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Studies on 17,24 kD Depleted Photosystem II Membranes[1]

I. EVIDENCES FOR HIGH AND LOW AFFINITY CALCIUM SITES IN 17,24 kD DEPLETED PSII MEMBRANES FROM WHEAT *VERSUS* SPINACH

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ABSTRACT

Analyses were made of the effects of extraction of the 17,24 kilodalton extrinsic proteins from spinach *versus* wheat photosystem II (PSII) membranes on Ca abundance and O_2 evolution capacity determined in the absence and presence of either Cl^- or Ca^{2+}. Extraction of these proteins from spinach PSII routinely diminished steady state O_2 evolution by about 70% when assayed in the presence of sufficient Cl^-. Additionally, O_2 evolution of 17,24 kilodalton-less spinach PSII membranes showed about 2-fold more enhancement by Ca^{2+} than by Cl^- during assay. When the same extraction and assay procedures were applied to wheat PSII membranes, we observed, in contrast to 17,24 kilodalton-less spinach PSII, only about 50% inhibition of O_2 evolution and about 2-fold greater enhancement by Cl^- than by Ca^{2+}. Irrespective of differences in the magnitude of enhancement of O_2 evolution by Ca^{2+} *versus* Cl^- in spinach *versus* wheat, the K_m values for Cl^- (about 1.7 millimolar) and Ca^{2+} (about 1.5 millimolar) were similar for both type preparations. The abundance of Ca specifically associated with fully functional PSII (about 2 and about 3 Ca/200 chlorophyll for spinach and wheat, respectively) was diminished to about 1 per 200 chlorophyll upon 17.24 kilodalton protein depletion. Further treatment of wheat 17,24 kilodalton-less PSII in darkness with 2 molar NaCl/1 millimolar ethyleneglycol-bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid/20 micromolar A23187[2] made O_2 evolution highly dependent on Ca^{2+} addition, much like the 17,24 kilodalton-less spinach PSII. Analyses of this Ca^{2+} effect on O_2 evolution revealed both high (K_m about 65 micromolar) and low (K_m about 1.5 millimolar) affinity Ca^{2+} sites in wheat 17,24 kilodalton-less PSII. The results suggest that during 17,24 kilodalton extraction by NaCl, spinach PSII is more susceptible than wheat PSII to loss of high affinity Ca and irreversible inhibition of O_2 evolution.

The realization of O_2 evolving everted thylakoid vesicles (1, 21) and Triton X-100 prepared PSII membranes (3, 24), also having everted membrane orientation (14, 24), has led to intensive research focused on defining the polypeptides essential for efficient functioning of the PSII/water oxidizing complex. Parallel research has been directed towards identifying which polypeptides ligate Ca^{2+}, the polynuclear Mn complex, and Cl^-, elements known to be essential for O_2 evolution (11, 13, 18).

It seems clear that the 17,24 kD PSII extrinsic polypeptides are not directly required in the ligation of the polynuclear Mn complex (11, 13, 18, 34) or for cycling of the S-states[2] of O_2 evolution (12, 35–37). It also is apparent (11, 13, 18, 19) that these polypeptides somehow modulate the concentration of Cl^- required for the $S_2 \rightarrow S_3$ (20, 25, 36, 45) or the $S_3 \rightarrow (S_4) \rightarrow S_0 + O_2$ transitions. (40). On the other hand, it is not at all clear what functions the 17,24 kD polypeptides may have on Ca ligation by the PSII/water oxidizing complex. According to Ghanotakis *et al.* (15, 16), these polypeptides promote high affinity Ca^{2+} binding in spinach PSII membranes; however, other workers (6, 7) indicate both high and low affinity Ca^{2+} effects on O_2 evolution in spinach 17,24 kD-less PSII membranes. Moreover, indirect evidence suggests that the Ca bound with high affinity in 17,24 kD depleted PSII membranes can be dissociated reversibly by either flash illumination in the presence of EDTA (12) or by continuous illumination of PSII membranes in the presence of 1 to 2 M NaCl (6, 7). According to Dekker *et al.* (12), Ghanotakis *et al.* (15), and Jansson *et al.* (21), such dissociation results in a reversible decoupling of Z from the S-state complex. In contrast, the data in Boussac *et al.* (7) suggest the dissociation results in a reversible perturbation of the $S_3 \rightarrow (S_4) \rightarrow S_0 + O_2$ transition. In either case, the loss of high affinity Ca results in diminished O_2 evolution capacity.

Early studies on effects of 17,24 kD depletion on steady state O_2 evolution by everted thylakoid vesicles and spinach PSII membranes indicated losses ranging from none (31) to 50 to 90% (4–7, 15, 16, 21, 25, 27) when determined in the presence of saturating concentrations of Cl^-. Subsequently, it was shown (6, 7, 15, 16) that addition of 10 to 20 mM Ca^{2+} to 17,24 kD-less, Cl^- sufficient spinach PSII membranes increased rates of O_2 evolution as much as 4- to 6-fold to rates sometimes nearly equivalent to those observed with unextracted membranes. However, no Ca^{2+} additions were necessary to observe substantial period-4 oscillations of the S-states with only about 25% disconnection of the S-state complexes from PSII traps (12).

Conceivably, at least some of the rather large variability in extent of loss of O_2 evolution following depletion of the 17,24 kD proteins could reflect inadvertent dissociation of high affinity Ca^{2+} during extraction. The variability might also reflect irreversible secondary perturbations to either the oxidizing or reducing ends of PSII (12, 37, 44).

With wheat PSII membranes, we have consistently observed ≤50% loss of O_2 evolution in any light regime (without Ca^{2+} additions) following extraction of the 17,24 kD proteins (9, 37). Depending on the method of extraction, either no or a maximum of about 40% disconnection of PSII traps from the S-state complex is observed (21, 35, 37, 44). The significant differences in magnitude of loss of steady state O_2 evolution accompanying extraction of the 17,24 kD proteins from everted spinach thyla-

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[2] Abbreviations: S_0, S_1, S_2, S_3, S_4, transitional states of the S-state water oxidizing catalyst; A23187, a lipophilic divalent cation ionophore; Z, the secondary electron donor of photosystem II; PBQ, phenyl-*p*-benzoquinone; VO_2, rate of O_2 evolution in saturating light; FeCN, potassium ferricyanide; LHCP, light harvesting Chl protein complex; PMSF, phenylmethylsulfonyl fluoride.

koid vesicles (1) and spinach PSII (4–7, 15, 16, 21, 25) *versus* wheat PSII membranes (9, 37) prompted the studies reported here.

We made direct comparisons of the effects of 17,24 kD extraction from spinach *versus* wheat PSII membranes on rates of O_2 evolution determined in the absence and presence of Cl^- and Ca^{2+}. Additionally, analyses were made of the effect of extractions of these polypeptides on specifically ligated PSII Ca. Our results show heterogeneous ligation among three Ca^{2+} per PSII trap/S-state complex with two of the three being required for water oxidation. A high affinity Ca^{2+} site is more conserved in 17,24 kD-less membranes from wheat than from spinach, thus offering partial explanation for some of the apparent conflict in the literature dealing with functions of the 17,24 kD proteins in the PSII trap/S-state complex.

MATERIALS AND METHODS

Preparation and Extraction of O_2 Evolving PSII Membranes. The procedures for preparation and storage of O_2-evolving PSII membranes containing PSII extrinsic proteins (TMF-2) and membranes depleted of the 17,24 kD extrinsic proteins by NaCl during Triton X-100 extraction (NaCl-TMF-2) have been described (37). Wheat (*Triticum aestivum* var Oasis) preparations were made from greenhouse grown 7 to 9 d old seedlings. Spinach (*Spinacia oleracea*) was purchased locally. Optimal yields and O_2 evolution activity were obtained using Triton:Chl(w:w) ratios of 20:1 and 15:1 for wheat and spinach, respectively.

Extraction of the 17,24 kD proteins by NaCl extraction of isolated TMF-2 was done at 0.5 mg Chl/ml using either 1 M NaCl/50 mM Mes-NaOH (pH 6.2) for durations indicated in Figure legends or 2 M NaCl/50 mM Mes-NaOH (pH 6.0) for 30 min. In the latter case, the extracted membranes were pelleted (30,000*g*/15 min) then washed once with the same extraction buffer. In both cases, the extracted membranes were washed once and resuspended (≥2 mg Chl/ml) in buffer A (0.4 M sucrose/50 mM Mes-NaOH, pH 6.2/15 mM NaCl) then used directly or after storage at −80°C.

In some cases, isolated TMF-2 (1 mg Chl/ml) was extracted for 30 min using buffer B (0.4 M sucrose/20 mM Hepes-NaOH, pH 7.5/5 mM $MgCl_2$/10 mM Na-ascorbate) and the concentrations of urea indicated in "Results." Following urea extraction, the pelleted membranes were washed once (1 mg Chl/ml) and resuspended in buffer B then assayed directly.

When Mn and/or Ca analyses were to be made, the various PSII membranes were suspended (1 mg Chl/ml) and incubated (15 min at 4°C) in buffer C (0.3 M sorbitol/15 mM NaCl/40 mM Mes-NaOH, pH 6.5/1 mM EGTA/ 20 μM A23187 prepared in doubly deionized H_2O. Following incubation and centrifugation (30,000*g*/15 min), the membranes were resuspended (>1 mg Chl/ml) in buffer C except for omission of EGTA and A23187. In such experiments and those described below, all glassware/ syringes used had been soaked in 2 N HCl then rinsed with doubly deionized H_2O.

Extraction of NaCl-TMF-2 (0.5 mg Chl/ml) with buffer D (2 M NaCl/40 mM Mes-NaOH, pH 6.5/1 mM EGTA/20 μM A23187 prepared in double deionized H_2O) was done in darkness at 4°C for 1 h. Following centrifugation, the membranes were washed three times in buffer C (A23187 omitted) before resuspension (≥2 mg Chl/ml) in buffer C (EGTA/A23187 omitted).

Reconstitution of NaCl-TMF-2 with 17,24 kD Polypeptides. NaCl-TMF-2 was washed and resuspended in 0.4 M sucrose/30 mM NaCl/5 mM Mes-NaOH (pH 6.2), then incubated (0.5 mg Chl/ml) at 4°C in darkness for ≥15 min in 0.2 M sucrose/15 mM NaCl/5 mM Mes-NaOH (pH 6.2) with saturating amounts of various protein fractions.

The wheat 17,24 kD proteins and spinach 17,24,33 kD proteins used in the reconstitution experiments were extracted from TMF-2 by 30 min incubations in 2 M NaCl (pH 6.0) (15) and 1 M Tris (pH 9.3) (46), respectively. Concentration and dialysis of crude proteins were carried out as described (42) except that all steps were performed at pH 6.2. Spinach proteins were further purified by ion exchange chromatography (1, 27) in 20 mM Na-phosphate buffer (pH 6.9) and subsequently dialyzed *versus* 5 mM Mes-NaOH (pH 6.2). Protein fractions were stored at −80°C. The degree of purity of spinach or wheat protein fractions had no effect on reconstitution of NaCl-TMF-2 provided that saturating amounts of the 17,24 kD proteins were present.

Other Procedures. Rate measurements of O_2 evolution were made polarographically in the presence of 300 μM PBQ and 1 mM FeCN as previously described (37). When Cl^- and Ca^{2+} concentration effects on V_{O_2} were determined, NaCl was omitted from the standard assay buffer (37) and replaced with the concentration of NaCl or $CaCl_2$ indicated in individual Figures. In the case of Figure 5, the indicated $CaCl_2$ concentration was supplemented with an appropriate amount of NaCl to maintain the Cl^- concentration constant at 30 mM. When V_{O_2} measurements were made with 2 M NaCl/1 mM EGTA/20 μM A23187 extracted NaCl-TMF-2, the polarograph vessel and syringes employed were washed first with 1 mM EGTA then with doubly deionized H_2O. Such extracted membranes or those preincubated (250 μg Chl/ml) in buffer C (EGTA/A23187 omitted) with designated Ca^{2+} concentrations were assayed in the standard assay mixture containing either 1 mM EGTA or 15 mM $CaCl_2$ (with omission of the NaCl in the latter case). Unless otherwise noted, all V_{O_2} values given were determined under Cl^- saturated conditions and in the absence of Ca^{2+} addition. Preliminary experiments established that any observed enhancement of V_{O_2} by Ca^{2+} was specific for this cation (15).

The polypeptide composition of PSII membranes was analyzed by SDS-PAGE on 10 to 20% gradients of polyacrylamide as described by Chua (10) using the conditions of Neville (32, 37). Relative polypeptide abundances were determined by densitometer analyses of Coomassie blue stained gels (37).

Mn was determined by flameless atomic absorption (42). Ca determinations were made by flame atomic absorption on samples subjected to total digestion (37, 42) or to hot HCl extraction essentially as described by Ghanotakis *et al.* (16). Equivalent results were obtained from both procedures. All glassware employed in the Ca/Mn analyses was acid washed (42).

RESULTS

Extraction of the 17,24 kD Proteins by NaCl/Triton X-100 and by Urea: Effects on Protein Solubilization, V_{O_2} and PSII Mn. Figure 1 shows the effects of V_{O_2}, Mn, and 17,24 kD protein abundances of PSII membranes resulting from the addition of increasing NaCl concentrations during Triton X-100 preparation of these membranes from wheat chloroplasts. The data shown were obtained from two preparations with different V_{O_2} values and different 17,24 kD abundances in the unextracted control PSII membranes. All values are normalized to the preparation yielding V_{O_2} of 544 μmol O_2/mg Chl·h (open symbols). Nevertheless, the data show that 75 and 200 mM NaCl sufficed to give half-maximal solubilization of the 17 and 24 kD proteins, respectively, independent of their initial abundances. Note also: (a) ≥ about 500 mM NaCl caused complete extraction of both proteins without extraction of PSII Mn; (b) irrespective of differences in V_{O_2} values and 17,24 kD abundances of parent membranes, the V_{O_2} values converged to an about 50% value with complete extraction of the 17,24 kD proteins; and (c) the 17 kD protein is more easily solubilized than the 24 kD protein, and extensive loss of the 17 kD species can occur with little decrease of V_{O_2}. This latter result corroborates conclusions in the literature (11, 13, 18).

A similar partial loss of V_{O_2} occurs with selective extraction of

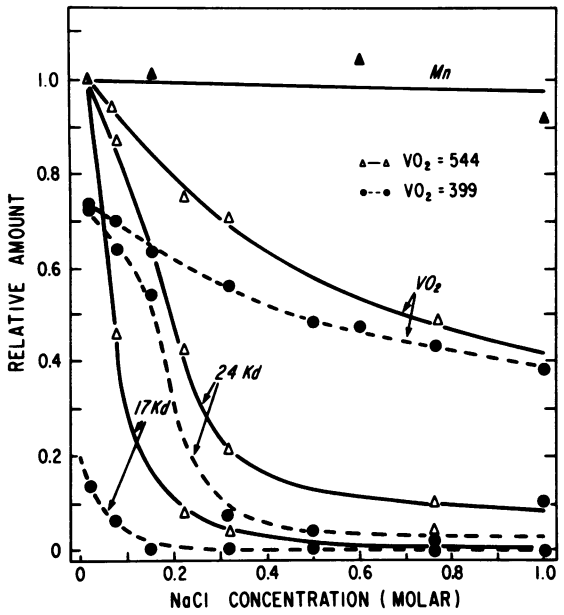

FIG. 1. Effects on V_{O_2}, PSII Mn, and 17,24 kD extrinsic protein abundances in wheat NaCl-TMF-2 prepared with increasing concentrations of NaCl. Wheat NaCl-TMF-2 was prepared (37) with the NaCl concentrations given on the abscissa. The relative abundances of the 17,24 kD proteins were determined by densitometric scanning of Coomassie blue stained SDS-PAGE profiles with normalization to the 47 kD band. V_{O_2} was determined in the standard assay buffer (37) containing 30 mM NaCl. Relative Mn abundance of 1.0 corresponds to 7 Mn/200 Chl.

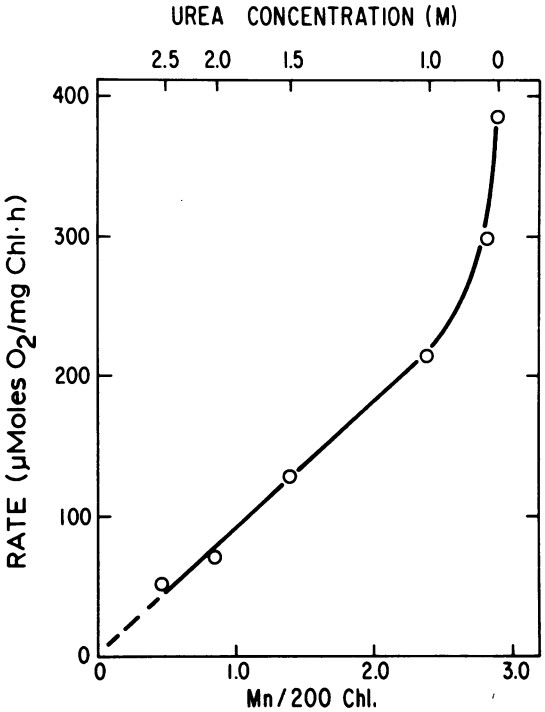

FIG. 2. Effects of V_{O_2} and Mn abundance from extraction of wheat TMF-2 with urea. Membranes were extracted ("Materials and Methods"), and then V_{O_2} was determined. SDS-PAGE analyses of membranes extracted at ≤1.0 M urea showed selective solubilization of the 17,24 kD proteins. At ≥1.0 M urea, solubilization of the 33 kD protein also occurred (see "Results" for details).

the 17,24 kD proteins from wheat TMF-2 by urea (Fig. 2). Figure 2 shows a precipitous decrease in V_{O_2} with only a small extent of solubilization of functional Mn by extraction at ≤1 M urea. With urea concentrations >1 M, the data show a decrease of V_{O_2} proportionate to the decrease of PSII Mn. Extrapolation of the two portions of the biphasic curve shows a 35% loss of V_{O_2} without any solubilization of the Mn. With about 1.0 M urea, V_{O_2} was diminished by 45% with only about 17.5% solubilization of Mn.

SDS-PAGE analyses of the urea extracted wheat TMF-2 showed: (a) at ≤1.0 M urea, the polypeptides solubilized were 17,24 kD ≫33 kD; however, the solubilization of the 17,24 kD proteins was not as complete as shown in Figure 1; and (b) at ≥1.0 M urea, increasing solubilization of the 33 kD protein occurred. Thus, we relate the precipitous partial decrease in V_{O_2} to the solubilization of only the 17,24 kD proteins and the more extensive loss of V_{O_2} to solubilization of the PSII Mn and the 33 kD protein, which are more essential for V_{O_2} than the 17,24 kD proteins (11, 13, 18).

The data of Figure 2 seemingly contrast to those of Murata *et al.* (30). They showed that extraction of spinach PSII with 2.5 M urea at pH 6.5 caused complete loss of V_{O_2} and the 33 kD protein but only about 60% and 30 to 40% solubilization of Mn and the 17,24 kD proteins, respectively. However, the experiments summarized in Figure 2 were done at pH 7.5, a condition which tends to partially solubilize both the 17,24 kD proteins and PSII Mn even in the absence of urea (unpublished).

Time-Course of Decrease of V_{O_2} of Spinach *versus* Wheat TMF-2 during Extraction of the 17,24 kD Proteins by 1 M NaCl. Figure 1 and Refs. 9, 37, and 44 indicate a limit value of about 50% decrease of V_{O_2} following complete extraction of the 17,24 kD proteins by NaCl during Triton X-100 preparation of wheat PSII membranes. These results contrast to reports (4–7, 15, 16; see however Refs. 27, 31, 35) indicating ≥70% loss of V_{O_2} following extraction of isolated spinach PSII membranes with ≥1 M NaCl.

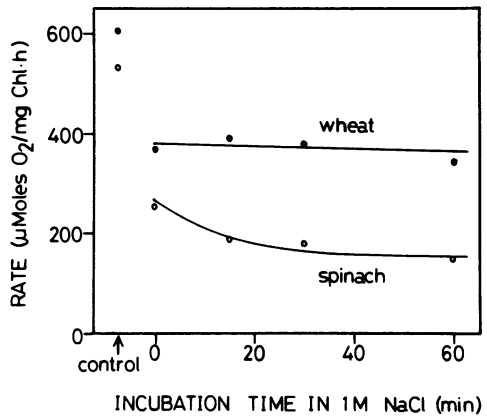

FIG. 3. Time course of decrease of rates of O_2 evolution by wheat and spinach TMF-2 subjected to 1 M NaCl extraction at pH 6.2. The times indicated on the abscissa do not include time (7 min) for centrifugation and resuspension of the extracted membranes. The standard assay for V_{O_2} was used. For other details see "Materials and Methods."

In the experiments of Figure 3, TMF-2 from wheat and spinach were subjected to parallel 1 M NaCl extractions under identical conditions for the various times shown. Almost 40% loss of V_{O_2} from wheat TMF-2 occurred in 15 min. In contrast, spinach PSII membranes showed almost 65% loss of V_{O_2} after 15 min of extraction. Moreover, no significant additional loss was observed for either type preparation with prolonged incubations (up to 2 h, data not shown). SDS-PAGE analyses of the extracted wheat and spinach membranes showed that maximal, though incomplete, solubilization of the 17,24 kD proteins occurred in both preparations after only 15 min extraction with 1 M NaCl. Simi-

Table I. *Effects of Chloride and Calcium on Vo_2 of 17,24 kD-less PSII Membranes from Spinach and Wheat*

Preparation	Rate of O_2 Evolution			Increase in Rate Due Specifically to Addition of	
	No Additions	Plus 30 mM NaCl	Plus 15 mM $CaCl_2$	30 mM Cl^-	15 mM Ca^{2+}
			μmol/mg Chl·h		
Wheat					
TMF-2	528	572	606	44	34
NaCl-TMF-2[a]	57	299	415	242	116
NaCl Extracted TMF-2[b]	54	258	369	204	111
Spinach					
TMF-2	508	538	505	30	
NaCl-TMF-2[a]	67	163	384	96	221
NaCl Extracted TMF-2[b]	33	115	247	82	132

[a] Extraction of TMF-2 with 0.5 M/0.75 M NaCl during isolation with Triton X-100 (37). [b] TMF-2 (0.5 mg Chl/ml) was extracted for 30 min in darkness with 2 M NaCl/50 mM Mes-NaOH, pH 6.0 ("Materials and Methods").

larly, maximal Cl^- and/or Ca^{2+} enhancements of Vo_2 of both wheat and spinach membranes occurred after only 15 min extraction.

Chloride and Calcium Requirements for Maximal Expression of Vo_2 in Spinach *versus* Wheat 17,24 kD-Less PSII Membranes. Table I shows the effects of Cl^- and Ca^{2+} additions of Vo_2 of wheat and spinach 17,24 kD-less PSII membranes obtained by two different procedures. Also shown are data for parent TMF-2 from which the 17,24 kD-less membranes were prepared. The lack of appreciable increase of Vo_2 by either Cl^- or Ca^{2+} additions to parent membranes corroborates previously reported results for wheat (9, 37, 44) and spinach (11, 13, 18) PSII membranes. Similarly, the very low rates of Vo_2 observed in the absence of Cl^- or Ca^{2+} additions to 17,24 kD-less PSII membranes from either wheat or spinach are consistent with reports in the literature (11, 13, 18, 44).

Note that irrespective of the procedure used for depletion of the 17,24 kD proteins from spinach, the Ca^{2+} stimulation of Vo_2 was 1.6- to 2.3-fold greater than the Cl^- stimulation of Vo_2. These observations qualitatively agree with those in Refs. 6, 7, 15, 16, and 25. In contrast, wheat 17,24 kD-less PSII membranes show a Cl^- stimulation of Vo_2 which is 1.8- to 2.1-fold greater than that by Ca^{2+}. Indeed, in the presence of sufficient Cl^-, Vo_2 of wheat 17,24 kD-less PSII membranes was typically increased by only 15 to 30% by Ca^{2+}. The contrasting responses of Vo_2 of spinach *versus* wheat 17,24 kD-less PSII membranes to Cl^- *versus* Ca^{2+} were consistently observed in many experiments like those shown in Table I. The ratio of increase of Vo_2 specifically by Ca^{2+} relative to the increase specifically by Cl^- was 2:1 and 1:2 for 17,24 kD-less spinach and wheat PSII membranes, respectively, and Vo_2 by spinach (with/without 17,24 kD proteins) was always less than by wheat (with/without 17,24 kD proteins) irrespective of assay conditions. The larger observed enhancement of Vo_2 by Ca^{2+} with 17,24 kD-less spinach conceivably could reflect a greater Ca^{2+} dependent rate limitation of Vo_2 in spinach *versus* wheat thereby diminishing the magnitude of Cl^- stimulation of Vo_2 in spinach membranes.

Figure 4 shows the Cl^- concentration dependency of Vo_2 for wheat and spinach NaCl-TMF-2. Despite the difference in magnitude of NaCl enhanced Vo_2 for spinach *versus* wheat membranes, both preparations were enhanced maximally by 30 mM NaCl. The NaCl concentrations for half-maximal effect were very similar (1.8 and 2.8 mM NaCl for wheat and spinach 17,24 kD-less PSII membranes, respectively).

Figure 5 is a plot of the Ca^{2+} concentration dependency of Vo_2 of wheat and spinach 17,24 kD-less PSII membranes. Again, despite the observed differences in magnitude of Ca^{2+} enhanced Vo_2 for spinach *versus* wheat membranes, the $CaCl_2$ concentrations required for half-maximal and maximal effects proved to be 1.5 and 15 mM, respectively, for both preparations. These values are consistent with those previously reported for 17,24 kD-less spinach PSII membranes (6, 15, 16, 28).

The similar NaCl and $CaCl_2$ concentration dependencies for

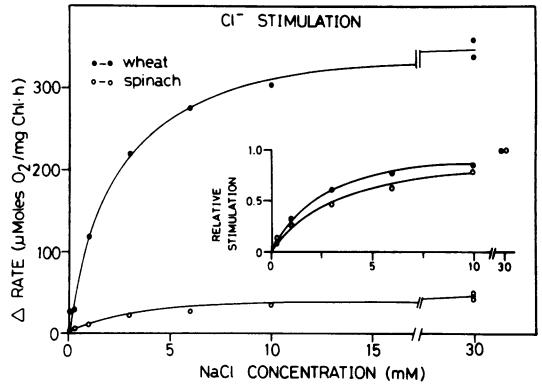

FIG. 4. NaCl concentration dependency of stimulation of Vo_2 for wheat and spinach NaCl-TMF-2. Vo_2 was determined in the standard assay buffer using the concentrations of NaCl indicated on the abscissa. Inset: Results of the main figure normalized to rates measured in the presence of 30 mM NaCl. Residual Vo_2 in the absence of NaCl (39 and 31 μmol O_2/mg Chl·h for wheat and spinach NaCl-TMF-2, respectively) was subtracted from each measurement.

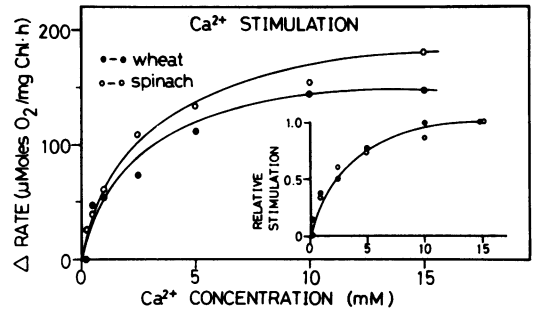

FIG. 5. Ca^{2+} concentration dependency of stimulation of Vo_2 for wheat and spinach NaCl-TMF-2. Vo_2 was determined in the standard assay buffer containing the Ca^{2+} concentration (as $CaCl_2$) indicated on the abscissa. Sufficient NaCl was included during assay to maintain a 30 mM Cl^- concentration. Inset: Results of the main figure normalized to rates measured in 15 mM $CaCl_2$. Residual Vo_2 with 30 mM NaCl only (392 and 97 μmol O_2/mg Chl·h for wheat and spinach NaCl-TMF-2, respectively) was subtracted from each measurement.

stimulation of V_{O_2} imply that Cl^-/Ca^{2+} affect mechanisms common to both wheat and spinach PSII membranes. The differing magnitudes of Cl^- *versus* Ca^{2+} stimulation of V_{O_2} in spinach *versus* wheat 17,24 kD-less PSII membranes imply: (a) differing extents of solubilization/perturbation of perhaps the 33 kD extrinsic protein (23, 26, 29, 33–35), or (b) greater depletion from spinach than from wheat 17,24 kD-less PSII membranes of the PSII Ca required for connecting the PSII/S-state complex (12, 15) or for the $S_3 \rightarrow (S_4) \rightarrow S_0 + O_2$ transition (7).

SDS-PAGE Analyses of Wheat *versus* Spinach PSII Membranes and Reconstitution Analyses of V_{O_2} with the 17,24 kD Extrinsic Proteins. Figure 6 shows the polypeptide composition of the various type preparations used in the analyses of Table I. Comparison of lane 1 *versus* lane 4 (spinach *versus* wheat TMF-2) reveals greater complexity of bands in the 21 to 24 kD region with spinach than with wheat TMF-2, and different profiles in the LHCP region (about 25–30 kD). The greater complexity in spinach TMF-2 also was observed when prepared from freshly picked leaves in the presence of protease inhibitors (0.4 mM PMSF, 2 mM benzamidine, 2 mM ϵ-amino caproic acid).

Lanes 2 and 3 (spinach) and lanes 5 and 6 (wheat) show the polypeptide profiles of NaCl-TMF-2 and 2 M NaCl extracted TMF-2, respectively. Inspection of these lanes *versus* lanes 1 and 4 (unextracted) reveals: (a) both procedures effectively solubilized major bands in the 17 and 24 kD region as well as proteins of less abundance in the 10 to 20 kD region, (b) neither procedure effected any apparent solubilization of the 33 kD extrinsic protein from either spinach or wheat PSII membranes, (c) NaCl-TMF-2 type preparations were more extensively depleted of the 17 and 24 kD and low abundance polypeptides than 2 M NaCl extracted TMF-2, and (d) spinach TMF-2 shows an about 24 kD band (not observed in wheat TMF-2), the removal of which cannot be correlated with results in Table I and Figures 3 to 5. The NaCl extractable spinach protein which does correlate with the Cl^-/Ca^{2+} effects on V_{O_2} has an SDS-PAGE mobility corresponding to 23 kD *versus* 24 kD for the corresponding wheat protein. Nevertheless, as shown in Table II, the 17,24 kD PSII polypeptides from wheat *versus* spinach are functionally interchangeable for the reconstitution of V_{O_2} while simultaneously decreasing the Cl^-/Ca^{2+} requirements relative to 17,24 kD-less PSII membranes. We thus find no evidence in the data of Figures 3 to 5 and Table I which would support a supposition relating the observed differences in magnitudes of Cl^- and Ca^{2+} enhancement of V_{O_2} by wheat *versus* spinach 17,24 kD-less membranes to differences in their polypeptide composition.

PSII Ca Abundance of Wheat *versus* Spinach PSII Membranes. Table III shows typical results from the effects of NaCl and $CaCl_2$ additions on V_{O_2} of TMF-2, NaCl-TMF-2, and NaCl-TMF-2 additionally extracted with 2 M NaCl/EGTA/A23187. Also shown are results from experiments in which specifically

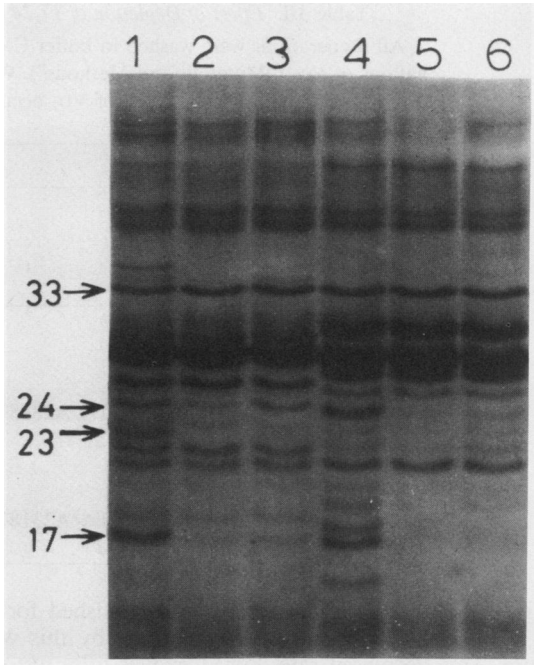

FIG. 6. SDS-PAGE analyses of TMF-2, 1 M NaCl-TMF-2, and 2 M NaCl-washed TMF-2 from wheat and spinach. Lanes 1, 2, and 3 are TMF-2, 1 M NaCl-TMF-2, and 2 M NaCl-washed TMF-2 of spinach, respectively. Lanes 4, 5, and 6 are the corresponding preparations from wheat. Samples equivalent to 75 μg protein were solubilized as described in Radmer *et al.* (37).

bound Ca was measured. The Ca/200 Chl abundances represent Ca abundances in membranes following their washing in buffer C containing 1 mM EGTA and 20 μM A23187 ("Materials and Methods") to remove any nonspecifically bound Ca. Such washing of TMF-2 type preparations from either wheat or spinach did not diminish V_{O_2} values determined either in the absence or presence of Ca^{2+}. Note the low Ca abundance of about 2.9 and 1.8 Ca/200 Chl for wheat and spinach TMF-2, respectively. These values are appreciably less than the abundance of about 168 Ca/200 Chl (15) and a higher value (31) reported previously for spinach PSII membranes.

As shown, wheat and spinach NaCl-TMF-2 not subjected to EGTA/A23187 washing before assay of V_{O_2} showed 1.4- and 2.4-fold enhancement, respectively, by Ca^{2+} addition to assays. The EGTA/A23187 washing of either wheat or spinach NaCl-TMF-2 (not the 2 M NaCl/EGTA/A23187 extraction shown on line 3, Table III A/B) did not diminish the absolute rates of O_2 evolution determined in the presence of Ca^{2+}. However, V_{O_2}

Table II. *Effects on V_{O_2} of Reconstitution of Wheat and Spinach NaCl-TMF-2 with 17,24 kD Proteins*

NaCl-TMF-2 Source	Protein Addition Source of 17,24 kD Proteins[a]	Rate of O_2 Evolution[b]		
		No additions	Plus 30 mM NaCl	Plus 15 mM $CaCl_2$
		μmol/mg Chl·h		
Wheat	None	53	316	482
	Wheat	369 (316)[c]	376 (60)	503 (21)
	Spinach	310 (257)	355 (39)	482 (0)
Spinach	None	47	105	280
	Wheat	265 (218)	224 (119)	283 (3)
	Spinach	256 (209)	236 (131)	314 (34)

[a] See "Materials and Methods" for details of protein isolation and reconstitution of NaCl-TMF-2. [b] V_{O_2} was determined in the standard assay buffer and, where noted, with addition of 30 mM NaCl or 15 mM $CaCl_2$. [c] Numbers in parentheses represent increase in V_{O_2} over no addition of proteins under the various assay conditions.

Table III. *Effect of Depletion of 17,24 kD Proteins and PSII Ca on Enhancement of V_{O_2} by Ca^{2+}*

All preparations were washed in buffer C containing 1 mM EGTA and 20 μM A23187 following determinations of V_{O_2} ("Materials and Methods"). Without this wash of wheat or spinach TMF-2, 12 to 15 Ca/200 Chl were observed. No decrease of V_{O_2} occurred by decrease of this Ca abundance to the levels shown for TMF-2.

Preparation		Rate of O_2 Evolution			
Source	Type	plus 30 mM NaCl	plus 15 mM $CaCl_2$	Increase by Ca^{2+} over 30 mM NaCl	Ca/200 Chl
		μmol/mg Chl·h			*ratio*
A. Wheat	1. TMF-2	746	747	+1	2.92 ± 0.98
	2. NaCl-TMF-2	299	415	+116	1.10 ± 0.20
	3. 2 M NaCl/EGTA/A23187 NaCl-TMF-2	88	422	+334	0.83 ± 0.07
B. Spinach	1. TMF-2	620	605	−15	1.83 ± 0.43
	2. NaCl-TMF-2	163	384	+221	1.05 ± 0.35
	3. 2 M NaCl/EGTA/A23187 NaCl-TMF-2	41	248	+207	0.95 ± 0.25

determined in the absence of Ca^{2+} was diminished for wheat NaCl-TMF-2 (but not spinach NaCl-TMF-2) by this washing procedure. This decreased rate, however, was fully restored by Ca^{2+} additions to assays. The magnitude of this Ca^{2+} effect is about 2-fold greater than shown in Table III for wheat NaCl-TMF-2 not washed with EGTA/A23187. The EGTA/A23187 washing of both spinach and wheat membranes depleted of the 17,24 kD polypeptides diminished the Ca abundance to only about 1 Ca/200 Chl, a value which is appreciably less than the previously reported about 104 Ca/200 Chl for 17,24 kD-less spinach PSII membranes (15). Apparently, in Refs. 15 and 31 adventitious as well as specifically ligated PSII Ca was measured. If our determinations in Table III adequately discriminate between nonspecifically and specifically ligated Ca, then we conclude only about 2 Ca/PSII reaction centers are susceptible to release following 17,24 kD protein solubilization from wheat TMF-2 and that these 2 Ca/PSII are required for either coupling of the PSII trap to the S-state complex (12, 15) and/or for the $S_3 \rightarrow (S_4) \rightarrow S_0 + O_2$ transition (7).

Finally, additional exhaustive dark extraction of wheat NaCl-TMF-2 with EGTA/A23187 in the presence of 2 M NaCl diminished V_{O_2} in the absence of Ca^{2+} addition and invoked a large (about 4.8-fold) enhancement of V_{O_2} by Ca^{2+}. The Ca^{2+} enhanced rate of O_2 evolution was equivalent to that of parent NaCl-TMF-2, indicating complete recovery from the effects of the additional 2 M NaCl/EGTA/A23187 extraction. With spinach NaCl-TMF-2, the same extraction also decreased V_{O_2} in the absence of Ca^{2+} addition, but the enhanced rate was about 36% less than the rate observed prior to the 2 M NaCl/EGTA/A23187 extraction; moreover, the absolute magnitude of the stimulation of V_{O_2} by Ca^{2+} was not increased. Apparently, the additional 2 M NaCl/EGTA/A23187 extraction of spinach NaCl-TMF-2 caused some secondary irreversible effects on the PSII/S-state complex which did not occur with the same extraction of wheat NaCl-TMF-2.

Evidence for High and Low Affinity Ca Sites in PSII. Figure 7 shows data obtained with wheat NaCl-TMF-2 which had been subjected to the 2 M NaCl/EGTA/A23187 extraction. The lower curve was obtained by preincubation (60 min at 4°C in darkness) of the 17,24 kD-less membranes (260 μg Chl/ml) with the Ca^{2+} concentrations shown before V_{O_2} determinations in the presence of 1 mM EGTA to chelate any Ca^{2+} not bound with high affinity. Subsequently, the preincubated samples were reassayed in the presence of 15 mM Ca^{2+} (upper curve).

The lower curve reflects stimulation of V_{O_2} from religation of Ca^{2+} at a high affinity site (K_m about 65 μM) and shows saturation at 250 μM, values very similar to those obtained by Boussac *et al.* (6, 7) with spinach PSII membranes depleted of Ca^{2+} and the 17,24 kD polypeptides by light incubation in 2 M NaCl then dark incubation in EGTA.

The upper curve reflects effects from a combination of Ca^{2+} binding at high and low affinity sites dependent on the extent of saturation of the high affinity sites during the preincubation and/or during the 45 s equilibration time of membranes with 15 mM $CaCl_2$ in the polarograph. (Ca^{2+} binding at the high affinity site is time-dependent [$t_{1/2}$ > 45 s] while the binding at the low affinity site is rapid [<10 s]. These behaviors contribute to the unusual shape of the upper curve.) In experiments not shown, we evaluated the K_m of the low affinity Ca^{2+} binding site in 2 M NaCl/EGTA/A23187 extracted membranes after religation of Ca^{2+} to the high affinity site and obtained a K_m of about 1.5 mM Ca^{2+}. This value is consistent with those obtained independently in Figure 5 and Refs. 6, 15, 16, and 28.

Figure 7, inset, is a plot of the increase in V_{O_2} due to religation of Ca^{2+} at the high affinity site in the 2 M NaCl/EGTA/A23187 extracted 17,24 kD-less wheat PSII membranes relative to the increase of V_{O_2} due to religation of Ca^{2+} at both the high and low affinity sites. This plot shows that the maximal enhancement of V_{O_2} from religation of Ca^{2+} at the low affinity site is only about 1.85-fold, a smaller relative increase than the 5.4-fold enhancement resulting from religation of Ca^{2+} at only the high affinity site (Fig. 7, main figure). However, the absolute magnitudes of V_{O_2} enhancement by Ca^{2+} at the high and low affinity sites are approximately equivalent.

These analyses (Fig. 7) of the Ca^{2+} enhancement of V_{O_2} permit estimates of the extent of depletion of the high and low affinity Ca from wheat 17,24 kD-less membranes not subjected to the additional 2 M NaCl/EGTA/A23187 extraction (Table I). The NaCl-TMF-2 and 2 M NaCl extracted TMF-2 from wheat showed 1.39- and 1.43-fold increase in V_{O_2}, respectively, from addition of 15 mM Ca^{2+} to the Cl^- sufficient assay buffer. Comparison of the results in Table I with those in Figure 7 suggests that both the NaCl-TMF-2 and 2 M NaCl extracted TMF-2 from wheat contained all the high affinity Ca and a significant fraction of the low affinity Ca despite complete or near complete depletion of the 17,24 kD proteins. This same comparison but with spinach NaCl-TMF-2 and 2 M NaCl extracted TMF-2 suggests that these membranes, in our hands, are completely depleted of the low affinity Ca and partially depleted of the high affinity Ca.

Our analyses imply that some significant fraction of the high affinity Ca site of our spinach NaCl-TMF-2 was irreversibly modified. Indeed, preincubations (30 min) of spinach NaCl-TMF-2 with 1 mM $CaCl_2$ to populate any high affinity Ca site

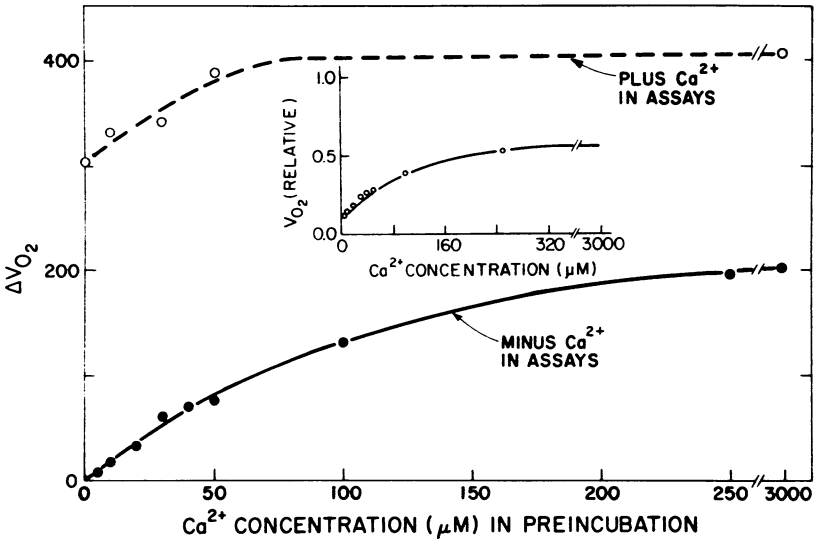

FIG. 7. Effect on V_{O_2} from the preincubation of 2 M NaCl/EGTA/A23187 extracted wheat 17,24 kD-less PSII membranes from $CaCl_2$. NaCl/EGTA/A23187 extracted wheat NaCl-TMF-2 (2 M) was preincubated (60 min at 4°C) with the $CaCl_2$ concentrations shown before V_{O_2} assays in the standard assay buffer containing 1 mM EGTA (solid symbols) then in standard assay buffer containing 15 mM $CaCl_2$ (open symbols). The inset records the increase in V_{O_2} resulting from preincubation only relative to the maximum observed increase in V_{O_2} resulting from both the preincubation and the assay in the presence of 15 mM $CaCl_2$. V_{O_2} is expressed as μmol O_2/mg Chl·h.

(Fig. 7) were largely unsuccessful yielding an increase of V_{O_2} of only ≤90 μmol O_2/mg Chl·h. This maximum value contrasts to an increase of about 200 μmol O_2/mg Chl·h observed on population of the high affinity Ca site in wheat NaCl-TMF-2 (Fig. 7).

DISCUSSION

It is apparent that the 17,24 kD extrinsic proteins of the PSII reaction center/water oxidizing complex are not absolutely essential for charge stabilization by the reaction center (11–13, 18, 35, 37, 44), the coupling of the reaction center to the S-state water oxidizing complex (12, 35, 37, 44), and the transitions from $S_0 \rightarrow S_4$ leading to O_2 evolution (6, 7, 12, 35, 37). It is equally apparent that the procedures for extraction of these proteins from Triton-prepared PSII membranes result in some secondary modifications of the above mentioned reactions manifested even following reconstitution of extracted membranes with these proteins and/or the addition of excess Cl^- and Ca^{2+} (4–7, 12, 13, 18, 35, 37, 44). The literature also agrees that depletion of these polypeptides modifies the Cl^- concentration requirements for O_2 evolution (11, 19, 36). On the other hand, the influence of the 17,24 kD proteins on Ca^{2+} binding to the PSII/S-state complex and the effect on specific PSII/S-state complex reactions resulting from depletion of the 17,24 kD proteins and Ca^{2+} from spinach PSII membranes remain controversial.

Qualitatively, our steady state V_{O_2} determinations made with spinach PSII membranes depleted of the 17,24 kD proteins by two different NaCl extraction procedures verify partially the results/conclusions obtained previously with 17,24 kD-less spinach PSII membranes (15, 16). Namely, they show (Figs. 3–5; Tables I–III) a ≥70% loss of V_{O_2} at quantum yield (data not shown) and high light intensities in Cl^- sufficient conditions and a significant increase (about 2.3-fold) in V_{O_2} by Ca^{2+} addition. This ≥70% loss of V_{O_2} is appreciably greater than predicted from flash induced UV-absorbance changes of S-state transitions (12) or measured by O_2 flash yields with 17,24 kD-less spinach PSII membranes in the absence of Ca^{2+} and without EDTA (35).

In contrast, only about 50% loss of V_{O_2} (measured in continuous light at either quantum yield or saturating light intensity, or in a train of actinic flashes and also in absence of Ca^{2+} addition) is observed on 17,24 kD protein depletion from wheat PSII membranes (37). These observations are very similar to those reported recently by Ono and Inoue (35) with 17,24 kD-less spinach PSII membranes in the presence of EDTA. Ca^{2+} addition to our wheat 17,24 kD-less PSII membranes yielded only minimal (about 30%) increase of V_{O_2} (Figs. 3–5; Tables I–III), and similarly, Ca^{2+} addition to EDTA preincubated 17,24 kD-less spinach PSII membranes of Ono and Inoue (35) gave only about 41 to 48% increase of V_{O_2} measured in continuous or flash illumination.

The following observations made here in parallel experiments with wheat and spinach 17,24 kD-less PSII membranes give some rationale for the contrasting differential requirements of V_{O_2} for Ca^{2+} and Cl^-, and for the widely differing dependency of V_{O_2} on Ca^{2+} reported with spinach membranes (4–7, 12, 15, 16, 25, 28, 31, 35). First, the contrasting behaviors between wheat and spinach 17,24 kD-less PSII membranes are unrelated to either an observed difference in the mobility of the 24 kD polypeptide or wheat *versus* spinach PSII membranes (Fig. 6) or to the presence in spinach but not wheat 17,24 kD-less PSII membranes of an additional polypeptide in the 24 kD region of SDS-PAGE gels. This conclusion is supported by data showing that the 17,24 kD proteins from either source are equally effective and interchangeable for reconstitution of V_{O_2} of spinach *versus* wheat 17,24 kD-less PSII membranes (Table II). Similarly, the differences are not due to a differential depletion of the 33 kD extrinsic protein which is required for expression of catalytic activity of the tetra-Mn complex (23, 24, 26, 29, 30, 33–35, 43) but partially replaceable by high Ca^{2+}/Cl^- concentrations (23, 29, 33, 35). Data supporting this conclusion are: (a) no observed differences in the relative abundance of the 33 kD protein in wheat *versus* spinach 17,24 kD-less PSII membranes; and (b) 33 kD 'reconstitution' of the 17,24 kD-less wheat or spinach PSII membranes did not modify the differential responses to added Cl^- and Ca^{2+}.

Second, despite the observed large differential enhancements of V_{O_2} by added Cl^- or Ca^{2+} to the wheat *versus* spinach 17,24 kD-less PSII membranes, no differences in $K_m^{Cl^-}$ or $K_m^{Ca^{2+}}$ were discerned between the two preparations. This result is compatible with common Ca^{2+} or Cl^- effects on V_{O_2} rate constraints in the 17,24 kD-less membranes and an apparent absence of Ca^{2+}/Cl^- interactions in the membranes.

Third, either extraction of wheat 17,24 kD-less PSII membranes with 2 M NaCl/EGTA/A23187 in darkness or with buffer

A plus 1 mM EGTA in a train of actinic flashes (data not shown) conferred marked dependency of V_{O_2} on added Ca^{2+} (Fig. 7). Kinetic analyses of this Ca^{2+} effect(s) gave clear evidence for both high (K_m about 65 μM) and low (K_m about 1.5 mM) affinity Ca sites in the 17,24 kD-less wheat PSII membranes. Very similar results have been obtained with spinach 17,24 kD-less PSII membranes obtained by NaCl washing of parent membranes in room light (6, 7). The observations (Table III) made with 17,24 kD containing PSII membranes showing no loss of V_{O_2} accompanying depletion of PSII Ca to a limit value of about 3 Ca/200 Chl by EGTA/A23187 tend to support the hypothesis invoking some function of 17,24 kD proteins in Ca binding by PSII membranes (16). Because the K_m value for the high affinity Ca site in both wheat and spinach 17,24 kD-less PSII membranes is about 5-fold less than the high affinity Ca site created by reconstitution of spinach 17,24 kD-less PSII membranes with these proteins (16), we accept the application of the hypothesis in Ref. 16 to our data only if such reconstitution decreased the K_m of the high affinity Ca site in 17,24 kD-less PSII membranes to a value of <65 μM.

In our hands, however, a high affinity Ca site is more stable in 17,24 kD-less PSII membranes from wheat than from spinach. The observation that the Triton/Chl ratio required for optimization of V_{O_2} (and yield of membranes) in the isolation of the membranes is greater with wheat than with spinach chloroplasts suggests differing integrity of membranes from the two sources. Based in part on data of Table III showing that Cl^-/Ca^{2+} enhancements of V_{O_2} by 17,24 kD-less PSII membranes from spinach *versus* wheat become similar only after a reversible depletion of Ca from the high affinity site in wheat 17,24 kD-less PSII membranes (Fig. 7), we conclude that Ca from this site in our spinach 17,24 kD-less PSII membranes has been dissociated during preparation possibly as a consequence of modification of the site, and/or that Ca at the site became dissociated during assay. We favor the first alternative based on the fact that V_{O_2} of our spinach 17,24 kD-less PSII membranes, in contrast to those of Boussac *et al.* (6, 7), was not increased significantly following preincubation with 1 mM Ca^{2+} (*cf.* with data for wheat 17,24 kD-less PSII membranes, Fig. 7).

We concur with the conclusions reached in Refs. 4–7, 12, 13, 18, 35, 37, and 44. Namely, depletion of the 17,24 kDa proteins unavoidably leads to some alteration(s) of the PSII/S-state microenvironment which cannot be totally reversed either by Ca^{2+} additions or reconstitution of membranes. The evidences are: (a) with wheat 17,24 kDa-less PSII membranes containing full abundance of the tetra-Mn complex, occurrence of high affinity Ca site(s), and sometimes unaltered abundance of the S_2-multiline EPR signal (37, 44), never have we observed V_{O_2} > about 70% of unextracted controls in assays sufficient in both Cl^- and Ca^{2+}; and (b) never have we been able to fully reconstitute V_{O_2} to control unextracted rates even though the reconstitution eliminates the sometimes observed 25 to 40% decoupling of PSII traps from S-state complexes in the extracted membranes (37). However, the presence of $CaCl_2$ during KCl extraction of the 17,24 kD proteins from spinach PSII membranes (25) apparently partially stabilizes the high affinity Ca site or the structural microenvironment required for efficient PSII/S-state functioning as evidenced by a diminished loss of V_{O_2} (about 90% *versus* about 50%) and a diminished enhancement of V_{O_2} by Ca^{2+} addition to assays.

Thus, in our data it is clear that the high affinity Ca site in 17,24 kD-less PSII membranes from wheat is more stable than the site in similar spinach membranes. The properties of the 17,24 kD-less wheat membranes conferring this greater stability remain unknown, but such properties may be the underlying differences between wheat (42) *versus* spinach (17) 17,24 kD-less PSII membranes in the reactivity of their water oxidizing tetra-Mn complex with lipophilic reducing agents. These different membrane properties may be distinct from the general effects of the 17,24 kD proteins on the microenvironment of the PSII/S-state complex and its Ca binding sites. These general effects are reflected in data showing reversible modification of the reactivity of the tetra-Mn complex with NH_2OH/NH_2NH_2 following 17,24 kD protein extraction/reconstitution (42), the modifications of Ca^{2+} binding by reconstitution of spinach 17,24 kD-less PSII membranes (16; confirmed in data not shown), the about 50% decrease of O_2 flash yields by EDTA addition to 17,24 kD-less spinach PSII membranes (35), and the reconstitution induced recoupling of any decoupled PSII trap/S-state 17,24 kD-less PSII (44).

Our data indicate only about 3 Ca per PSII trap/S-state complex are required for complete coupling between these complexes and advancement of S-states characteristic of the Kok (22) kinetic model of O_2 evolution. This conclusion is based on data obtained with wheat TMF-2 which consistently showed greater V_{O_2} and Ca abundance than spinach TMF-2 (Table III). This result implies highly specific loci of Ca ligation despite the numerous potential sites of ligation by *e.g.* carboxylic groups among the PSII extrinsic polypeptides (11, 13, 18). We tentatively assign about 2 Ca to the S-state complex and about 1 Ca to the PSII trap based on the following: (a) depletion of the 17,24 kD polypeptides by 2 M NaCl in the presence of EGTA/A23187 or washing of 17,24 kD-less membranes with EGTA/A23187 decreases Ca abundance from about 3 to about 1 Ca per reaction center (Table III) without depletion of the tetra-Mn complex; (b) depletion of the 17,24 kD and the 17,24,33 kD polypeptides by NH_2OH and Tris, respectively, along with depletion of the tetra-Mn complex also decreases Ca abundance to about 1 Ca per reaction center (43); and (c) more rigorous extractions (pH ≤2, 70°C heating), yielding loss of PSII donor photooxidations, eliminates the about 1 Ca per reaction center (data not shown). This latter observation may be relevant to observations obtained with cyanobacteria showing decoupling of P680 from Z following extensive depletion of Ca from cells (2, 8) or PSII membranes (38, 39). (After submission of this manuscript, a publication appeared [T Ohno, K Satoh, S Katoh 1986 Biochim Biophys Acta 852: 1–8] indicating only 1 Ca/PSII Reaction Center is essential for O_2 evolution by cyanobacterial membranes. The basis for the differences between the results/conclusions obtained with cyanobacteria membranes *versus* those here and in Refs. 6, 7, 12, 15, 16 but with PSII membranes from higher plants is not clear.)

Clearly, the ligation of Ca^{2+} in PSII membranes from wheat and spinach chloroplasts had heterogeneous properties. Such heterogeneity of Ca^{2+} ligation is observed in wheat 17,24 kD-less PSII membranes which apparently lack the 13 to 15 kD Ca binding protein isolated from spinach PSII membranes (41); thus, the chemical nature of the ligands determining heterogeneity of Ca ligation in higher plant PSII membranes remain unknown.

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LITERATURE CITED

1. ÅKERLUND H-E, C JANSSON, B ANDERSSON 1982 Reconstitution of photosynthetic water splitting in inside-out thylakoid vesicles and identification of a participating polypeptide. Biochim Biophys Acta 681: 1–10
2. BECKER DW, JJ BRAND 1984 A calcium/sodium requirement near reaction center II in *Anacystis nidulans*. *In* C Sybesma, ed, Advances in Photosynthesis Research, Vol 2. Martinus Nijhoff/Dr W Junk, The Hague/Boston, pp 659–662
3. BERTHOLD DA, GT BABCOCK, CF YOCUM 1981 A highly resolved, oxygen-evolving photosystem II preparation from spinach thylakoid membranes: epr and electron transport properties. FEBS Lett 134: 231–234
4. BLOUGH NV, K SAUER 1984 The effects of mono- and divalent salts on the O_2-evolution activity and low temperature multiline epr spectrum of photosystem II preparations from spinach. Biochim Biophys Acta 767: 377–381

5. BOSKA M, NV BLOUGH, K SAUER 1985 The effect of mono- and divalent salts on the rise and decay kinetics of epr signal in photosystem II preparations from spinach. Biochim Biophys Acta 808: 132–139
6. BOUSSAC A, B MAISON-PETERI, A-L ETIENNE, C VERNOTTE 1985 Reactivation of oxygen evolution of NaCl-washed photosystem II particles by Ca^{2+} and/or the 24 kD protein. Biochim Biophys Acta 808: 231–234
7. BOUSSAC A, B MAISON-PETERI, C VERNOTTE, A-L ETIENNE 1985 The charge accumulation mechanism in NaCl-washed and in Ca^{2+}-reactivated photosystem II particles. Biochim Biophys Acta 808: 225–230
8. BRAND JJ, P MOHANTY, DC FORK 1983 Reversible inhibition of photochemistry of photosystem II by Ca^{2+} removal from intact cells of *Anacystis nidulans*. FEBS Lett 155: 120–124
9. CAMMARATA K, N TAMURA, R SAYRE, G CHENIAE 1984 Identification of polypeptides essential for oxygen evolution by extraction and mutational analyses. *In* C Sybesma, ed, Advances in Photosynthesis Research, Vol I. Martinus Nijhoff/Dr W Junk, The Hague/Boston, pp 311–320
10. CHUA N-H 1980 Electrophoretic analysis of chloroplast proteins. Methods Enzymol 69: 434–446
11. CRITCHLEY C 1985 The role of chloride in photosystem II. Biochim Biophys Acta 811: 33–46
12. DEKKER JP, DF GHANOTAKIS, JJ PLIJTER, HJ VAN GORKOM, GT BABCOCK 1984 Kinetics of the oxygen-evolving complex in salt-washed photosystem II preparations. Biochim Biophys Acta 767: 515–523
13. DISMUKES GC 1986 The metal centers of the photosynthetic oxygen evolving complex. Photochem Photobiol 43: 99–115
14. DUNAHAY TG, LA STAEHELIN, M SEIBERT, PD OGILVIE, SP BERG 1984 Structural, biochemical and biophysical characterization of four oxygen-evolving photosystem II preparations from spinach. Biochim Biophys Acta 764: 179–193
15. GHANOTAKIS DF, GT BABCOCK, CF YOCUM 1984 Calcium reconstitutes high rates of oxygen evolution in polypeptide depleted photosystem II preparations. FEBS Lett 167: 127–130
16. GHANOTAKIS DF, JN TOPPER, GT BABCOCK, CF YOCUM 1984 Water-soluble 17 and 23 kD polypeptides restore oxygen evolution by creating a high-affinity binding site for Ca^{2+} on the oxidizing side of photosystem II. FEBS Lett 170: 169–173
17. GHANOTAKIS D, JN TOPPER, CF YOCUM 1984 Structural organization of the oxidizing side of PS-II. Exogenous reductants reduce and destroy the Mn-complex in PS-II membranes depleted of the 17 and 23 kD polypeptides. Biochim Biophys Acta 767: 524–531
18. GOVINDJEE, T KAMBARA, W COLEMAN 1985 The electron donor side of photosystem II: the oxygen evolving complex. Photochem Photobiol 42: 187–210
19. HOMANN PH 1985 The association of functional anions with the oxygen-evolving center of chloroplasts. Biochim Biophys Acta 809: 311–319
20. ITOH S, CT YERKES, H KOIKE, HH ROBINSON, AR CROFTS 1984 Effects of chloride depletion on electron donation from the water oxidizing complex to the photosystem II reaction center as measured by the microsecond rise of chlorophyll fluoresence in isolated pea chloroplasts. Biochim Biophys Acta 766: 612–622
21. JANSSON C, O HANSSON, H-E ÅKERLUND, L-E ANDREASSON 1984 EPR studies on the photosystem II donor side in salt-washed and reconstituted inside-out thylakoids. Biochem Biophys Res Commun 124: 269–276
22. KOK B, B FORBUSH, M MCGLOIN 1970 Cooperation of charges in photosynthetic O_2 evolution. I. A linear four step mechanism. Photochem Photobiol 11: 457–475
23. KUWABARA T, M MIYAO, T MURATA, N MURATA 1985 The function of 33-kD protein in the photosynthetic oxygen evolution system studied by reconstitution experiments. Biochim Biophys Acta 806: 283–289
24. KUWABARA T, N MURATA 1982 Inactivation of photosynthetic oxygen evolution and concomitant release of three polypeptides in the photosystem II particles of spinach chloroplasts. Plant Cell Physiol 23: 533–539
25. LEE J-Y, H BAN-DAR, P RONG-LONG 1985 The high affinity binding site for calcium on the oxidizing side of photosystem II. Biochem Biophys Res Commun 128: 464–469
26. MIYAO M, N MURATA 1983 Partial reconstitution of the photosynthetic oxygen evolution system by rebinding of the 33 kD polypeptide. FEBS Lett 164: 375–378
27. MIYAO M, N MURATA 1983 Partial disintegration and reconstitution of the photosynthetic oxygen evolution system: binding of 24 kilodalton and 18 kilodalton polypeptides. Biochim Biophys Acta 725: 87–93
28. MIYAO M, N MURATA 1984 Calcium ions can be substituted for the 24-kD polypeptide in photosynthetic oxygen evolution. FEBS Lett 168: 118–120
29. MIYAO M, N MURATA 1984 Role of the 33-kD polypeptide in preserving Mn in the photosynthetic oxygen-evolution system and its replacement by chloride ions. FEBS Lett 170: 350–354
30. MURATA N, M MIYAO, T KUWABARA 1983 Organization of the photosynthetic oxygen evolution system. *In* Y Inoue, AR Crofts, Govindjee, N Murata, G Renger, K Satoh, eds, The Oxygen Evolving System of Photosynthesis. Academic Press Japan, Tokyo, pp 213–222
31. NAKATANI HY 1984 Photosynthetic oxygen evolution does not require the participation of polypeptides of 16 and 24 kilodaltons. Biochem Biophys Res Commun 120: 299–304
32. NEVILLE DM JR 1977 Molecular weight determination of protein-dodecylsulfate complexes by gel electrophoresis in a discontinuous buffer system. J Biol Chem 246: 6328–6334
33. ONO T, Y INOUE 1984 Ca^{2+}-dependent restoration of O_2 evolving activity in $CaCl_2$-washed PS-II particles depleted of 33, 24 and 16 kD proteins. FEBS Lett 168: 281–286
34. ONO T, Y INOUE 1984 Reconstitution of photosynthetic oxygen evolving activity by rebinding of 33 kD protein to $CaCl_2$-extracted PSII particles. FEBS Lett 166: 381–384
35. ONO T-A, Y INOUE 1986 Effects of removal and reconstitution of the extrinsic 33, 24 and 16 kD proteins on flash oxygen yield in photosystem II particles. Biochim Biophys Acta 850: 380–389
36. PRESTON C, RJ PACE 1985 The S-state dependence of Cl^- binding to plant photosystem II. Biochim Biophys Acta 810: 388–391
37. RADMER R, K CAMMARATA, N TAMURA, O OLLINGER, G CHENIAE 1986 Depletion of photosystem II-extrinsic proteins. I. Effects on O_2- and N_2-flash yields and steady-state O_2 evolution. Biochim Biophys Acta 850: 21–32
38. SATOH K, S KATOH 1985 A functional site of Ca^{2+} in the oxygen-evolving photosystem II preparation from *Synechococcus* sp. FEBS Lett 190: 199–203
39. SATOH K, S KATOH 1985 Inhibition by ethylenediamine tetraacetate and restoration by Mn^{2+} and Ca^{2+} of oxygen-evolving activity in photosystem II preparation from the thermophilic cyanobacterium, *Synechococcus* sp. Biochim Biophys Acta 806: 221–229
40. SINCLAIR J 1984 The influence of anions on oxygen evolution by isolated spinach chloroplasts. Biochim Biophys Acta 764: 247–252
41. SPARROW RW, RR ENGLAND 1984 Isolation of a calcium binding protein from an oxygen-evolving photosystem II preparation. FEBS Lett 177: 95–98
42. TAMURA N, G CHENIAE 1985 Effects of photosystem II extrinsic proteins on microstructure of the oxygen-evolving complex and its reactivity to water analogs. Biochim Biophys Acta 809: 245–259
43. TAMURA N, GM CHENIAE 1986 Requirements for the photoligation of Mn^{2+} in PSII membranes and the expression of water-oxidizing activity of the polynuclear Mn-catalyst. FEBS Lett 200: 231–236
44. TAMURA N, R RADMER, S LANTZ, K CAMMARATA, G CHENIAE 1986 Depletion of photosystem II proteins. II. Analysis of the PSII/water-oxidizing complex by measurements of *N,N,N',N'*-tetramethyl-*p*-phenylenediamine oxidation following an actinic flash. Biochim Biophys Acta 850: 369–379
45. THEG SM, PA JURSINIC, PH HOMANN 1984 Studies on the mechanism of chloride action on photosynthetic water oxidation. Biochim Biophys Acta 766: 636–646
46. YAMAMOTO Y, K TABATA, Y ISOGAI, M NISHIMURA, S OKAYAMA, K MATSUURA, S ITOH 1984 Quantitative analysis of membrane components in a highly active O_2-evolving photosystem II preparation from spinach chloroplasts. Biochim Biophys Acta 767: 493–500