Y-2: Y-1 without ascorbate diluted 25×	(5) Suspend pellet in Y-3; adjust Chl concn. to 1.0 mg/ml (6) Add equal volume 0.5% digitonin in Y-3; stir 5 min on ice
Y-3: Y-1 without ascorbate	(7) Dilute 10-times with Y-3; centrifuge (10000× *g*, 30 min) (8) Suspend pellet in Y-3; adjust Chl concn. to 1.0 mg/ml (9) Add equal volume 0.4% Triton X-100 in Y-3; stir 5 min on ice (10) Dilute 10-times with Y-3; centrifuge (12000× *g*, 30 min) (11) Suspend pellet in Y-3; store at −80°C

MDT (Ref. this study)

Solutions	Procedure
M-1: 0.4 M NaCl 2 mM $MgCl_2$ 0.2% BSA 20 mM Tricine (pH 8.0)	(1) Grind 100 g deveined spinach in 250 ml M-1 in Waring Blender (2) Filter through four layers cheesecloth, centrifuge out debris (300× *g*, 1 min) (3) Centrifuge supernatant from (2) to pellet broken chloroplasts (4000× *g*, 10 min) (4) Wash pellet once in M-2 (5) Suspend pellet in M-3; adjust Chl concn. to 1.0 mg/ml
M-2: 0.15 M NaCl 5 mM $MgCl_2$ 0.2% BSA 20 mM Tricine (pH 8.0)	(6) Add equal volume 0.5% digitonin in M-3: stir 5 min on ice (7) Dilute 10-times with M-3; centrifuge (10000× *g*, 30 min) (8) Suspend pellet in M-3; adjust Chl concn. to 1.0 mg/ml (9) Add equal volume 0.4% Triton X-100 in Y-3; stir 5 min on ice (10) Dilute 10-times with M-3; centrifuge (12000× *g*, 30 min)
M-3: 0.33 M sorbitol 15 mM NaCl 4 mM $MgCl_2$ 10 mM Mes (pH 6.5)	(11) Suspend pellet in M-3; store at −80°C

used for membrane solubilization and electrophoresis. Therefore, the electrophoretic pattern of the pigmented bands alone does not appear to be an accurate measure of the pigment-protein composition of thylakoid membranes or membrane fractions.

The peptide composition of the four PS II membrane preparations is shown in Fig. 2. The PS II fractions were all enriched in the Chl *a*/*b*-LHC (24–25 kDa, [38], the PS II reaction-center proteins (42–50 kDa [39,40]) and two or more peptides of 20–22 kDa of unknown function. There is also a prominent band at 32 kDa in all samples. This band is sometimes seen as a doublet. These two comigrating peptides are probably the 32 kDa herbicide-binding protein [41] and the 33 kDa

peptide [42,43] associated with manganese binding and O_2 evolution in PS II [19,44]. Two peptides at 16 and 23 kDa are present in varying quantities in the four preparations studied. A number of laboratories have recently correlated peptides of these size classes, as well as the 33 kDa peptide, with O_2 evolution in PS II-enriched samples [12,19,45].

Several peptides are dramatically depleted in all four PS II preparations relative to intact thylakoids, notably two major bands at approx. 55 and 62 kDa and a doublet at about 19 kDa. The 55 and 62 kDa bands represent the α and β subunits of the CF_1 segment of the CF_0-CF_1 ATP synthetase complex [46]. The P-700 binding apoprotein of the PS I reaction center complex is seen as a faint band just above CF_1-α [47,48]. The two peptides at 19 kDa are probably components of the PS I-specific antenna complex, LHC-I [49,50].

Cytochrome *f* was identified on the gels by the diaminobenzidine staining procedure of [34]. The gel in Fig. 2 reveals different amounts of cytochrome *f* remaining in the four PS II preparations. Cytochrome b_6 was not detectable by this method, as the technique is specific for cytochromes with covalently bound heme groups.

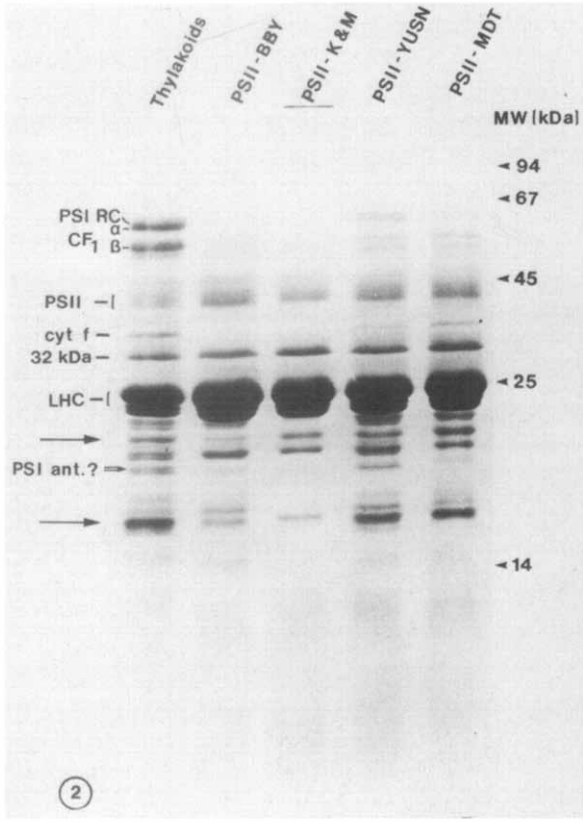


Fig. 2. Coomassie blue-stained 10–15% Laemmli gel of spinach thylakoid membranes and the four PS II membrane preparations. The PS II samples are enriched in the light-harvesting complex peptides, one or more proteins associated with the PS II reaction center, a prominent band at 32 kDa (see text) plus two or more peptides between 20–22 kDa of unknown function. The samples are depleted to varying degrees in cytochrome *f*, the α and β subunits of the CF_1-ATPase, the PS I reaction center, and a doublet at approx. 19 kDa, which may be part of the PS I antenna. The arrows at 16 and 23 kDa indicate peptides reported to be involved in O_2 evolution.

Freeze-fracture and freeze-etch morphology

Freeze-fractured thylakoid membranes exhibit four distinct fracture faces, two of which (EFs and PFs) are associated with stacked, grana membrane regions, and two (EFu and PFu) that are related to unstacked, stroma membranes. Each fracture face can be recognized by the characteristic size range and density of its intramembrane particles (see Fig. 3). In vitro preservation of this spatial differentiation of thylakoid membranes requires more than 3 mM $MgCl_2$ or more than 150 mM NaCl in the suspending medium [30,51]. At lower salt con-

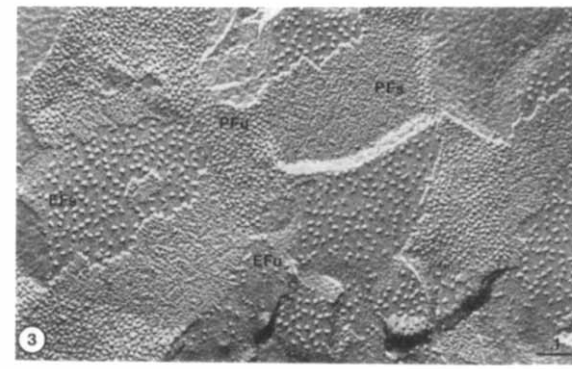
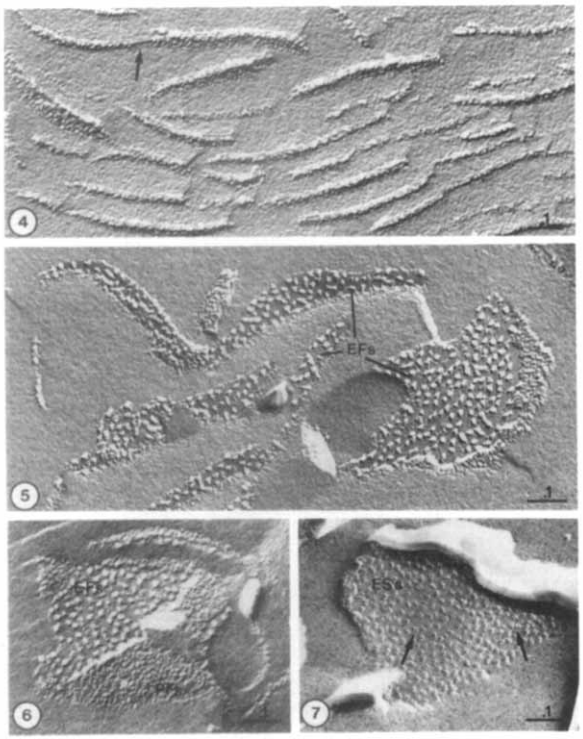


Fig. 3. Freeze-fracture micrograph of control pea thylakoids suspended in a medium containing 5 mM $MgCl_2$ to preserve grana stacks. The fracture faces of stacked grana membrane regions are designated EFs and PFs, those of unstacked, stroma membrane regions as EFu and PFu. Note the similarity of the EFs and PFs faces of these control thylakoids with the fracture faces of the membranes that constitute the oxygen-evolving PS II preparations (see Figs. 5, 6 and 9–12). Magnification: ×45 000.

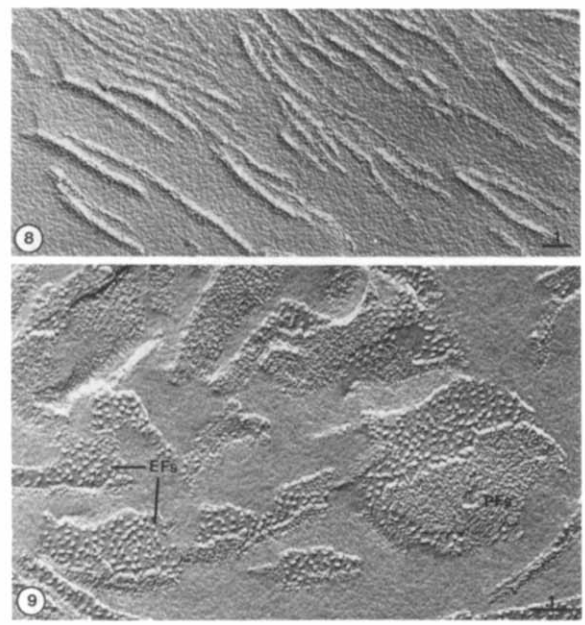
centrations, the membranes become unstacked, and intermixing of the different categories of particles is observed [30,52].

All of the published procedures for making oxygen-evolving PS II preparations from spinach involve detergent treatment of thylakoid membranes in the presence of mono- or divalent ion concentrations sufficient to preserve at least some membrane stacking. This fact, plus the observation that all oxygen-evolving PS II preparations from higher plant chloroplasts can be pelleted at relatively low centrifugation speeds, led us to predict that these preparations represent highly enriched grana thylakoid fractions, produced by differential solubilization of stacked and unstacked membrane regions. The following freeze-fracture observations both confirm this prediction and reveal several more subtle differences between the samples obtained by the different procedures.

The grana origin of the O_2-evolving PS II preparations is most clearly seen in the BBY (Figs. 4–7) and the K&M (Figs. 8 and 9) samples. In both of these preparations, the stacked (paired) membranes appear as flat, sheet-like membrane fragments essentially devoid of unstacked marginal membrane regions (Figs. 4 and 8; see also Ref. 26). The diameter of the majority of these membrane fragments is approx. 0.5 μm, which is close to the diameter of grana stacks of spinach and pea



Figs. 4–7. Freeze-fracture (-etch) micrographs of a fresh BBY-PS II preparation. Fig. 4 shows that the preparation consists of flat, double-membrane sheets with free ends, which resemble stacked grana membranes devoid of margins. The arrow points to two of these sheets that appear to be connected through a single short membrane. The grana origin of the membrane fragments is supported by the appearance of their fracture faces (Figs. 5 and 6), which resemble closely the EFs and PFs regions of freeze-fractured control thylakoid membranes (compare with Fig. 3). The large EFs particles are generally assumed to be structural equivalents of PS II-light harvesting complexes. Fig. 7 reveals the etched surface (ESs) of an isolated BBY-PS II stacked membrane sheet. The subunit structure of the protuding membrane particles (presumptive PS II units; arrows) is virtually indistinguishable from their appearance in control membranes. Magnifications: Fig. 4, ×53000; Fig. 5, ×58000; Fig. 6, ×58000; Fig. 7, ×67000.



Figs. 8 and 9. Freeze-fractured membranes of a fresh K&M-PS II preparation. In Fig. 8 each cross-fractured membrane fragment appears to consist of two relatively short and tightly appressed membrane fragments that resemble stacked grana thylakoids devoid of margins. Typical fracture faces (EFs and PFs) of such grana-resembling membrane fragments are shown in Fig. 9. In structural terms, this preparation resembles the BBY preparation (Figs. 4–6). Magnifications: Fig. 8, ×40000; Fig. 9, ×58000.

chloroplasts. Only occasionally are two fragments seen to be connected laterally by a single membrane (Fig. 4, arrow). When viewed face-on, these membrane fragments reveal typical EFs and PFs faces and virtually no EFu and PFu faces (Figs. 5, 6 and 9). The particles of these EFs and PFs faces fall into the same size ranges and have the same density as those seen on control thylakoids, supporting the notion that the membrane fragments are grana-derived and have not arisen from random aggregation of detergent-solubilized membrane complexes.

The true surfaces of frozen membranes can be visualized by removal of the ice from a membrane surface by etching prior to applicates of the metal shadow [30]. The PS II-LHC aggregation, which produce large EF particles on fractured membranes [53], protrude from the membrane to produce large particles on the lumenal membrane surface (ES) with a characteristic substructure [30]. Freeze-etching reveals this distinctive PS II structure on the ESs surface of the PS II membrane samples examined here (Fig. 7), indicating that the detergent treatments have not destroyed the characteristic ultrastructure of the grana membranes. The location of the ESs surface with respect to the central plane of the appressed membranes, plus the polarity of the EFs and PFs fracture faces, indicates that the exposed surface of the membrane fragments corresponds to the lumenal surface of grana thylakoids.

The difference in appearance of the BBY and K&M preparations (Figs. 4–6, 8 and 9) versus the YUSN and MDT preparations (Figs. 10 and 11) is rather striking. In all of our freshly prepared O_2-evolving PS II samples exposed to digitonin, we have observed rounded fragments of appressed membranes and not flat, sheet-like structures. Despite their curvature, the fracture faces of these membrane fragments can easily be categorized as being of the EFs or PFs type (Figs. 10 and 11). The curved membranes do not seem to form closed, vesicular structures as evidenced by the presence of numerous free ends of crossfractured membranes (Fig. 10, arrows). In general, the YUSN-PS II membrane fragments are larger than those obtained by the MDT technique (compare Figs. 10 and 11).

If the curved YUSN or MDT membrane preparations are allowed to stand at room temperature, or are subjected to several freeze-thaw cycles during storage, the membrane fragments tend to fuse laterally and the membrane sheets become flattened (Fig. 12). We found this phenomenon to be useful in the evaluation of the extent of stroma membrane 'contamination' of the purified grana membranes, since the distinct PFu particles of the stroma membranes tend to aggregate at the interface between adjacent, fused grana membranes (Fig. 12, PFu marked region).

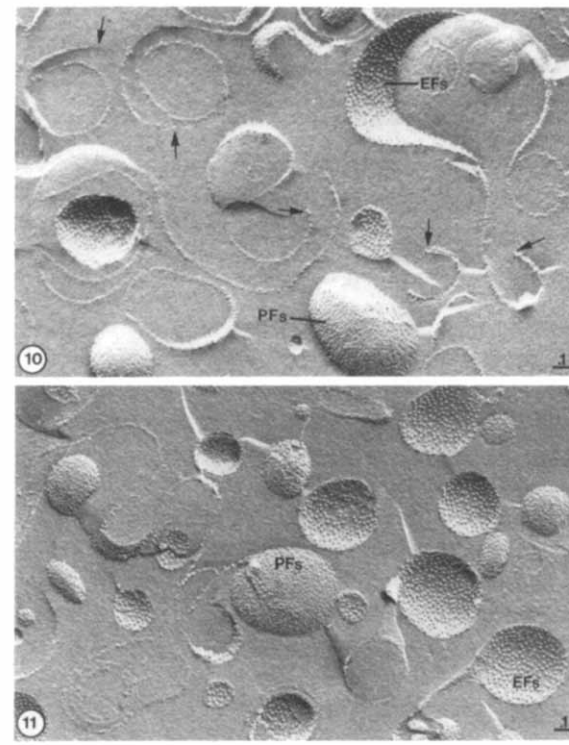


Fig. 10. Freeze-fracture micrograph of a fresh YUSN-PS II preparation. Note the rounded configuration of the stacked membrane fragments, which tend to be larger than those observed in the BBY (Fig. 4) and the K&M (Fig. 8) preparations. The membranes exhibit typical EFs and PFs fracture faces. Note the many free ends of the membrane fragments (arrows), indicating that most of the curved membranes do not form closed vesicular structures. Magnification: ×29000.

Fig. 11. Freeze-fractured membranes of a MDT-PS II preparation. The rounded, vesicle-like configuration of the stacked membrane fragments, which display typical EFs and PFs faces is reminiscent of the YUSN-PS II preparation (see Fig. 10). However, the average diameter of the curved membrane sheet is smaller in the MDT preparation. Magnification: ×29000.

Fig. 12. Freeze-fractured membranes of a YUSN-PS II preparation after three months storage at −80°C and several freeze-thaw cycles associated with the removal of sample aliquots for experiments. Note the large, flattened sheet-like appearance of the membranes, caused by the lateral fusion of membrane fragments. In such sheets, remnants of stroma thylakoids with typical 10–11 nm PFu particles (purported PS I particles) can be recognized between the laterally fused grana membrane fragments (EFs and PFs). In addition, the lack of a step (arrow) between the EFs face and the PFs faces on the right (arrow) indicates that two membranes with opposite polarity have fused in that region. Magnification: ×53000.

Absorbance spectra

The four types of O_2-evolving PS II preparation are indistinguishable by their room-temperature absorbance spectra. All the preparations show 2–3 nm blue shifts in both the 440 and 680 nm peaks observable in chloroplasts. In addition, the 470 and 650 nm shoulders of Chl *b* in chloroplasts were better defined in the oxygen-evolving PS II preparations. These same results have been reported for the YUSN preparation and actual spectra can be found in Fig. 1 of Ref. 27.

Biochemical and biophysical properties

Table II compares a number of properties representative of the O_2-evolving PS II membrane preparations with those of broken chloroplasts. Under our conditions, the specific rate of O_2 evolution under saturating steady-state light was greater than that observed in chloroplasts for the YUSN and MDT preparations, and lower than

TABLE II

A COMPARISON OF FOUR SPINACH PS II PREPARATIONS WITH THYLAKOIDS

Parameter	Thylakoid membranes	BBY [a]	K&M	YUSN	MDT
Steady-state O_2 evolution (μmol $O_2 \cdot$ mg Chl$^{-1} \cdot$ h^{-1})	298	202	159	402	390
Mehler reaction – O_2 uptake (μmol $O_2 \cdot$ mg Chl$^{-1} \cdot$ h^{-1})	290	21	43	22	46
EPR-detectable Signal I [b]	1.00	0.22	0.04	0.47	0.22
Low-temperature fluorescence (height of F_{735} peak)	1.00	0.27	0.07	0.48	0.23
Y_3 per unit Chl [c]	2.95	1.22	0.919	3.04	3.28
O_2 evolution stability at 22 °C in dark ($t_{1/2}$ of Y_3 in h) [d]					
On O_2 rate electrode	0.5	0.25 (1.5) [e]	NA	1.5	7.75
Off O_2 rate electrode	2.0	0.3 (20.5) [e]	19.5	13.3	22.5
Cytochrome *f* (diaminobenzidine-stained gels)	+ + +	+	−	+ +	+ +

[a] These results were obtained using BBY preparations extracted with only one Triton X-100 treatment. Less PS I, lower steady-state rates and abnormal flash yield sequences are observed if two extractions are used as in Ref. 17.

[b] Intensities of signal *I* were estimated from the relationship $I(H)^2Y$ [65], where *I* is intensity, *H* is the peak-to-peak width of the first-derivative Signal *I* spectrum (8 G) and *Y* is that part of the peak-to-peak height of the spectrum attributable to ferricyanide. Instrument conditions: field set, 3396.8 G; modulation frequency, 100 KHz; modulation amplitude, 5 Gpp; microwave power, 20 mW; scan rate, 6.25 G/min; time constant, 4 s; receiver gain, $5 \cdot 10^4$; temperature, 22°C; Chl concentrations, 1.8–4.5 mg/ml.

[c] Specific flash yield for O_2 production obtained on the third flash with an O_2 rate electrode.

[d] Data obtained with an O_2 rate electrode. Samples aged either on or off the electrode prior to assaying for Y_3.

[e] Stability of this preparation increases to the values in parentheses if a wash centrifugation step is inserted after Triton treatment and prior to final dilution in storage buffer.

chloroplasts for the BBY and K&M preparations. All four PS II preparations exhibited far less methyl viologen-mediated O_2 uptake than did chloroplasts. This has been interpreted as indicative of low PS I activity and, thus, absence of PS I pigments in O_2-evolving PS II preparations [27], but as we shall see, this is not the whole story.

EPR-detectable Signal I has been identified as oxidized P-700 [17]. Table II compares the amount of potassium-ferricyanide (10 mM)-induced Signal I present in each of the PS II samples relative to the thylakoid control value, (defined as 1.00). The relative amount of long-wavelength, low-temperature fluorescence (F_{735}) induced by exciting light at 430 nm is also compared in Table II. F_{735} under these conditions has been attributed to PS I pigments. Note that the EPR and fluorescence results are consistent with each other but not with the O_2-uptake data.

The patterns of O_2 evolution resulting from sequences of very short flashes of light have been used to probe the mechanism of O_2 evolution in algae, chloroplasts and O_2-evolving PS II preparations [54,55,28,29]. The maximum yield of O_2 production observed in dark-adapted samples on an O_2 rate electrode occurs after the third flash (Y_3). Table II compares the relative amount of Y_3 per unit chlorophyll in the various samples, and the results are qualitatively similar to the steady-state light results. The stability of the preparations differs greatly as determined by observing Y_3 values after different incubation times at room temperature in the dark. In Table II we have determined the $t_{1/2}$ for inactivation of O_2-evolution activity under two conditions: (1) when the samples were aged on the rate electrode itself and (2) when the samples were aged in a test-tube and then transferred to the rate electrode 5 min before a measurement. The data show that the PS II samples are more stable when aged off the electrode than on it. In addition, the O_2 rate electrode activity of the YUSN, K&M and MDT preparations is much more stable than the same activity in chloroplasts and the BBY preparation. If care is taken to remove excess Triton X-100 from the BBY preparation, it, too, becomes much more stable.

Discussion

The most striking morphological feature of all the O_2-evolving PS II preparations examined in this study is that they are not individual 'particles' as implied in several publications [17,19,20]. Instead, these PS II preparations are composed of appressed grana membrane fragments (Figs. 4–12) produced by the differential sensitivity of stacked (grana) and unstacked (stroma) thylakoid regions to detergents. This differential sensitivity is supported by spin-label studies [56,57] which show stroma membranes to be more fluid than grana regions, and by the observation that hydrophobic molecules such as detergents will selectively partition into membrane regions of increased fluidity [58,60]. Furthermore, freeze-fracture electron microscopy (Fig. 7) confirms that these membrane fragments exist in an 'inside-out' orientation, i.e., with the lumenal membrane surfaces exposed to the medium. Thus the detergent-derived PS II preparations are both structurally and functionally related to the grana-derived 'inside out' vesicles of Andersson and co-workers [11–13]. In contrast to the Andersson preparation, however, none of the PS II-enriched membrane fragments obtained by detergent treatment formed closed vesicular structures.

Analysis of the chlorophyll-protein and peptide composition of the O_2-evolving PS II preparations by gel electrophoresis also supports our conclusion that these samples are actually highly purified grana membranes (Figs. 1 and 2). Workers in other laboratories have reported the isolation of non-O_2-evolving PS II particles by detergent solubilization of thylakoids combined with sucrose density gradient centrifugation [41] or with a multistep procedure involving centrifugation, column chromatography and isoelectric focussing [39]. These particles contained significantly fewer peptides than intact thylakoids, consisting almost entirely of the PS II reaction center (42–48 kDa), the LHC peptides (25–27 kDa) and, in the Mullet and Arntzen preparation [41], the 32–34 kDa protein. The PS II preparations studied here contain most of the peptides present in intact thylakoids (Fig. 2). However, all four samples are noticeably enriched in peptides associated with PS II and the light-harvesting complex, and dramatically de-

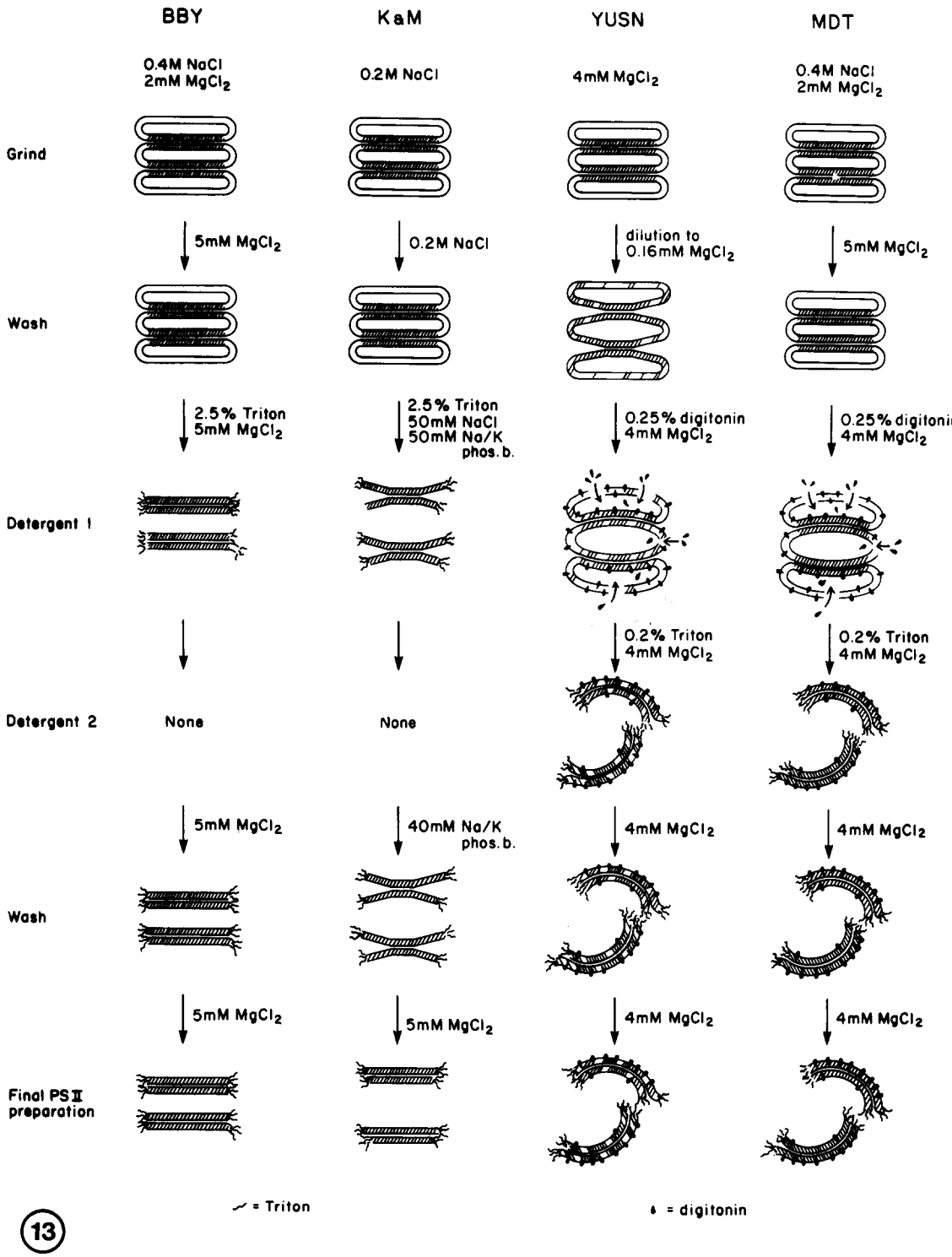


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Fig. 13. Diagram summarizing the effects of different salt conditions and detergent treatments on the configuration of thylakoid membranes during the preparation of the four oxygen-evolving PS II samples. For details, see Discussion. Note that the second wash step for BBY preparations included here was not included in the original procedure of Ref. 17. Monovalent salt concentrations are included only where they would have a significant effect on membrane stacking.

pleted in PS I peptides and the CF_1-ATPase. Thus, our results are in agreement with previous membrane fractionation studies which have localized PS I (more than 85%) and the CF_0-CF_1 ATPase (100%) in the unstacked stroma membranes, while the grana membranes are highly enriched in PS II and the Chl *a*/*b*-LHC [51,62,36].

The variations in structure, composition, and biochemical and biophysical properties among the four PS II preparations can be interpreted with respect to the differences in the salt and detergent conditions of the four isolation procedures (Table I). We have summarized these ideas in the diagram in Fig. 13. In both the BBY and the MDT preparations, high-salt conditions (more than 4 mM $MgCl_2$) are used throughout to stabilize the stacked grana membrane regions, and, thereby, enhance the differential solubility of grana and stroma thylakoids. The somewhat harsher detergent treatment of the BBY preparation yields a slightly more pure sample of stacked membranes than the MDT preparation, but this is partly offset by more damage to the O_2-evolving enzyme system.

The YUSN and K&M preparations differ from the BBY and MDT preparations in that the amount of membrane stacking is not held constant during the preparation of the YUSN and K&M samples. In the YUSN preparations, partial unstacking occurs as the divalent salt concentration is reduced from 4 mM $MgCl_2$ to 0.16 mM $MgCl_2$ before addition of the detergent. This unstacking leads to partial intermixing of grana and stroma membrane components (Fig. 12, Ref. 30). However, the return of the samples to a 4 mM $MgCl_2$, 0.25% digitonin solution leads to restacking of the membranes while detergent-solubilization is initiated. During this rapid restacking we would expect some stroma membrane components to become entrapped in the newly reformed grana regions, where they are somewhat protected from detergent action. This explains why the YUSN preparation has the greatest amount of contamination by stroma membrane components (Fig. 12; Table II).

The K&M preparation, finally, involves short exposure of the membranes to a relatively high concentration of Triton, while the membranes are in the process of becoming partially unstacked due to the lower concentration of monovalent salts in the detergent buffer. This treatment leads to the loss of both stroma membrane regions and of freshly unstacked peripheral regions of the appressed grana membranes. Although the K&M procedure produces a lower yield of oxygen-evolving PS II membrane fragments, in our hands these stacked membrane fragments possess the lowest amount of PS I contamination.

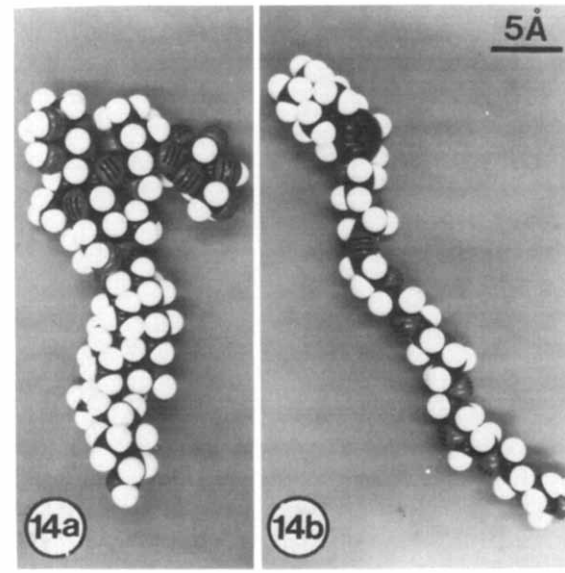

Fig. 14. Molecular models of digitonin (a) and Triton X-100 (b). Note the compact wedge configuration of the digitonin molecular and the more flexible configuration of the longer and thinner Triton molecule. The effects of these two detergents on thylakoid membranes are summarized in Fig. 13 and discussed in detail in the text.

One unexpected finding of our structural analysis is the difference in the configuration of the membrane fragments prepared using different detergents. The PS II preparations exposed to Triton X-100 possessed flat appressed membrane fragments reminiscent of grana membranes devoid of margins (Figs. 4 and 8), while those treated first with digitonin and then with Triton develop membranes with a curved configuration (Figs. 10 and 11). In Fig. 13 we have included a possible explanation for this digitonin-dependent formation of curved membrane fragments. Fig. 14 shows molecular models of the two detergents used in our study, digitonin and Triton X-100. Digitonin is an amphipathic molecule with a very bulky and hydrophobic headgroup, resulting in a pronounced

wedge shape. In contrast, Triton X-100 has a snake-like configuration with a smaller, less polar head, and a tail with one side more polar than the other. Triton would appear to have the ability to both readily penetrate and pass through the membrane. Digitonin, on the other hand, can be expected to insert itself initially only into the exposed outer leaflet of a closed membrane vesicle, thereby expanding that leaflet and causing membrane curvature and breakage. Vesicle lysis enables other digitonin molecules to gain access to the inner leaflet of the membrane. Upon addition of digitonin to thylakoid membranes, the lumenal surfaces of the appressed grana membrane become exposed to digitonin only after the detergent has ruptured the exposed stroma membranes, grana end membranes and margins; the appressed membrane surfaces remain inaccessible to digitonin action throughout detergent treatment. Disproportionate insertion of digitonin molecules into only one of the two membranes of an appressed region will cause that membrane to expand into a curved configuration. This leads to curvature and compression of the lipid and protein components in the adjacent membrane. The ability of digitonin to subsequently penetrate into the compressed membrane will be reduced due to the tighter packing of the lipids. Thus, the initially induced curvature of the appressed membrane fragments will be preserved.

The detergent conditions used to solubilize the membranes in each preparation appear to affect significantly the capacity of the sample to evolve oxygen. The two preparations with the highest rates of oxygen evolution (YUSN and MDT) were exposed to the mildest detergent treatment, i.e., sequential short exposure to low concentrations of digitonin and Triton, in contrast to high concentrations of Triton alone for up to 60 min in the BBY procedure. The electrophoretic data suggests that the digitonin/Triton treatment stabilizes O_2 evolution by preventing the loss of two or more peptides (16 and 23 kDa). As suggested by others [12,19,45], these peptides may be involved in the water-splitting reaction.

The cytochrome f/b_6 complex also exhibits differential sensitivity to the Triton vs. digitonin/Triton treatments. This complex has been reported to be equally distributed between grana and stroma membranes [63,64]. Analysis by gel electrophoresis (Fig. 2) indicates that although cytochrome *f* is depleted in all four PS II samples, there are still significant amounts present in the YUSN and MDT preparations. This could indicate a destructive effect of Triton on the cytochrome complex and/or a stabilizing effect of digitonin.

All four of the PS II preparations evolve oxygen in the light and exhibit maximum flash yields on the third of a series of short flashes (Table II) [28,29]. This, of course, demonstrates PS II electron transport and an O_2-evolving enzyme that functions normally.

The PS II preparations also contain varying amounts of PS I as determined by room-temperature EPR and low-temperature fluorescence assays (Table II). Consequently, the results obtained by biophysical means are in agreement with the electron microscopic and electrophoretic studies mentioned previously. However, the Mehler reaction requires an intact electron transport chain from P-700 to P-430 in order to observe PS I activity. Since the Mehler reaction results do not correlate with those using EPR and fluorescence (Table II), we conclude that the P-700 remaining in the PS II preparations cannot participate normally in the Mehler assay perhaps due to damage to or removal of peptides on the reducing side of PS I. It is important to note that we found larger amounts of PS I in the BBY preparation than did Berthold et al. [17]. This is probably due to our omission of the second Triton wash; we found this wash altered the O_2 flash yield patterns. Our experiments have confirmed that the second wash does greatly reduce the PS I present in the preparation.

Of special note in this study is the remarkable room-temperature stability of all the O_2-evolving PS II preparations compared to chloroplasts. Though consistent with previous observations using K&M [19] and YUSN [29] preparations, we demonstrate that increased stability of the O_2-evolving enzyme is a universal property of detergent-fractionated PS II membranes from spinach. This improved stability is probably not due to the elimination of proteolytic enzymes from the PS II environment, because 'inside-out' spinach vesicles are no more stable than chloroplasts (Andersson, B., personal communication). Thus, the detergents

used to isolate the PS II membranes (at residual concentrations) seem to stabilize the water-splitting function.

As in any comparative study, one must evaluate the relative advantages of the materials that are being compared. An actual value judgement, as to which preparation is 'the best', cannot be justified at present, since the different preparations have properties that might be used to advantage in different experimental situations. However, we summarize as below.

(A). The detergent-fractionated, O_2-evolving PS II appressed membrane preparations are easier to make with higher recovery yields, are more stable at room temperature, and exhibit higher oxygen evolution rates than the mechanically fractionated 'inside out' vesicles. On the other hand, the former contain residual detergent (though this does not seem to affect the mechanism of O_2 evolution [29]), while the latter are not exposed to detergents.

(B). All detergent-fractionated PS II membrane preparations contain light-harvesting complexes, PS II reaction centers, the 16 and 23 kDa peptides associated by others [12,19,45] with O_2 evolution, and a band seen on gels at 32 kDa which probably contains both the DCMU-binding site [41] and the putative manganese-binding peptide of [42,43]. All preparations also contain some PS I pigments (YUSN more than MDT more than BBY more than K&M).

(C). Both the YUSN and the MDT preparations contain cytochrome *f* (and presumably cytochrome *b*), while the BBY and K&M preparations contain little or none.

It is clear that development of these new methods for producing functionally active PS II preparations has opened up new opportunities for studying the photosynthetic water-splitting function and the oxidizing side of PS II. Hopefully, these opportunities will lead to a better understanding of this critical area of photosynthesis.

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Note added in proof

(A) Since submitting this paper, we have found that modification of the BBY preparation (Ref. 66, but still using one Triton extraction step) resulted in the isolation of PS II membranes with less PS I contamination and higher O_2 yields (between 300–400 μmol $O_2 \cdot$ mg $Chl^{-1} \cdot h^{-1}$) than reported in Table II. In addition, we have found that the Triton/Chl ratio, required to produce K&M PS II membranes which have minimum PS I contamination, varies with the market spinach which we use. Winter spinach requires a 15 : 1 ratio, while summer spinach requires a 22.5 : 1 ratio. Furthermore, elimination of potassium ferricyanide and $MgCl_2$ from the Hill assay resulted in O_2 yields from the K&M preparation of between 250 and 300 μmol $O_2 \cdot$ mg $Chl^{-1} \cdot h^{-1}$. Finally, we have obtained YUSN preparations, on occasion, that have less PS I contamination than indicated in Table II, but not as low as observed in the other preparations.
(B) Please note that the photographic plates have been reduced to approx. one half of their original size by the printer.

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