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Depletion of Photosystem II-extrinsic proteins. I. Effects on O_2- and N_2-flash yields and steady-state O_2 evolution

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Wheat O_2-evolving Photosystem II (PS II) membranes having a PS II unit of approx. 200 chlorophylls (Chl), approx. 4 Mn/200 Chl, less than 1 P-700/3000 Chl and an electron-acceptor pool of approx. 2.5 equiv./PS II were analyzed and compared with wheat PS II membranes depleted (at least 90%) of the 17 and 23 kDa proteins by NaCl extraction during Triton X-100 isolation of membranes. Extraction of these proteins caused approx. 50% decrease in O_2 evolution in any light regime and an increase of approx. 2 equiv./PS II of the electron-acceptor pool, but affected neither Mn abundance, photoreduction of DCIP by tetraphenylboron, or N_2 yield (from NH_2OH) from a single flash. Mass spectrometric analyses of O_2 flash yields in the presence of potassium ferricyanide showed that both chloroplasts and the unextracted PS II membranes yielded oscillations compatible with $S_0/S_1/S_2/S_3$ of 25:75:0:0 and α (0.1) and β (0.05). Depletion of 17 and 23 kDa proteins resulted in a two-fold increase in α, approx. 25–40% disconnection of the S state complex from the PS II trap complex but with no change in β. Preincubation of control or extracted PS II membranes with potassium ferricyanide permitted a significant double-hit on the first flash. In the absence of an added electron acceptor, N_2 flash yields were more sustained with 17 and 23 kDa depleted than with 17 and 23 kDa sufficient PS II membranes. In contrast, no significant O_2 flash yields were observed with extracted PS II preparations under these conditions (control PS II membranes showed a predictable O_2 pattern before damping after only 5–6 flashes). These results suggest that extraction of the 17 and 23 kDa proteins results in an increase of pool size on the PS II acceptor side (seen as unmasking 'Component C'). 'Component C' can mediate electron transfer from Q^- to Z^+ (S_2).

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Abbreviations: PS II, Photosystem II; Mes, 4-morpholine-ethanesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; P-700 and P-680, the reaction center chlorophyll of Photosystem I and II, respectively; CP1, chlorophyll *a* complex of Photosystem I; TMF-2, oxygen-evolving Triton X-100 prepared Photosystem II membrane fragment; DCIP, 2,6-dichlorophenolindophenol; Chl, chlorophyll; PBQ, phenyl-*p*-benzoquinone; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; DCBQ, 2,5-dichloro-*p*-benzoquinone; LHCP, light-harvesting chlorophyll protein; Y_n, oxygen yield after a flash number n; A_H, high-potential acceptor of Photosystem II; S_0, S_1, S_2, S_3, S_4, transitional states of the water-oxidizing catalyst; Q_A and Q_B, primary and secondary stable acceptor of Photosystem II, respectively; Z, the secondary electron donor of Photosystem II; C, postulated endogenous electron shuttle component; D, electron donor to $P\text{-}680^+$ when oxygen evolution is inhibited; Cyt, cytochrome.

Introduction

Recent research directed towards unraveling the molecular basis of the Kok–Joliot kinetic model of photosynthetic O_2 evolution has focused on defining the functions of several PS II polypeptides in the water-oxidizing complex (for reviews, see Refs. 1 and 2). Generally, much of this research has attempted to determine whether certain PS II polypeptides provide sites for ligation/stabilization of the tetra-Mn polynuclear complex and/or whether these proteins provide sites for binding of Cl^-, believed essential for the transitions of the higher S states.

Polypeptide analyses of mutants of *Scenedesmus* [3] and *Chlamydomonas* [4], having low amounts of functional Mn and water-oxidizing activity, have given evidence for functions of a 34 kDa and of 17 and 23 kDa proteins, respectively. Similarly, numerous extraction/reconstitution studies made with everted thylakoid membranes [5–9], cholate-extracted thylakoids [10,12] and Triton-prepared PS II membranes [9,11–20] have led to a number of proposed roles of the PS II extrinsic polypeptides (17, 23 and 33 kDa) in the water-oxidizing process.

The precise functions of these extrinsic polypeptides and the 34 kDa intrinsic polypeptide [3] remain to be established. It is clear that extractions of the 17 and 23 kDa proteins [4,9,10,12,19, 20] or the 17, 23 and 33 kDa proteins [21], by at least some extractants, do not deplete the tetra-Mn complex from the membranes. However, it is not at all clear what effects such extractions have on the coordination sphere of the Mn, the folding organization of the PS II intrinsic polypeptides, or the rate constants of reactions associated with the overall process of photosynthetic O_2 evolution.

The solubilization of 17, 23 and 33 kDa polypeptides from membranes by various procedures can result in as much as a 90% [7,9,15] or as little as a 50% [4,13] decrease in the rates of O_2 evolution measured in strong continuous light. These observed differences in rate may reflect variations in the extents of depletion of those polypeptides and/or manifestations of secondary effects of the extractions on the PS II complex, which may not be revealed by analyses of polypeptide solubilization. Such suppositions may underlie the variable extents of recovery of O_2 evolution following reconstitution of the extracted membranes with the 17 and 23 kDa proteins [5,13,16], only the 23 kDa protein [5,9,13,22], or by the addition of more than 5 mM $CaCl_2$ [14,15].

In an attempt to gain greater insights into the role(s) of the 17 and 23 kDa polypeptides in the O_2-evolution process and to determine whether the extraction procedure(s) for removal of these polypeptides resulted in secondary effects on the PS II complex, we measured: (1) patterns of flash-induced O_2 evolution in the presence and absence of a PS II electron acceptor; and (2) flash yields of N_2 (from NH_2OH) in the absence of a PS II electron acceptor; and (3) rates of O_2 evolution in continuous weak and strong light. Our results indicate that complete extraction of the 17 and 23 kDa polypeptides results in a less than 50% decrease in O_2 evolution in any light regime. Removal of these peptides does not result in the extraction of functional Mn or change of value of Kok's double-hit (β) parameter [23], but does cause a 2-fold increase in the value of Kok's 'miss' (α) parameter. No evidence was obtained to support the proposal [17,19] that removal of the 17 and 23 kDa proteins by NaCl washing of PS II membranes affects only the higher oxidation states of the water-oxidizing complex. Evidence was obtained, however, that removal of these polypeptides decreases charge stabilization of the hole-electron pair; this effect can be overcome by the use of a donor (NH_2OH) to fill the hole or an acceptor (potassium ferricyanide) to remove the electron.

Materials and Methods

Preparation of chloroplasts and oxygen-evolving Triton X-100 membrane fragments

Chloroplasts were prepared from 7–9 day old wheat seedlings grown in vermiculite in the greenhouse on a half-strength nutrient solution. The leaves were cut with scissors into segments of approx. 3 mm; then 70 g were blended for 20 s in 450 ml buffer A (0.35 M sucrose/50 mM Hepes-NaOH/2 mM $MgCl_2$ (pH 7.5), containing 0.1% bovine serum albumin and 0.2% sodium ascorbate). The homogenate was filtered through four layers of cheesecloth and then centrifuged (3000 ×

g for 4.5 min) (chloroplasts prepared from wheat in this buffer routinely gave a 2-fold greater O_2 evolution then when prepared in the NaCl/EDTA-containing buffer used in Ref. 24). The chloroplasts were resuspended in 20 mM Mes-NaOH (pH 6.2)/15 mM NaCl/5 mM $MgCl_2$/0.2% sodium ascorbate to give approx. 0.15 mg Chl/ml, and then immediately pelleted (3550 × g/7 min).

Fig. 1 describes the procedure routinely employed to prepare NaCl-TMF-2 devoid of the 17 and 23 kDa extrinsic polypeptides. The same procedure was used to prepare TMF-2 (containing the 17 and 23 kDa polypeptides) except for: (1) omission of NaCl additions where indicated, and (2) washing of the 30 000 × g for 15 min pellet at approx. 1 mg Chl/ml (40–50% yield). In both cases the washed chloroplasts were resuspended (more than 2 mg Chl/ml) in the same buffer and subjected to Triton X-100 treatment (c.f., the top part of Fig. 1). All operations were done at 4°C and in very dim room light. The final pellets were resuspended (more than 3 mg Chl/ml) in 0.4 M sucrose/20 mM Mes-NaOH (pH 6.2)/15 mM NaCl/5 mM $MgCl_2$ and either used immediately or stored at −80°C. We found no evidence to indicate freezing modified the properties of TMF-2 or NaCl-TMF-2.

This procedure for TMF-2 membrane preparation represents modifications of previously described procedures [24,25]. Decreasing the pH from that employed in Ref. 24 increased O_2-evolution activity of TMF-2 approx. 4.5 fold. Differential centrifugation and washings of the 30 000 × g pellets of pH 6.2 preparations resulted in removal of CP_1, and diminished P-700 abundance (from approx. 3 to less than 1 P-700/3000 Chls as determined by flash spectroscopy [26]).

Over the course of several years, involving many different preparations, we routinely obtained yields of TMF-2 and NaCl-TMF-2 (from chloroplasts on a Chl basis) of 40–50% and 20–30%, respectively, and activities of 500–700 and 250–350 O_2/Chl per h, respectively. On some occasions, however, the procedures for TMF-2 and NaCl-TMF-2 preparations gave anomalously low yields and diminished activities. Dunahay et al. [27] found it necessary to decrease Triton/Chl ratios in order to obtain an O_2-evolving pellet and minimal P-700

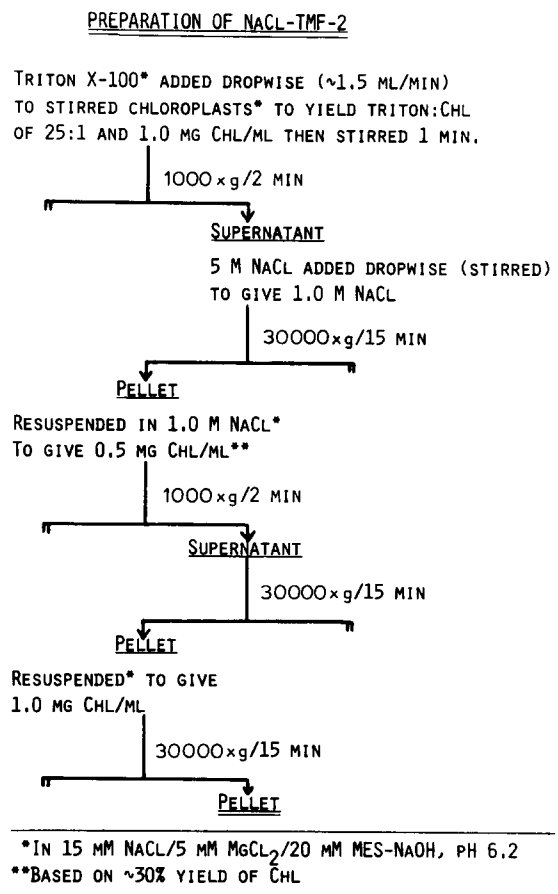

Fig. 1. Schematic diagram outlining procedures for the preparation of NaCl-TMF-2 from wheat chloroplasts. See text for details and TMF-2 preparations.

contamination in some cases. This variability may be attributable to differences between lots of Triton X-100 and/or the chloroplast starting material. Thus, on some occasions, the procedure of Fig. 1 was modified by using Triton/Chl of 20 : 1 and, where indicated, addition of 5 M NaCl to give only 0.5 M final concentration and subsequent washing of the 30 000 × g/15 min pellet with 0.75 M NaCl.

Rate measurements of oxygen evolution and DCIP photoreduction

Rate measurements of O_2 evolution in continuous light were made polarographically [10] using a vessel of 1.0 ml capacity and saturating light filtered through 5 cm of 2% $CuSO_4$, two Schott 116 and one OG-3 filters. The assay mixture for

chloroplasts (5 μg Chl/ml) contained 0.4 M sucrose/50 mM Hepes-NaOH (pH 7.5)/30 mM methylamine/1 mM FeCN and, where noted, 300 μM PBQ. The assay mixture for TMF-2 preparations (5 μg Chl/ml) contained 0.4 M sucrose/50 mM Mes-NaOH (pH 6.2)/1 mM FeCN/30 mM NaCl and, where noted, 300 μM PBQ. Neutral density filters were used to vary light intensity.

DCIP photoreduction was measured using an Aminco-DW-2 spectrophotometer (580–540 nm, dual-beam mode). Side illumination was provided by a Dolan-Jenner 180 light source. The light was focused onto the 1 cm light path cuvette and filtered through a Schott 116 and a Corning 2-59 filter. A Corning 4-95 filter masked the PM tube. The assay mixture contained 0.4 M sucrose/50 mM Mes-NaOH, (pH 6.2)/30 mM NaCl/26 μM DCIP/25 μg Chl per ml and, where indicated, 50 μM TPB. Absolute rates were determined using a millimolar extinction coefficient of 4.97.

Flash measurement of O_2 and N_2 yields

The mass spectrometric apparatus and measuring technique used was a modification of system described earlier [28]. The heart of the system is a 1 mm thick silicone rubber membrane that admits gases dissolved in the liquid phase to the mass spectrometer vacuum. Preliminary experiments showed that the Photosystem II preparations being studied would not settle adequately on the silicone membrane described in Ref. 28. (We also found it difficult to change conditions during the course of the experiment using that apparatus.) Consequently, we constructed the reaction vessel and ancillary equipment shown in Fig. 2, in which the chloroplast preparation was affixed to a captured membrane filter. With this apparatus, (1) preparations that do not settle effectively onto the inlet membrane can be monitored, and (2) samples can be rapidly subjected to various reaction mixtures and/or deposited on the reaction vessel in total darkness.

Samples were deposited on the membrane using a standard filtration apparatus (Millipore 25 mm filter holder). An aliquot of each sample (25 μg chlorophyll) was suspended in 1 ml of buffer containing 0.4 M sucrose/20 mM Mes-NaOH (pH 6.2)/15 mM NaCl/5 mM $MgCl_2$. The TMF-2 preparation was then drawn onto the sample membrane (0.45 μm Schleicher and Schuell membrane filter) from the suspension via an aspirator. The membrane with its deposited sample was then mounted in retaining rings and the assembly stored in buffer in the dark and 0°C until use (10–15 min).

Immediately before analysis 100 μl of reaction solution was placed on the mass spectrometer membrane (for O_2 measurements this was assay buffer (0.4 M sucrose/50 mM Mes-NaOH (pH 6.2)/30 mM NaCl): for N_2 measurements the buffer also contained 20 mM hydroxylamine). The membrane containing the deposited sample was placed on the inlet (after excess buffer was removed with a Kimwipe) and secured with a retaining nut. 400 μl of the same reaction solution was then added to the back side of the membrane. After the light shield was placed over the inlet assembly, a syringe containing 500 μl of 5 mM potassium ferricyanide in assay buffer was inserted into the reaction vessel through a septum port in the light shield. After a 3 min dark adaption, the fluid in the syringe was discharged into the reaction vessel. Flash yield data were obtained as soon as the signals stabilized (less than 1 min).

After the completion of the flash yield measurements, the membrane containing the deposited sample was carefully removed from the retaining rings using a scalpel, placed in a tube containing 5 ml of methanol, and stored at 0°C in the dark. Chlorophyll concentrations were determined in 100% methanol after filtration. Because the filters themselves dissolved in the methanol, quantitative recovery of the chlorophyll was assured.

All measurements were made at room temperature. To increase the sensitivity of the analyses, all N_2 evolution studies were conducted using hydroxyl[^{15}N]amine-HCl (98 atom% ^{15}N, obtained from Amersham (Arlington Heights, IL)).

Other methods

SDS-polyacrylamide gel electrophoresis analyses were done essentially as described by Chua [29] using a 12–20% linear gradient of acrylamide and a Neville buffer system [30]. Samples of 65 μg protein were solubilized in 50 mM dithiothreitol/50 mM Na_2CO_3/10% sucrose using 15 mg SDS per mg protein. Solubilization was done at 25°C for 25 min, then 70°C for 5 min. Protein de-

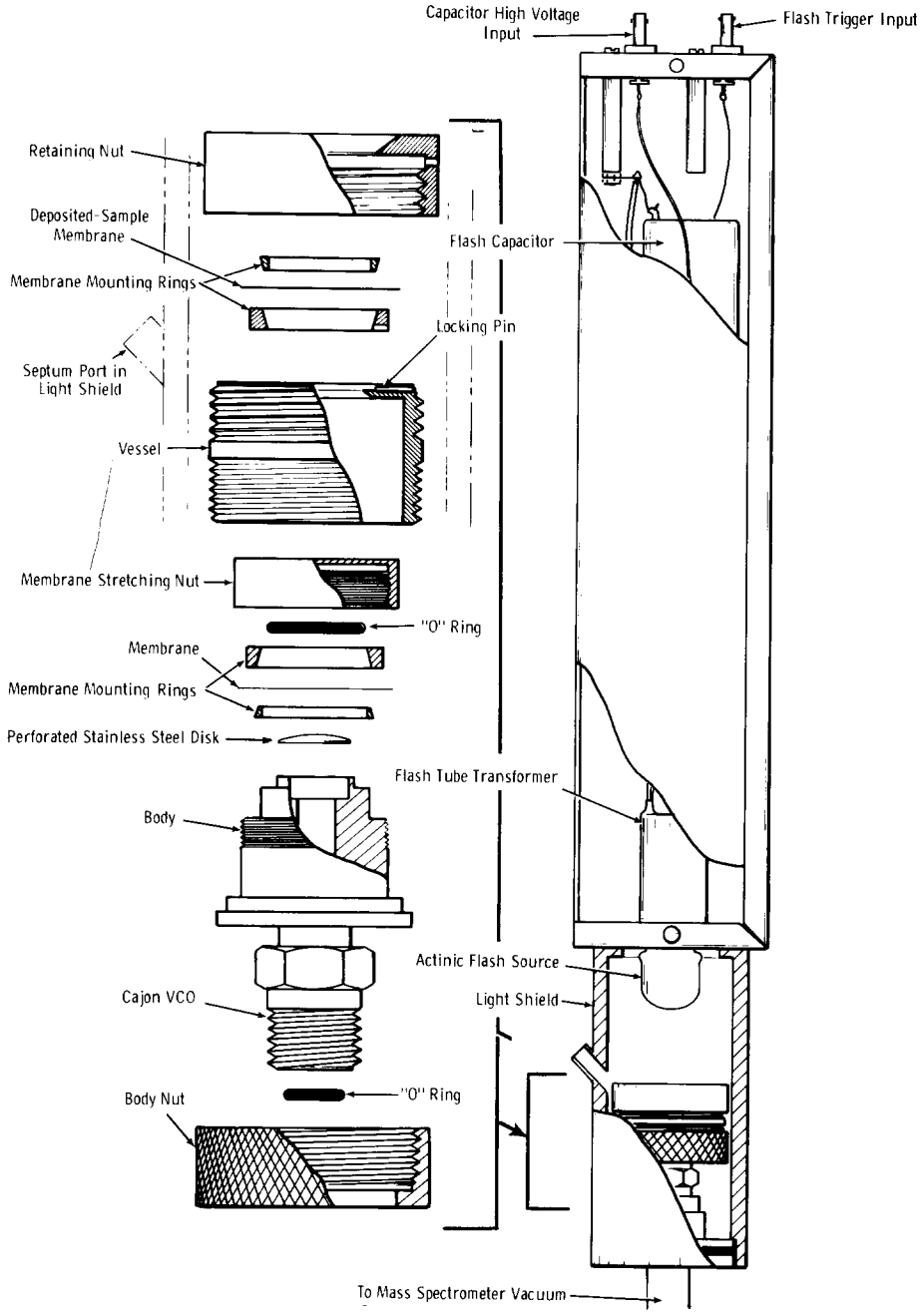

Fig. 2. Mass spectrometer reaction vessel and ancillary equipment for analyses of flash yields of O_2 and N_2.

terminations on the membrane preparations were made as described by Lowry et al. [31] in the presence of 0.1% (w/v) sodium deoxycholate. Densitometer analyses of the Coomassie blue stained gels were made at 575 nm using a Gelman Model ACD-15 instrument with 1 mm slit width. Mn determination procedures have been described [32].

Results

Biochemical characterization of preparations

Table I is a tabulation of the abundance of functional Mn (Mn/400 Chl), the relative abundances of the 17, 23 and 33 kDa extrinsic PS II polypeptides, and the rates of O_2 evolution in strong continuous light, typically observed for chloroplasts, TMF-2, and NaCl-TMF-2. Note that TMF-2 is approx. 2-fold enriched in functional Mn relative to the parent chloroplasts. Thus, the procedure which extracts the 17 and 23 kDa proteins does not diminish the Mn abundance of approx. 8 Mn/400 Chl. This Mn abundance is functional, since it is not decreased by washing with buffer containing 20 μM A23187 and 1 mM EDTA, but is depleted by NH_2OH or Tris extraction to levels less than 1 Mn/400 Chl [4]. Additionally, TMF-2 and NaCl-TMF-2 showed no hexaquo Mn^{2+} EPR signal and both frequently yielded equivalent amounts of the low-temperature multiline EPR signal of the S_2-state (see Ref. 33, data not shown).

Despite the routinely observed approx. 2-fold enrichment of the Mn-S-state complex in TMF-2, rates of O_2 evolution in strong light did not show proportionate increases when assayed in optimum conditions with PBQ. The corresponding enrichment of rates of O_2 evolution (TMF-2/chloroplast) ranged from 1.22- to at most 1.75-fold. In the absence of PBQ, O_2 evolution by TMF-2 or NaCl-TMF-2 was severely limited, as mentioned previously [19].

The data of Table I show that the O_2 evolution rate of NaCl-TMF-2 (more than 90% depleted of the 17 and 23 kDa proteins during preparation) was diminished by only approx. 50% when measured in strong light, a similar (48%) decrease in O_2 evolution was also observed for NaCl-TMF-2 measured at quantum yield intensities (see Fig. 3). The consistent approx. 50% decrease in O_2 evolution observed with NaCl-TMF-2 in high or low light agrees well with results reported by Miyao and Murata [13] with spinach PS II membranes depleted of these proteins by extraction with 1 M NaCl for 30 min at pH 6.5. Our results are also in approximate agreement with results obtained by 2 M NaCl extraction (pH 6.0, 30 min) of spinach PS II membranes [16,18]. In earlier experiments [15] using saturating light, 80% inhibition was observed with near total depletion of the 17 and 23

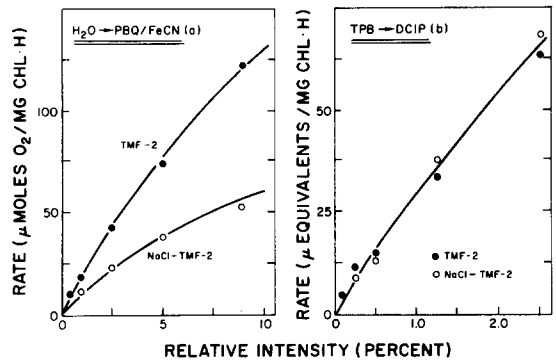

Fig. 3. Rates of PS II electron transport as a function of light intensity for TMF-2 (closed circles) and NaCl-TMF-2 (open circles). (A) O_2 evolution measured polarographically with PBQ. (B) DCIP photoreduction from TPB. Intensities in A cannot be directly equated with those in B.

TABLE I

RATES OF OXYGEN EVOLUTION, Mn ABUNDANCE, AND RELATIVE ABUNDANCE OF PS II-EXTRINSIC POLYPEPTIDES IN O_2-EVOLVING PREPARATIONS

The range of values shown represent values obtained from approx. 50 different preparations from wheat seedlings. Considerable variation in rates of O_2 evolution of preparations from market spinach was observed, although the maximum rates were never greater than those shown. Mn is linearly correlated with O_2 evolution (see text). Densitometric analyses of Coomassie Blue-stained SDS-polyacrylamide gel electrophoresis (see text) gave the relative abundance of polypeptides indicated.

Type of preparation	Rate of oxygen evolution (μmol O_2/mg Chl per h)		Mn/400 Chl	Relative abundance of polypeptides		
	without PBQ	with PBQ		17 kDa	23 kDa	33 kDa
Chloroplasts	238–446	375–480	4.2–4.6	+	+	+
TMF-2	24– 42	500–700	7.5–8.5	+	+	+
NaCl-TMF-2	25– 52	230–350	7.5–8.5	–	–	+

kDa polypeptides; however, more recent results (with total depletion of these proteins) indicate less inhibition (approx. 65%) when assayed in saturating [22] or in weak light [17,18] and in the absence of $CaCl_2$ additions. This range of inhibition produced by 2 M NaCl extraction of PS II membranes is similar to that obtained with 250 mM NaCl extraction of very dilute everted spinach thylakoid vesicles at pH 7.5 [5–7,22]. Partial (50–90%) solubilization of the 23 kDa was correlated with a partial and similar loss of O_2 evolution, in contrast to the rapid depletion of the 17 kDa.

When TMF-2 and NaCl-TMF-2 preparations were analyzed by SDS polyacrylamide gel electrophoresis, visual inspection of the Coomassie blue staining patterns indicated complete removal of the 17 and 23 kDa polypeptides without loss of the 33 kDa protein (Table I). We attempted to quantitate these results by densitometric scanning of the banding patterns (see Fig. 4), integration of the area under the peaks, and normalization to the abundance of a band which showed essentially no variation by extractions which inactivate O_2 evolution. The approx. 47 kDa reaction center polypeptide [34,35] and bands in the LHCP region were found to be suitably invariant and indicative of a 'unit size'. The particular NaCl-TMF-2 preparation shown in Fig. 4 was depleted of approx. 21%, 88% and 100% of the 33, 23 and 17 kDa proteins, respectively. These determinations are compromised, however, by a significant level of background staining and variability in resolution. We estimate typical NaCl-TMF-2 preparations to be at least 90% depleted of the 17 and 23 kDa proteins with not more than 10% loss of the 33 kDa species; however, preparations which definitively show 100% depletion of both the 17 and 23 kDa bands may sometimes also show up to 25% loss of the 33 kDa. Nonetheless, solubilization of the 33 kDa can be only partly responsible for the observed 50% decreased O_2 evolution in NaCl-TMF-2. Our data suggest that at most only a 50% decrease in O_2 evolution, and perhaps less, can be attributed specifically to the removal of the 17 and 23 kDa proteins.

NaCl-TMF-2 contained only one polypeptide (approx. 20 kDa) between the LHCP region and the approx. 10 kDa doublet band (Fig. 4). We sometimes observed several minor components in the 10–25 kDa region in TMF-2; spinach PS II membranes [16] show several significant bands in the 20–25 kDa region even after 2 M NaCl washing. There appears to be no clear relationship between these differences in polypeptide composi-

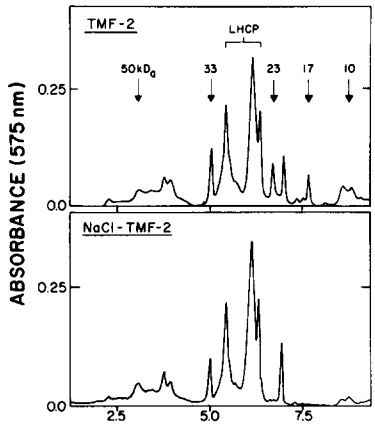

Fig. 4. Densitometer scans of Coomassie Blue-stained 12–20% SDS-polyacrylamide gel electrophoresis. Samples equivalent to 9 μg Chl. Arrows indicate approximate molecular mass (in kDa). LHCP identifies bands associated with the light-harvesting complex.

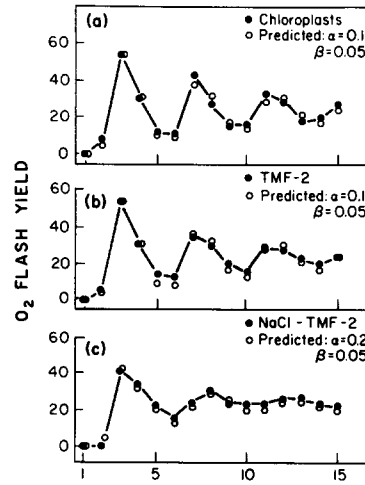

Fig. 5. Observed and predicted relative O_2 flash yields with spinach chloroplasts (a), TMF-2 (b), and NaCl-TMF-2 (c) in the presence of added electron acceptor (5 mM potassium ferricyanide) added immediately prior to analysis. All experiments were done with the same chlorophyll concentration (25 μg Chl/sample).

tion and the diminished O_2-evolving capacity of NaCl-TMF-2, or observed differences between NaCl-TMF-2 and 2 M NaCl washed PS II membranes to, e.g., Ca^{2+} stimulation and sensitivity to chemical reductants. It does seem clear, however, from data of Fig. 3 that neither the complete extraction of the 17 and 23 kDa nor of any minor polypeptide in the 10–25 kDa region affects the relative quantum yield of TPB/DCIP photoreduction by PS II. This result confirms previous conclusions [17] obtained (sometimes) with PS II preparations less extensively depleted of the 17 and 23 kDa polypeptides.

Analyses of O_2 flash yields with TMF-2 and NaCl-TMF-2

The preceding analyses suggest that there may be some direct functions of the 17 and 23 kDa proteins in the O_2-yielding reactions *. Accordingly, we analyzed O_2-flash-yield patterns by procedures free of complications sometimes encountered [36,37] with the Joliot bare electrode.

Fig. 5 shows the O_2 flash yields obtained when broken chloroplasts, TMF-2 and NaCl-TMF-2 were subjected to a series of saturating flashes (3 s spacing). Accompanying each sequence are the predicted flash yields obtained using the model of Kok et al. [23]. In these experiments potassium ferricyanide (final concentration, 2.5 mM) was added immediately (within 30 s) before illumination. This procedure minimized the high Y_2 value (usually ascribed to a double hit [23]) and extended the O_2 flash-yield oscillation of the TMF-2 preparations (see below). Note that the regular TMF-2 preparation displays the same flash-yield kinetics as the chloroplasts, and is consistent with the predictions of the model of Kok et al. [23]. These observations suggest that Photosystem II is unperturbed in these preparations using these criteria. In no instance did we observe the anomalously high yield of O_2 on the first flash reported by Seibert and Lavorel [36,38] with PS II membranes on a Joliot electrode in the absence of a PS II electron acceptor.

A comparison of the O_2 flash yields obtained with NaCl-TMF-2 vs. the unextracted TMF-2 preparations, however, shows distinct differences. In particular, the miss factor (α) was increased 2-fold without any change in the double-hit factor (β) by depletion of the 17 and 23 kDa proteins from TMF-2.

Somewhat similar conclusions have been deduced from measurements using O_2-evolving PS II membranes subjected to 2 M NaCl washing [18]; however, this procedure also decreased the double-hit factor by 2-fold. We conclude, as in Ref. 18, that complete depletion of the 17 and 23 kDa proteins does not prohibit the advancement of the higher S-states.

As shown in Fig. 6, preincubation of the TMF-2 and NaCl-TMF-2 with potassium ferricyanide for 3 min before the onset of the O_2-yield measurements markedly altered the O_2-flash-yield patterns from both types of preparation: (1) Y_2 was increased significantly, and (2) the O_2 yield of subsequent flashes showed patterns distinctly different from those observed in the data of Fig. 5. These effects of potassium ferricyanide preincubation could reflect (1) an oxidation of a secondary acceptor [39] during preincubation, thereby permitting an increased double hit on the first flash, or (2) diminished rates of deactivation of the S_2 state due to the presence of the oxidant.

The predicted flash yields based on the two hypotheses are also shown in Fig. 6. We favor the double-hit explanation based on indirect argument; namely, the invariance, in our hands, of the Y_2/Y_3 ratio. This ratio does not appear to change with the duration of the dark preincubation in a manner consistent with the known rates of S_2 and S_3 deactivation [38]. We therefore conclude that the secondary acceptor exists functionally in TMF-2 and is not greatly perturbed in NaCl-TMF-2.

Analyses of N_2 and O_2 flash yields in absence of electron acceptors

Table II is a compilation of the relative steady-state yields of O_2 (from H_2O) and N_2 (from hydroxylamine) obtained with the different TMF-2 preparations in the presence of an added electron

* In Refs. 17 and 19, it was suggested that inhibition by NaCl washing only affects the higher (at least S_2) oxidation states of the water-oxidizing complex. It is not entirely clear in Ref. 17, however, if sufficient Cl^- was used in the assays. These conclusions were not supported by analyses of the period-4 oscillatory ultraviolet absorbance change or luminescence [18].

acceptor. Note that neither NaCl extraction nor Tris extraction decreased the population of functional reaction centers, as assayed by N_2 evolution from hydroxylamine. O_2 evolution, on the other hand, was strongly affected by both Tris extraction and NaCl extraction. Tris extraction completely destroyed the O_2-evolving capabilities of the preparation; this is in accord with numerous earlier reports. NaCl extraction resulted in an approx. 50% decrease in O_2-evolution capability when measured by flash yields; these data compare well with the steady-state rates shown in Table I.

In an earlier report [40] we estimated the pool size on the acceptor side by an analysis of the O_2-flash-yield sequence in the absence of an added electron acceptor. Here we use a modification of this technique to determine the acceptor pool size in TMF-2 and NaCl-TMF-2.

Fig. 7a shows results obtained when N_2 yields were measured with TMF-2 and NaCl-TMF-2 in the absence of an added electron acceptor. Note that, although the initial N_2-flash yields were identical, subsequent N_2-flash yields were appreciably different in the two cases. With TMF-2, the flash yield was diminished by approx. 92% after only five flashes; however, with NaCl-TMF-2, the flash yield was diminished by only approx. 56 and 86% after 5 and 12 flashes, respectively, in the absence of an added electron acceptor. When N_2-flash yields were measured in the presence of an electron acceptor (data not shown), both type preparations gave more sustained flash yields, the flash yield diminishing by only approx. 43% after 25 flashes in either type preparation.

TABLE II

RELATIVE STEADY-STATE YIELDS OF O_2 (FROM H_2O) AND N_2 (FROM NH_2OH) OBTAINED WITH DIFFERENT TMF-2 PREPARATIONS IN THE PRESENCE OF AN ADDED ELECTRON ACCEPTOR

The first flash yield of N_2 was taken as steady state. Tabulated values are in units of mV (average steady-state peak height) per μg Chl.

	O_2	N_2
TMF-2	0.48	1.03
NaCl-TMF-2	0.24	1.00
Tris-extracted	0.0	1.02

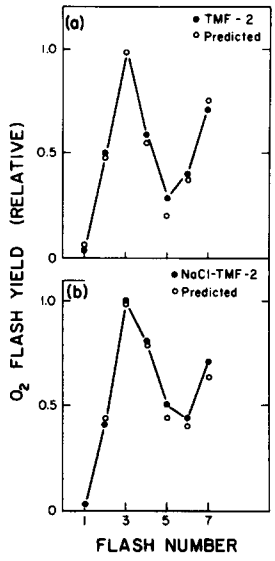

Fig. 6. Observed and predicted O_2-flash yields with TMF-2 (a) and NaCl-TMF-2 (b) after 3 min preincubation with potassium ferricyanide. For comparison of the experimental data with those predicted by the double-hit hypothesis, we assumed $S_0/S_1/S_2/S_3$ to be 25:75:0:0, $\alpha = 0.1$ (a) and 0.2 (b) on all flashes, $\beta = 0.35$ (a) and 0.25 (b) on the first flash, but $\beta = 0.05$ on subsequent flashes, and calculated the S-state distributions normalized to Y_3. An equivalent fit with the TMF-2 experimental data was obtained using the Incomplete Deactivation hypothesis, assuming $S_0/S_1/S_2/S_3$ initially to be 17:58:25:0 and $\alpha = 0.1$ and $\beta = 0.05$ on all flashes.

The differences in O_2-flash yields between TMF-2 and NaCl-TMF-2 under these conditions are even more profound than the differences in N_2-flash yields (Fig. 7). TMF-2 showed flash yields of O_2 through five flashes, while measurable flash

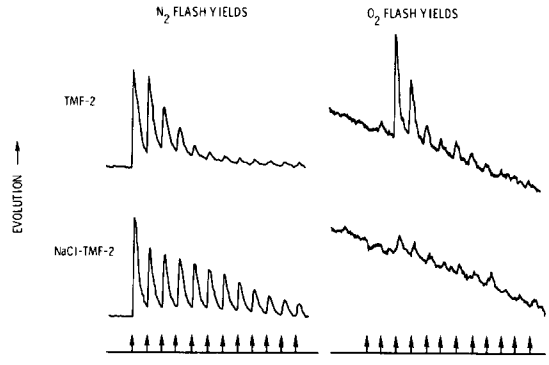

Fig. 7. Flash yields of N_2 and O_2 observed with TMF-2 and NaCl-TMF-2 in the absence of added acceptor. Flashes are denoted by arrows.

yields of O_2 were essentially absent in the case of NaCl-TMF-2.

These differences are strikingly apparent in Fig. 8, which is a compilation and calculation of some of the data of Fig. 7. The upper panels present the number of equivalents per flash oxidized by PS II computed from the N_2 flash yield data. (We assumed initial α values of 0.1 for both TMF-2 and NaCl-TMF-2). These values were then used to predict the O_2 flash yields ($\beta = 0.05$ for both TMF-2 and NaCl-TMF-2). A comparison of the predicted and observed values is given in the lower panels of Fig. 8. Note the good fit obtained for the data of the unextracted TMF-2 sample, suggesting that the two cases, i.e., N_2 evolution and O_2 evolution, are comparable with respect to electron flow. On the other hand, when the same method was applied to the data obtained with NaCl-TMF-2 there is no correlation between the observed and computed values: indeed, there was little O_2 evolution observed whatsoever. This slower damping of N_2 flash yields with NaCl-TMF-2 conceivably could reflect a modification of the PS II acceptor side with accompanying turnover of the acceptor side by molecular oxygen. However, mass spectrometer measurements ($^{18}O_2$ enriched atmosphere) gave no evidence for the reduction of O_2 in the N_2 flash yield measurements. This result, coupled with the absence of catalase in the NaCl-TMF-2 (or TMF-2) preparations (not shown) eliminates any supposition invoking turnover of the NaCl-TMF-2 PS II acceptor side by molecular oxygen.

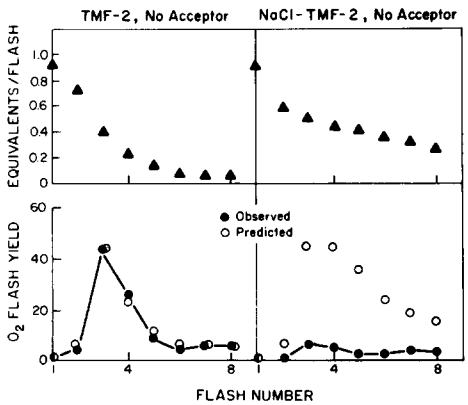

Fig. 8. Compilation and computation of data of Fig. 7 (see text).

Discussion

Analyses of O_2 evolution parameters in TMF-2 preparations using continuous light or a train of actinic light flashes in the presence of suitable PS II electron acceptors gave the following conclusions: (1) both chloroplasts and TMF-2 showed equivalent oscillations of flash yields of O_2, with $\alpha = 0.1$ and $\beta = 0.05$ in both cases; (2) preincubation of TMF-2 with potassium ferricyanide before the onset of actinic flashes permitted a significant double hit, indicating the presence of oxidized $A_H(R)$ [39]; and (3) despite the approx. 2-fold enrichment of functional Mn in TMF-2 relative to chloroplasts, no proportionate corresponding increase in maximum obtainable rates of O_2 evolution could be observed with use of PBQ/ferricyanide as electron acceptors.

Similar analyses of O_2 evolution parameters in NaCl-TMF-2 indicated: (1) steady-state rates were diminished only approx. 50% in any light regime (flash vs. continuous weak or high light); (2) O_2 flash yield measurements showed that α was increased 2-fold ($\alpha = 0.2$) with no change of β ($\beta = 0.05$) by the extraction of the 17 and 23 kDa proteins. A 25–40% decoupling of the S state complex from PS II traps sometimes occurred during preparation of NaCl-TMF-2, predicated on the difference between observed vs. predicted ($\alpha = 0.2$, $\beta = 0.05$) steady-state O_2 flash yields and on studies of TMPD oxidation following a single light flash (Tamura, N., Radmer, R., Lantz, S., Cammarata, K. and Cheniae, G., unpublished results). Such a disconnection may be a result of a variable, slight solubilization and/or perturbation of the 33 kDa polypeptide (see Fig. 4). A similar disconnection was observed in 2 M NaCl-extracted spinach PS II membranes [18].

In the N_2-flash-yield measurements, we tacitly assume: (1) NH_2OH donation to P-680$^+$ is exclusively via the alternate donor D [41] (donation via Z would be totally inhibited [42] by the 20 mM NH_2OH used here); and (2) negligible Q^-/P-680$^+$ charge recombination occurs in the presence of NH_2OH, since P-680$^+$ reduction by D is fast ($t_{1/2} \approx 23$ μs [43]) compared to Q^-/P-680$^+$ charge recombinations ($t_{1/2} \approx 600$ and 200 μs for control and for 17 and 23 kDa depleted PS II, respectively [44]). Thus, in the absence of an added electron

acceptor, a summation of N_2 yields from a train of flashes gives an approximate measure of the PS II electron acceptor pool. Data of Fig. 8 yielded values of approx. 2.5 and approx. 4.5 electron equivalents for TMF-2 and NaCl-TMF-2, respectively. The small pool size (TMF-2) is in substantial agreement with estimates made in Refs. 45 and 46; see, however, Refs. 36, 38 and 47. An increase of pool size by depletion of 17 and 23 kDa proteins (NaCl-TMF-2) has not been reported previously.

Analyses of flash-induced luminescence changes with PS II membranes extracted with 2 M NaCl [18] showed such extraction of the 17 and 23 kDa proteins abolished the two-electron PS II acceptor gate and conferred a requirement for DCBQ in a potassium ferricyanide acceptor system for observing period-4 oscillations of S states [18]. Our 17 and 23 kDa-depleted PS II showed period-4 O_2 oscillations in potassium ferricyanide only ($\alpha = 0.2$, $\beta = 0.5$) but not in the absence of any added acceptor. However, TMF-2 with a smaller PS II acceptor pool gave predictable ($\alpha = 0.1$, $\beta = 0.05$) O_2 yields even in the absence of any added acceptor system.

To explain the contrasting O_2 flash yield data (TMF-2 vs. NaCl-TMF-2) measured in the absence of any added electron acceptor system and the increased pool of electron acceptors in NaCl-TMF-2, we postulate: (1) The NaCl-TMF-2 preparation procedure causes 'activation' of a charge recombination pathway (wavy line, Fig. 9); and (2) component C^- (approx. 2 equiv./PS II) competes favorably with Z^+ reduction by the S-state complex and/or causes deactivation of S_2 and S_3. We further postulate that potassium ferricyanide 'short-circuits' the cyclic electron transfer, thus permitting predictable O_2 flash yields observed with NaCl-TMF-2 in the presence (Fig. 5), but not the absence of potassium ferricyanide (Figs. 7 and 8).

We considered the possibility that the $Q^-/C/Z^+(S_2)$ cyclic path normally operates in TMF-2, but is manifested more in NaCl-TMF-2 as a consequence of a possible decrease in rates of S-state advancement. We reject this explanation: (1) C^- reduction of $Z^+(S_2)$ occurs with NaCl-TMF-2 in single flash measurements of TMPD oxidation by PS II made in the presence of DCMU (Tamura, N., Radmer, R., Lantz, S., Cammarata, K. and Cheniae, G., unpublished results); and (2) neither NaCl-TMF-2 nor TMF-2 contains functional S-state complexes in the N_2 (NH_2OH)-flash-yield measurements yet our results (Figs. 7 and 8) show NaCl-TMF-2 > TMF-2 in size of the PS II electron acceptor pool.

The chemical identity of C is unknown. Jansson et al. [48] proposed activation of a charge-recombination pathway not unlike ours (Fig. 9) to explain an approx. 2-fold increase and a 60% decrease of $SigII_f$ and S_2-EPR signal, respectively, following NaCl extraction of inside-out thylakoids. They considered but rejected a pathway involving Cyt *b*-559, a PS II component which can undergo rapid oxidation. Our data do not exclude identification of C with Cyt *b*-559.

Flash-yield data with TMF-2 and NaCl-TMF-2 in this study were obtained in the presence of sufficient Cl^- for the S-state transitions; moreover, Ca^{2+} has little (less than 30%) effect on V_{O_2} with either type preparation in contrast to those employed in Refs. 15 and 18.

While reconstitution of NaCl-TMF-2 with 17 and 23 kDa proteins increased V_{O_2} and eliminated the partial disconnection, the reconstitution neither decreased $\alpha = 0.2$ nor abolished the $Q^-/C/Z^+)S_2)$ cyclic path (Tamura, N., Radmer, R., Lantz, S., Cammarata, K. and Cheniae, G., unpublished results). Though reconstitution eliminated the partial and variable extent of disconnection, only a maximum of 25–40% disconnection of PS II traps from the S-state complex was observed with at least 90% extraction of the 17 and 23 kDa proteins. Moreover, some NaCl-

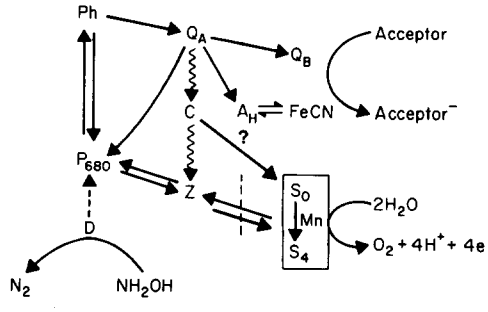

Fig. 9. Schematic representation of relevant PS II components. Dashed line, disconnection due to 17 and 23 kDa extraction. Wavy arrow, activated pathway in NaCl-TMF-2.

TMF-2 preparations show normal abundance of the S_2-multiline EPR signal [33]. All our observed effects on the PS II/S-state complex from extraction of the 17 and 23 kDa proteins thus appear to be a consequence of secondary effects from the extraction procedure(s).

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