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Depletion of Photosystem II-extrinsic proteins. II. Analysis of the PS II / water-oxidizing complex by measurements of *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine oxidation following an actinic flash

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The effects of selective extractions of Photosystem II (PS II) extrinsic proteins (with and without extraction of PS II Mn) on the coupling between the PS II trap and the S-state complex were determined by analysis of steady-state O_2 evolution, chemical reactivity of PS II Mn with *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD), and the kinetics of TMPD oxidation after a single short actinic flash. Unextracted PS II membranes showed only a slow approx. 60 ms component of TMPD oxidation ($S_2 + TMPD \rightarrow S_1 + TMPD^+$), yielding an estimate of a PS II unit of 200–230 chlorophylls. Partial and complete inactivation of V_{O_2} by Tris or NH_2OH gave a decrease of the S_2 component and a complementary increase of a fast approx. 600 μs component of TMPD oxidation ($Z^+ + TMPD \rightarrow Z + TMPD^+$). $CaCl_2$ extraction of the 17, 23 and 33 kDa proteins, without solubilization of Mn from PS II membranes, made the fast component predominant, indicating most (up to 80%) of the traps were disconnected from the S-states; however, such extraction also made the Mn susceptible to rapid solubilization by TMPD. Extraction of only the 17 and 23 kDa proteins (NaCl-TMF-2) caused approx. 25% disconnection of the S-state complex, approx. 50% loss of V_{O_2} and an increase of charge loss reactions. Cl^- depletion of NaCl-TMF-2 caused an increased extent of disconnection of the S-state complex and made PS II Mn susceptible to solubilization by TMPD. Reconstitution of NaCl-TMF-2 with the 17 and 23 kDa proteins abolished the approx. 25% disconnection and increased V_{O_2}, but did not abolish the charge loss path attributed to component C (Radmer, R., Cammarata, K., Tamura, N., Ollinger, O. and Cheniae, G. (1986) Biochim. Biophys. Acta 850, 21–32).

Introduction

Much of the current research on the PS II/S-state water-oxidizing complex is directed towards defining the precise functions of the PS II extrinsic peripheral 17, 23 and 33 kDa proteins (see reviews, Refs. 1–3). This research has paralleled efforts to ascertain the specific functions of the ligated functional Mn and Cl^- long known to be

Abbreviations: PS II, Photosystem II; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea: PS II/S-state complex, the Photosystem II trapping center in association with the Mn catalyst of water oxidation: TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine: TMF-2, oxygen-evolving Triton X-100 prepared Photosystem II-membrane fragments; Z, the secondary electron donor of Photosystem II; Chl, chlorophyll; S_0, S_1, S_2, S_3, S_4, transitional states of the water-oxidizing catalyst; Mes, 4-morpholineethanesulfonic acid; C, postulated endogenous electron shuttle component.

essential in the water oxidizing reactions [4–8]. Some of this research also has focused on the possible role(s) of some of these proteins in the binding of Ca^{2+}, now considered to be essential for O_2 evolution [9–13].

The effects of extraction of the 17 and 23 kDa and/or the 17, 23 and 33 kDa proteins on steady-state rates of O_2 evolution have been informative, particularly when applied with reconstitution of PS II membranes with these proteins [1–3]. Additional and more incisive insights have been gained from analyses employing short duration flashes to probe the effects of these proteins on single turnover events of the reactions associated with the oxidation of water [13–17]. Such measurements permit some discrimination between direct vs. indirect functions of the extrinsic proteins and allow identification of specific reaction(s) affected by extraction/reconstitution studies. Currently, however, appreciable controversy exists regarding the specific functions of the extrinsic proteins, particularly with respect to their requirements for obtaining the $S_1 \rightarrow S_2$ transition as determined by EPR analysis of the mutiline signal [18,19] vs. analyses by other procedures [20].

Analysis of the $S_3 \rightarrow (S_4) \rightarrow S_0 + O_2$ transitions in the 17 and 23 kDa depleted PS-II membranes by measurements of the ultraviolet absorbance change accompanying these transitions [14,16] or by direct measurements of O_2 oscillations in a train of actinic flashes [21] have yielded some rather consistent results: (1) in the presence but not the absence of a suitable PS II electron acceptor, the O_2 evolution system operates, albeit with a 2-fold increase in the α miss factor [16,21]; (2) approx. some 25% of the PS II trap/S-state complexes suffer disconnection during the extraction of the 17 and 23 kDa proteins [16]; (3) the rate of the transition, $S_3 \rightarrow (S_4) \rightarrow S_0 + O_2$, is slowed approx. 5-fold by extraction of the 17 and 23 kDa proteins, but this effect appears not to be specifically related to functions of these proteins [16]; and (4) the photooxidation of PS II artificial electron donors by a flash or continuous light is not diminished [21]. Observations 1 and 2 appear to be related to the reported increased charge recombination via P-680$^+$, the oxidized species of reaction center chlorophyll *a*, and Q_A^-, the reduced species of secondary e^- acceptor [20], and the modification of the kinetics of P-680$^+$ reduction by the secondary e^- donor, Z, resulting from extraction of the 17 and 23 kDa proteins [22].

In attempts to verify and extend our conclusions reached in Ref. 21 regarding direct vs. indirect roles of the 17 and 23 kDa proteins in O_2 evolution and to examine the role of the 33 kDa protein on the $S_1 \rightarrow S_2$ transition, we measured the kinetics of TMPD oxidation by PS II following an actinic flash. As shown by Velthuys [23], this measurement offers considerable utility in the analysis of the competency of PS II traps and the PS II trap/S-state complex.

Materials and Methods

Preparation of oxygen-evolving TMF-2 and TMF-2-depletion of the 17 and 23 and 17, 23 and 33 kDa extrinsic proteins. TMF-2 was prepared from chloroplasts from 7–9-day-old wheat seedlings [24] by modifications of procedures described in Refs. [25 and 26]. TMF-2 membranes devoid (more than 90%) of the 17 and 23 kDa polypeptides (NaCl-TMF-2) were obtained by NaCl extraction of TMF-2 during their preparation in Triton X-100 [21]. After preparation, TMF-2 and NaCl-TMF-2 were resuspended (more than 3 mg Chl/ml) in 0.4 M sucrose/15 mM NaCl/5 mM $MgCl_2$/20 mM Mes-NaOH (pH 6.2) and either used directly or stored at −80°C. Procedures for extraction of TMF-2 with NH_2OH and Tris have been described [24]. Sodium chloride-(1 M)-extracted TMF-2 was prepared by treating TMF-2 (1 mg Chl/ml) for 30 min at pH 6.2.

The extraction of the 17, 23 and 33 kDa polypeptides from TMF-2 ($CaCl_2$-TMF-2) was performed by a modification [23] of the procedure of Ono and Inoue [27].TMF-2 was extracted twice (1 mg Chl/ml for 30 min) with 0.32 mM sucrose/12 mM NaCl/1.0 M $CaCl_2$/40 mM Mes-NaOH, (pH 6.5). Following incubation, the membranes were pelleted (30000 × *g* for 15 min) and resuspended (more than 3 mg Chl/ml) in the above buffer containing only 50 mM $CaCl_2$ and were either used directly or stored at −80°C. No evidence was obtained indicating that freezing modified the properties of $CaCl_2$-, NaCl- and TMF-2 preparations.

Chloride-depleted NaCl-TMF-2 was obtained

by washing NaCl-TMF-2 with 0.4 M sucrose/50 mM Mes (pH 6.2). The NaCl-TMF-2 was diluted to approx. 300 μg Chl/ml then centrifuged (30 000 × *g* for 15 min). This step was repeated once at approx. 300 μg Chl/ml and again at approx. 600 μg Chl/ml before resuspension of the pellet (more than 3 mg Chl/ml) in 0.4 M sucrose/50 mM Mes (pH 6.2).

Spectrophotometric assay of TMPD oxidation kinetics. The spectrophotometric procedure and measuring technique used in these experiments was originally developed by Velthuys [23]. The TMPD absorbance change after a flash was measured at 570 nm as described below.

The sequence of events and the appropriate timing used in the spectrophotometric measurements is shown in scheme I. The data for each sweep was recorded in a transient recorder (Biomation 802) running in the delayed sweep mode with zero delay. The recorder was armed and triggered by a SYM-1 microcomputer. The same microcomputer was also used to coordinate the experimental protocol: during the kinetic measurement it sent out logic pulses at the proper intervals to various parts of the system. After each flash cycle the microcomputer was used to read out and store the data in the memory of the Biomation 802. After completing a prescribed number of cycles, the computer was used to subtract one curve from another or output the data directly onto a strip chart recorder or onto magnetic tape.

Lamp on $\xrightarrow{1\text{s}}$ Shutter open $\xrightarrow{15\text{ ms}}$ S/H to HOLD $\xrightarrow{3\text{ ms}}$
S/H output ON $\xrightarrow{2\text{ ms}}$ Arm and trigger recorder $\xrightarrow{30\text{ ms}}$
flash $\xrightarrow[\text{(kinetic)}]{300\text{ ms}}$ systems off, data to Sym-1

Scheme I. Sequence of events and timing for spectrophotometric assays. S/H, sample-and-hold circuit.

The final reaction mixture for the spectrophotometric measurements contained (unless otherwise noted) the reaction buffer employed in rate measurements of O_2 evolution, 1 mM TMPD, 5 μM DCMU and PS II preparation equivalent to 225 μg Chl/ml. The TMPD stock solution was made freshly daily, and added along with the DCMU to the cuvette just prior to the assay. PS II preparation was added last, in complete darkness, with the cuvette in an opaque protective holder. The cuvette with 0.2 cm light path was then shaken well to mix the reactants (final vol. of 400 μl) and placed in the spectrophotometer cuvette holder where it was allowed to incubate for 60 s prior to measurement. Care was taken to ensure that no light reached the cuvette between the time PS II preparation was added and the time of the measurement. All measurements were made at room temperature.

Preincubation with TMPD. $CaCl_2$-TMF-2 was incubated (200 μg Chl/ml for 10 min) in 0.4 M sucrose/15 mM NaCl/50 mM Mes-NaOH (pH 6.5) containing TMPD concentrations designated in the figures. Following incubation, the mixture was diluted 5-fold with the above buffer, then centrifuged (35 000 × *g* for 10 min). The resulting pellets were resuspended (approx. 40 μg Chl/ml) in 0.4 M sucrose/50 mM Mes-NaOH, (pH 6.5) then pelleted and resuspended (approx. 500 μg Chl/ml) in this same buffer before analysis of Mn abundance and rates of O_2 evolution. Rates of O_2 evolution were determined immediately after recovery of the TMPD-preincubated $CaCl_2$-TMF-2.

Preincubation of TMF-2, NaCl-TMF-2, and Cl^--depleted NaCl-TMF-2 with TMPD (see individual Figs.) was similarly performed with the following exceptions: (a) the incubation buffer was 0.4 M sucrose/50 mM mes-NaOH (pH 6.2); (b) the TMPD-HCl salt employed was freed of Cl^- just prior to use with an anion exchange resin in the OH^- form; and (c) the dilution and wash buffer was 0.4 M sucrose/15 mM NaCl/5 mM $MgCl_2$/1 mM EGTA/20 mM Mes-NaOH (pH 6.2). The final pellets were resuspended (approx. 800 μg Chl/ml) in the dilution and wash buffer without EGTA before determination of the rates of O_2 evolution and Mn abundance.

Other methods. Rate measurements of O_2 were made polarographically [24]. The procedures for Mn determinations and SDS-polyacrylamide gel electrophoresis analyses have been described [21].

The 17 and 23 kDa proteins were obtained according to Ref. 24. The crude protein fraction was either used directly for reconstitution of NaCl-TMF-2 activities [24] or purified by standard ion-exchange chromatography [28,29] before their use in the reconstitution of NaCl-TMF-2.

Reconstitution was carried out by incubating NaCl-TMF-2 (500 μg Chl) and 100 μg of the protein(s) in 1 ml of 0.4 M sucrose/50 mM Mes-NaOH (pH 6.2) for 15–30 min at 4°C. Preliminary experiments established that this amount of protein was 2-fold in excess of the amount required to obtain maximum reconstitution of rates of O_2 evolution in the standard assay buffer.

Results

TMPD oxidation kinetics, and their correlation with O_2 evolution capacity

Fig. 1 shows the kinetics of TMPD oxidation observed after the first three flashes of a TMF-2 preparation with high O_2 evolution capabilities. Note there was little appreciable TMPD oxidoreduction observed on flashes other than the first (compared to the data obtained in isolated chloroplasts [23]). This is consistent with the total lack of PS I activity (less than 1 P-700, the reaction center Chl of Photosystem I, per 3000 Chl) in these preparations and the presence of DCMU in the assays.

The data in the bottom right panel (Fig. 1) were obtained by a point-by-point subtraction of the kinetics of the third flash from that of the first. This procedure will be taken as the norm. This corrected curve shows a homogeneous first order rise in TMPD oxidation as has been reported earlier for the case of isolated chloroplasts [23]. In both cases, it can be ascribed to the reaction $S_2 + TMPD \rightarrow S_1 + TMPD^+$.

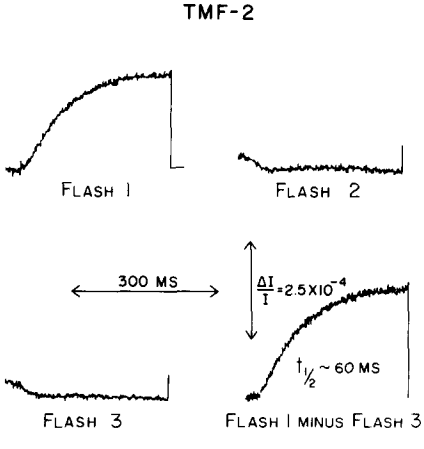

Fig. 1. Kinetics of TMPD oxidation with TMF-2.

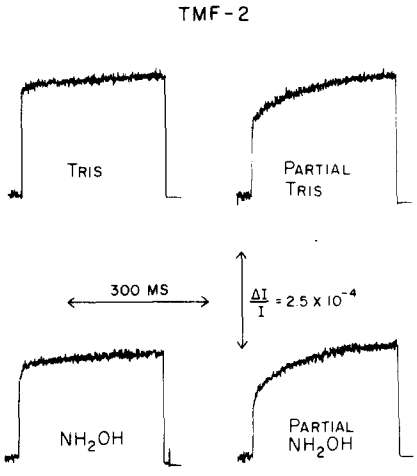

Fig. 2. Kinetics of TMPD oxidation with TMF-2 after full and partial extraction with Tris or NH_2OH.

Fig. 2 shows the TMPD oxidation kinetics observed when TMF-2 preparations were subjected to full and partial extraction by Tris or NH_2OH. Such extraction of TMF-2 with NH_2OH or Tris results in a removal of Mn and the PS II extrinsic proteins from the water oxidizing Mn-S-state complex proportionate to the extent of inactivation [24]. Additionally, the total inactivation by Tris is accompanied by complete solubilization of the 17, 23 and 33 kDa proteins [1–3,24], whereas inactivation by NH_2OH causes greater solubilization of the 17 and 23 kDa than does the 33 kDa protein [24].

As shown in Fig. 2, the TMPD oxidation kinetics were dominated by a fast ($t_{1/2}$ approx. 600 μs) kinetic phase in the fully extracted preparations. Partial extraction of TMF-2 preparations resulted in biphasic oxidation kinetics with fast and slow ($t_{1/2}$ approx 60 ms) kinetic components.

A comparison of the kinetic phases of TMPD oxidation with the oxygen evolution capability of the same preparations in continuous light is given in Table I. We note that: (1) the amplitude of the slow kinetic phase correlates with oxygen evolution capability; (2) the amplitude of the total signal – slow plus fast – remains relatively constant; and (3) the amplitude of the fast phase of TMPD oxidation observed in these extracted systems is proportional to the extent of inactivation of the S-state complex. This fast phase (less than 1

TABLE I

COMPARISON OF V_{O_2} WITH THE TWO KINETIC PHASES OF TMPD OXIDATION

From data of Figs. 1 and 2

Treatment	Normalized V_{O_2} (100 is 667 μmol O_2/mg Chl per h)	Normalized amplitudes of TMPD oxidation				
		total		fast (<1 ms)		Slow (≈ 60 ms)
		observed	predicted [a]	observed	observed/ predicted	
Unextracted	100	100	0	0	–	100
Tris-extracted	5	111	149	102	0.73	9
Partial Tris-extracted	31	111	132	70	0.77	41
NH_2OH-extracted	6	100	146	86	0.65	14
Partial NH_2OH-extracted	38	104	126	52	0.70	52

[a] The predicted values were calculated assuming $S_0/S_1/S_2/S_3$ to be 25:75:0:0, α to be 0.1 and β to be 0.05 [21] and the extent of 'disconnection' of PS II traps from the S-state complex expected based on the relative amplitude of the observed slow phase of TMPD oxidation.

ms) of TMPD oxidation presumably reflects a reaction closer to the reaction center, e.g., the reaction $Z^+ + TMPD \rightarrow Z + TMPD^+$, where Z and Z^+ are the reduced and oxidized species of the secondary e^- donor of PS II, respectively.

We observed (Table I) that the amplitude of the fast phase of TMPD oxidation in variously extracted TMF-2 preparations with residual O_2 evolution capacity was less than expected based on the remaining S_2 abundance; for example, with Tris-TMF-2 the observed fast-phase amplitude was approx. 23 to 35% less than predicted. Since both Tris and NH_2OH extraction retard P-680$^+$ reduction by Z [20,22,30–31], we hypothesize that this difference reflects an increased charge recombination (via a $Q_A^- + P\text{-}680^+ \rightarrow Q_A + P\text{-}680$ reaction, which occurs with half-times of approx. 80–900 μs [20,22,30–33] in chloroplasts and PS II membranes, respectively) competing with the fast phase of TMPD oxidation ($t_{1/2}$ approx. 600 μs). Thus, as suggested earlier [23], the TMPD-based PS II assay has the attractive feature of an apparent ability to assess the quality of a given PS II preparation with respect to O_2 evolution capability, and with parallel measurements of Mn ligated to the S-state complex, to distinguish between loss of connection between PS II traps and S-state complexes vs. inactivation of S-state complexes.

TMPD oxidation by $CaCl_2$-TMF-2

Extraction of TMF-2 with 1.0 M $CaCl_2$ causes loss of the 17, 23 and 33 kDa PS II extrinsic proteins without appreciable solubilization of Mn, but decreases O_2 evolution capacity [27]. The upper panel of Fig. 3 shows the TMPD oxidation kinetics obtained with $CaCl_2$-TMF-2 prepared from the TMF-2 preparations used in the experiments of Fig. 1. The fast phase constituted 80% of the total amplitude of TMPD oxidation by $CaCl_2$-TMF-2 and the total amplitude was 95% of that observed with TMF-2.

However, $CaCl_2$-TMF-2 displayed some unusual behavior in the presence of TMPD. First, as shown in the bottom panel of Fig. 3, the rapid

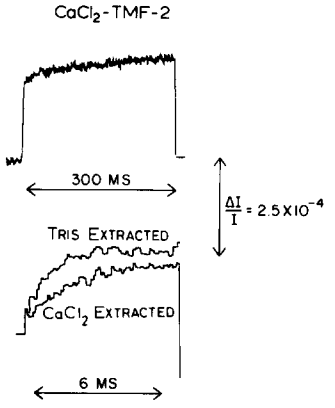

Fig. 3. Top, kinetics of TMPD oxidation with $CaCl_2$-TMF-2. Bottom, Comparison of intial phase of TMPD oxidation kinetics with $CaCl_2$-TMF-2 and Tris-extracted TMF-2.

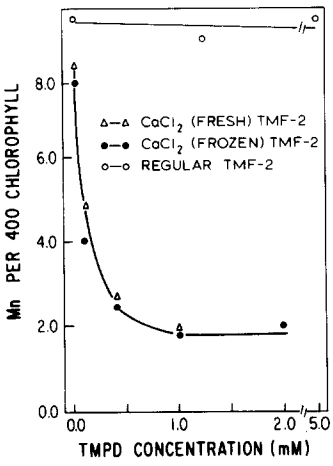

Fig. 4. Mn-extraction susceptibility to TMPD of TMF-2 and $CaCl_2$-TMF-2.

phase of TMPD oxidation (lower trace) was significantly slower ($t_{1/2}$ approx. 1000 μs) in $CaCl_2$-TMF-2 than in Tris extracted TMF-2 ($t_{1/2}$ approx. 600 μs, upper trace). In the bottom panel of Fig. 3, NH_2OH extracted TMF-2 showed kinetics equivalent to Tris extracted TMF-2 (data not shown). Second, as shown in Fig. 4, the ligated Mn in $CaCl_2$-TMF-2 is highly susceptible to extraction following brief incubation with TMPD. These data show that although $CaCl_2$ extraction of TMF-2 or subsequent preincubation and wash in 0.4 M sucrose/15 mM NaCl/50 mM Mes-NaOH (pH 6.5) caused no significant loss of Mn, preincubation of $CaCl_2$-TMF-2 in this same buffer with TMPD caused as much as an approx. 75% solubilization of the original approx. 8 Mn/400 Chl. We estimate that 1 mM TMPD at 200 μg Chl/ml would solubilize about 30% of the 8 Mn/400 Chl during the course of our TMPD assay (about 1 min).

The TMPD-induced inactivation of the S-state complex in $CaCl_2$-TMF-2 complicates interpretations of its TMPD oxidation kinetics. However, the contribution of the fast phase of TMPD oxidation (80%) is significantly greater than predicted (30%) from the kinetics of TMPD-induced inactivation of the S-state complex. We therefore conclude that $CaCl_2$ extraction of TMF-2 causes disconnection of the S-state enzyme from PS II traps in no more than 80% of the PS II trap/S-state complexes, thereby prohibiting S_1-to-S_2 transitions.

TMPD oxidation by NaCl-TMF-2

Flash measurements of TMPD oxidation kinetics by 17 and 23 kDa depleted-PS-II membranes were made in an effort to determine what functions, if any, these extrinsic polypeptides might have in primary-charge stabilization in PS II traps and in the $S_1 \rightarrow S_2$ transition.

Fig. 5 shows TMPD oxidation kinetics observed with NaCl-TMF-2 in a variety of conditions. The upper left hand panel shows the kinetics for NaCl-TMF-2 in the presence of 30 mM NaCl, a concentration sufficient to saturate the Cl^- requirements for O_2 evolution. The two striking features of this kinetic trace are: (1) the total amplitude is dominated by the slow kinetic phase (approx. 74% of total), although a small (approx. 26% of total) component of the fast kinetic phase also is observed; and (2) the total amplitude of TMPD oxidation with NaCl-TMF-2 is less than that observed with TMF-2 (Fig. 1). With 1 M NaCl extracted TMF-2 (trace not shown) very similar relative contributions of the fast and slow components to the total oxidized TMPD were observed, but in this case the total amplitude approached that observed with TMF-2 (see Table II).

The upper right hand panel shows the TMPD

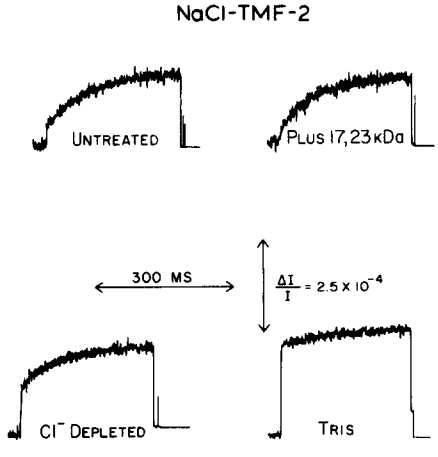

Fig. 5. Kinetics of TMPD oxidation with untreated NaCl-TMF-2, after readdition of the 17 and 23 kDa proteins, after Cl^- depletion and after Tris extraction.

TABLE II

EFFECTS FROM EXTRACTION/RECONSTITUTION OF THE 17 AND 23 kDa PROTEINS ON TMPD OXIDATION, V_{O_2} AND S_2 ABUNDANCE

Preparation	Normalized V_{O_2} (100 is 667 μmol O_2/mg Chl per h)	Normalized amplitudes of TMPD oxidation					Normalized S_2 abundance
		total		fast (< 1 ms)		slow ($\approx$ 60 ms)	
		observed	predicted	observed	observed predicted		
TMF-2	100	100	–	0	–	100	100 [a]
1 M NaCl-extracted TMF-2	57	92	101	26	0.7	65	66 [b]
NaCl-TMF-2	67	77	101	20	0.57	57	66 [b]
Reconstituted NaCl-TMF-2	80	66	88	0	0	66	88 [c]
NaCl-TMF-2, Cl-depleted, minus Cl^-	16	92	112	50	0.72	42	44 [d]
As line 5 but plus Cl^-	54	93	113	49	0.71	55	44 [d]

[a] Calculated assuming $S_0 : S_1 : S_2 : S_3$ in dark to be 25 : 75 : 0 : 0, α to be 0.1 and β to be 0.05.
[b] Calculated assuming $S_0 : S_1 : S_2 : S_3$ in dark to be 25 : 75 : 0 : 0, α to be 0.2 and β to be 0.05, and 25% disconnection of S-state enzyme from PS II traps.
[c] Calculated as in footnote b except for 0% disconnection of the S-state enzyme.
[d] Calculated as in footnote b except 50% disconnection of the S-state enzyme.

oxidation kinetics observed with 17 and 23 kDa reconstituted NaCl-TMF-2. The fast phase was completely abolished by reconstitution and the slow phase component was increased by only about 15%.

Comparison of the trace obtained with Cl^--depleted NaCl-TMF-2 (lower left panel) with NaCl-TMF-2 shows that Cl^- depletion caused: (1) an approx. 2-fold increase in the fast-phase component; (2) a decrease in the S_2 phase of TMPD oxidation; and (3) an increase in the total amplitude of TMPD oxidation relative to NaCl-TMF-2 such that the total amplitude was nearly equivalent to that observed with TMF-2. Neither readdition of Cl^- nor 17 and 23 kDa proteins to Cl^--depleted NaCl-TMF-2 affected the above characteristics of Cl^--depleted NaCl-TMF-2 (see Table II).

The anomalously low total amplitude of TMPD oxidation by NaCl-TMF-2 also was increased by Tris extraction (0.8 M, pH 8.0) (lower right panel). As with Tris-extracted TMF-2 (Fig. 2), the fast phase was predominant.

These comparisons in Fig. 5 are shown more clearly in Table II, which is a tabulation of the kinetic phases of TMPD oxidation, oxygen-evolution capability of TMF-2, NaCl-TMF-2 and TMF-2 subjected to 1 M NaCl extraction and the S_2 abundances in these preparations following a single actinic flash. All parameters have been normalized to the slow phase of TMF-2. (The parameters were chosen to yield an accurate fit to oscillations of O_2 yields in a sequence of actinic flashes measured with TMF-2 and NaCl-TMF-2 in the presence of an added electron acceptor [21]). Also tabulated are predicted values of TMPD oxidation and the observed/predicted ratio for the fast TMPD oxidation component.

The calculated abundance of S_2 correlates well with the slow phase of TMPD oxidation by TMF-2 extracted with 1 M NaCl or NaCl-TMF-2. Clearly, the $S_1 \rightarrow S_2$ transition occurs in the majority (approx. 75%) of the S-state complexes in NaCl-TMF-2. We suspect that the disconnection of about 25% of the S-state complexes from PS II traps is somehow related to the depletion of the 17 and 23 kDa proteins, since reconstitution of NaCl-TMF-2 with these proteins essentially eliminated the disconnection. However, the observed increase in S_2 abundance after reconstitu-

tion was still less than predicted (no disconnection, but α was still equal to 0.2).

The predicted S_2 abundances also were comparable to the steady-state O_2 evolving activities (measured in the presence of electron acceptors) with the exception of Cl^--depleted NaCl-TMF-2. This exception probably reflects the Cl^- requirements in the $S_2 \rightarrow S_3$ [6,7] and/or the $S_3 \rightarrow (S_4) \rightarrow S_0 + O_2$ [8] transitions.

The values of the observed/predicted ratios for the fast phase of TMPD oxidation obtained with 1 M NaCl extracted TMF-2 and Cl^--depleted NaCl-TMF-2 ($+/-$ Cl^-, Table II) are identical to those shown in Table I for Tris- or NH_2OH-extracted TMF-2. These values are less than 1.0, a result we interpret to reflect a diminished rate of P-680$^+$ reduction by Z and an increased charge recombination via $Q_A^- + \text{P-680}^+ \rightarrow Q_A + \text{P-680}$.

Effects of preincubation of NaCl-TMF-2 with TMPD on rates of O_2 evolution and abundance of Mn in S-state complex

Some of the anomalies of TMPD oxidation by Cl^- insufficient NaCl-TMF-2 possibly were a consequence of TMPD reactivity with the PS II complex similar to that observed using $CaCl_2$-TMF-2 (Fig. 4).

Fig. 6 illustrates the effects of TMPD preincubation of TMF-2, Cl^--sufficient NaCl-TMF-2, and Cl^--depleted NaCl-TMF-2 on subsequent rates of oxygen evolution. These data show a hierarchy among these preparations for loss of oxygen evolution capacity with increasing concentrations of TMPD: Cl^--depleted NaCl-TMF-2 > Cl^- sufficient NaCl-TMF-2 > TMF-2. TMF-2 suffered no loss even at 5 mM TMPD (10 min) and no loss at less than 1.2 mM TMPD during 60 min incubation. In contrast, Cl^--depleted NaCl-TMF-2 incurred approx. 80 and 55% losses of O_2 evolution capacity with 5 and 1 mM TMPD, respectively.

Fig. 7 shows results of two separate experiments (with two different preparations of Cl^--depleted NaCl-TMF-2) in which we measured oxygen evolution capacity and the Mn abundance of the S-state complex as a function of time of incubation with 2.5 mM TMPD, a concentration nearly optimal for maximal effects on these type TMF-2 (Fig. 6). These data show an initial rapid about

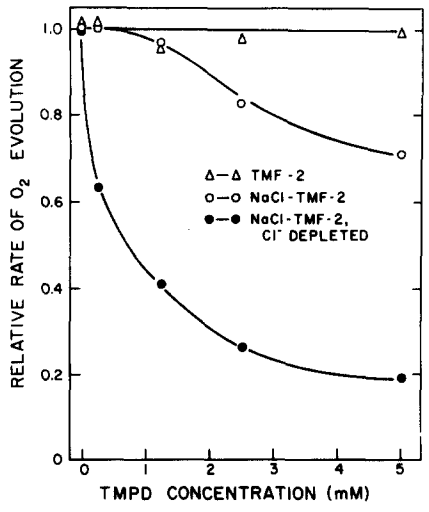

Fig. 6. O_2-evolution susceptibility to TMPD of TMF-2 and NaCl-TMF-2, Cl^--sufficient and Cl^--depleted.

15%, decrease (in less than 1 min) of both O_2 evolution capacity and functional Mn, followed by slower decreases of both these parameters. In both the rapid and slow kinetic phases, the decrease in O_2 evolution capacity is highly correlated with the decrease in functional Mn. The inset of Fig. 7, a semi-log plot of the slow phase of the loss of O_2 evolution capacity, shows that TMPD-induced de-

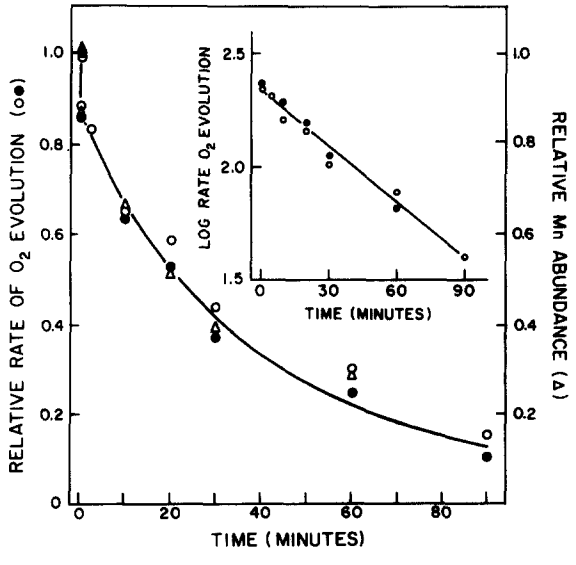

Fig. 7. O_2-evolution capability and Mn abundance of Cl^--depleted NaCl-TMF-2 after incubation with 2.5 mM TMPD.

struction of the S-state enzyme in Cl^- depleted NaCl-TMF-2 occurred with apparent first order kinetics and a half-time of approx. 27 min at 4°C with 2.5 mM TMPD. With Cl^--depleted NaCl-TMF-2 to which 15 mM NaCl was added before incubation with TMPD, we observed an 8% decrease of O_2 evolution capacity in less than 1 min but no further decrease throughout 90 min of incubation (data not shown). These data seemingly contrast to those of Ghanotakis et al. [34], who found that depletion of only the 17 and 23 kDa proteins from spinach PS II-membranes by 2 M NaCl washing modified the membranes such that the Mn-S-state enzyme became inactivated during equilibration with certain lipophilic redox reagents, even in Cl^--sufficient buffer.

Discussion

Fig. 8 is a schematic representation illustrating our interpretations of the kinetic components of TMPD oxidation by the PS II/S-state complex following a single actinic flash. Like Velthuys [23], we assign the 60 ms component to the reaction, $S_2 + TMPD \rightarrow S_1 + TMPD^+$, and the approx. 600 μs component to the reaction, $Z^+ + TMPD \rightarrow Z + TMPD^+$.

The underlying bases for these assignments rests in part on the following observations: (1) unextracted TMF-2 (≤ 1 P-700/3000 Chl, ≈ 4 Mn/200 Chl, ≥ 600 O_2/Chl per h) which yields Y_{O_2} patterns consistent with $S_0/S_1/S_2/S_3$ of 25 : 75 : 0 : 0 and α, β equal to 0.1 and 0.05, respectively, like the parent chloroplasts [21], showed only the S_2-60 ms component; and (2) the S_2-60 ms component and the Z^+-600 μs component decreased and increased, respectively, proportionate to the extents of inactivation of V_{O_2} by NH_2OH or Tris (Table I).

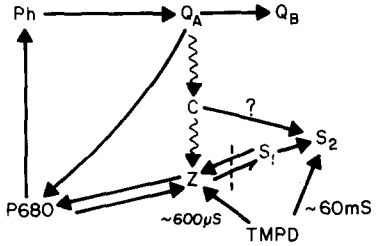

Fig. 8. Schematic representation of relevant PS II components and their relationship to TMPD oxidation.

The high correlation summarized in Tables I and II between V_{O_2} and the amplitude of the S_2-phase of TMPD oxidation permit determination of the PS II unit size in our wheat TMF-2 preparations. Using $\epsilon_{mM} = 11.8$ for $TMPD^+$ (pH 6.2), the dark distribution of the S-states and the α, β parameters deduced in Ref. 21, and the ΔI $(4.2–4.6) \cdot 10^{-3}$ observed in Fig. 1, we estimate the PS II unit for wheat TMF-2 to be 200–230 Chl. This unit size is comparable to estimates made with spinach PS II preparations but determined by other procedures [35–37].

Analysis of the kinetic phases of TMPD oxidation by the PS II/S-state complex when coupled with analysis of V_{O_2}, Y_{O_2} and the ligated Mn of PS II, therefore permit discrimination between centers inactivated (due to Mn loss) and those in which the S-state complex is disconnected from PS II traps. The data (see interpretations below) also yield information pertaining to charge loss processes in the PS II/S-state complex, and support the $Q_A^-/C/Z^+(S_2)$ pathway proposed to explain the contrasting O_2/N_2 flash yield patterns observed with TMF-2 vs. NaCl-TMF-2 in the absence of an added electron acceptor [21].

We conclude that the chemical reactivity/solubilization of PS II Mn by TMPD (or HQ) from dark equilibrated PS II membranes is modified in 17, 23 and 33 kDa-deficient and in Cl^--depleted 17 and 23 kDa-deficient PS membranes (see, however, Ref. 34). The rate of Mn solubilization at TMPD concentrations employed in the flash kinetic measurements was sufficiently high with 17, 23 and 33 kDa-deficient membranes (but not with Cl^--sufficient and Cl^--depleted 17 and 23 kDa-deficient PS II membranes) to complicate interpretations regarding any functions of these proteins in connecting PS II traps to the S-state complex [16–19]. Our data suggest that no more than 80% of the S-state complexes become disconnected during $CaCl_2$ extraction of the 17, 23 and 33 kDa proteins. Virtually complete decoupling has been reported by Toyoshima et al. [18] who analyzed the S_2-EPR multiline signal in $CaCl_2$-extracted PS II preparations. In contrast, Ono and Inoue [17] have concluded from their studies of the oscillatory behavior of thermoluminescence in

$CaCl_2$-TMF-2 that extraction of the 33 kDa protein does not appreciably prohibit the $S_0 \rightarrow S_3$ transitions; however, the $S_3 \rightarrow (S_4) \rightarrow S_0 + O_2$ transition is abolished. The underlying basis for these discrepancies is not apparent.

The analyses reported here of the effects of extraction of the 17 and 23 kDa proteins on the kinetic phases of TMPD oxidation by the PS II/S-state complex gave evidence for two seemingly unrelated effects: (1) irrespective of the two different procedures in attempts to specifically and completely extract these proteins, a disconnection of about 25% of the S-state complex occurred; the remaining 75% of the PS II/S-state complexes carried out the $S_1 \rightarrow S_2$ transition, albeit with a 2-fold increase in α [16,21]; and (2) preparation of NaCl-TMF-2 caused an apparent activation of a charge loss process resulting in less total TMPD oxidation than expected based on S_2 abudance and charge recombination by $Q_A^- + P\text{-}680^+ \rightarrow Q_A + P\text{-}680$.

The 25% disconnection (no S-state Mn loss) observed here is comparable in magnitude to results reported in Refs. 16 and 17 (see however Refs. 20 and 22) following extraction of the 17 and 23 kDa proteins. We do not ascribe any direct role of these proteins to the coupling of PS II traps with the S-state complex [22], since the extent of decoupling (25%) was not proportionate to the extent of depletion (at least 90%) of these proteins. Moreover, in some NaCl-TMF-2 preparations we observe normal S_2-multiline abundance (unpublished data). We ascribe the elimination of the 25% disconnection by reconstitution to be a consequence of alteration(s) of intrinsic components of the PS II/S-state complex but without altering the 2-fold increased α value [16,21].

We previously explained [21] the contrasting O_2/N_2 flash yields observed with TMF-2 vs. NaCl-TMF-2 preparations in terms of an activation in NaCl-TMF-2 of a PS II cyclic path shown by the wavy line of Fig. 8. Here we use this same proposed pathway to explain the deficits of total TMPD oxidation and S_2 abundance seen particularly in reconstituted NaCl-TMF-2 and less obviously in NaCl-TMF-2 (Table II). In these single flash measurements made in the presence of DCMU, we suggest that C is reduced by TMPD during dark preequilibration and that the reduction of $Z^+(S_2)$ by C^- is faster than $Z^+(S_2)$ oxidation of TMPD following the flash. We further suggest that this charge-loss process is lost following Cl^- depletion, Cl^- depletion and reconstitution and Tris extraction of NaCl-TMF-2. In these instances, the deficits of total TMPD oxidation and S_2 abundance observed with reconstituted NaCl-TMF-2, and to a lesser extent with NaCl-TMF-2 are not observed (Table II). According to these interpretations, these treatments of NaCl-TMF-2 either modify the redox potential of C such that TMPD can no longer reduce C during dark preequilibration or they cause a physical disconnection of the $C^-/Z^+(S_2)$ pathway.

Acknowledgements

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