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Evidence for an association between a 33 kDa extrinsic membrane protein, manganese and photosynthetic oxygen evolution. I. Correlation with the S_2 multiline EPR signal

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The removal of peripheral membrane proteins of a molecular mass of 17 and 23 kDa by washing of spinach Photosystem-II (PS II) membranes in 1 M salt between pH 4.5 and 6.5 produces a minimal loss of the $S_1 \rightarrow S_2$ reaction, as seen by the multiline EPR signal for the S_2 state of the water-oxidizing complex, while reversibly inhibiting O_2 evolution. The multiline EPR signal simplifies from a '19-line' spectrum to a '16-line' spectrum, suggestive of partial uncoupling of a cluster of 3 or 4 Mn ions to yield photo-oxidation of a binuclear Mn site. Alkaline salt washing progressively releases a 33 kDa peripheral protein between pH 6.5 and 9.5, in direct parallel with the loss of O_2 evolution and the S_2 multiline EPR signal. The 33 kDa protein can be partially removed (20%) at pH 8.0 prior to manganese release. Salt treatment releases four Mn ions between pH 8.0 and 9.5 with the first 2 or 3 Mn ions released cooperatively. A common binding site is thus suggested in agreement with earlier EPR spectroscopic data establishing a tetranuclear Mn site. At least two of these Mn ions bind directly at a site in the PS II complex for which photooxidation by the reaction center is controlled by the 33 kDa protein. The washing of PS II membranes with 1 M $CaCl_2$ to affect the release of the 33 kDa protein, while preserving Mn binding to the membrane (Ono, T.-A. and Inoue, Y. (1983) FEBS Lett. 164, 255–260), is found to leave some 33 kDa protein undissociated in proportion to the extent of O_2 evolution and S_2 multiline yield. These depleted membranes do not oxidize water or produce the normal S_2 state without the binding of the 33 kDa protein. A method for the accurate determination of relative concentrations of the peripheral membrane proteins using gel electrophoresis is presented.

Introduction

There is keen interest in, yet limited knowledge of, the organization of manganese within the complex responsible for the oxidation of water to O_2 in photosynthetic membranes. Manganese has been indirectly implicated as the site for water binding and direct observations of oxidation state changes have indicated a clear redox function (reviewed in Refs. 1 and 2).

Of the four Mn ions located within the smallest protein-membrane complexes capable of high O_2

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Abbreviations: Chl, chlorophyll; PS II, Photosystem II; PPBQ, *p*-phenylbenzoquinone; LDS, lithium dodecylsulfate; Mes, 4-morpholineethanesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; Ches, 2-[*N*-cyclohexylamino]-ethanesulfonic acid.

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evolution activity, there is evidence for their organization into two distinct types based upon their lability to denaturing conditions [3–7]. A close arrangement of at least two Mn ions separated by a shared ligand is well established [8] and this pair shows clear evidence for coupling to another paramagnetic center believed to comprise one or both of the other Mn ions [9,10,13].

Furthermore, there is evidence that two of the four ions can be replaced by divalent ions in the reactivation of electron donation from exogenous donors (but not significant O_2 evolution) [5,7]. It is not yet clear if this pair of cations must also be Mn in order to observe optimal O_2 evolution rates. There is some speculation that this labile pair may be required for a structural rather than redox function. In contrast, electronic spectroscopic data have been interpreted as evidence for a redox function for all four Mn ions in water oxidation [11,12]. Evaluation of these diverse lines of evidence on the function and organization of manganese has been interpreted in favor of a model in which the catalytic unit is comprised of two pairs of closely interacting Mn ions [9,13].

Three extrinsic membrane proteins associated with O_2 evolution can be dissociated from Photosystem-II (PS-II) membranes (reviewed in Ref. 19). Two of these subunits have apparent molecular masses of 16–18 kDa and 23–24 kDa and participate in the regulation of Cl^- affinity, a required physiological anion. The 23 kDa protein plays a more important role and can be replaced by Ca(II) [15–17] and by Cl^- at a 20–50-fold higher concentration than is physiologically optimal [17,18].

The third subunit has a mass of 33 kDa. Manganese can be released with this protein depending upon the conditions used for dissociation from the membrane. Alkaline Tris washing releases this protein and 2 Mn ions from membranes initially depleted of the 17- and 23-kDa proteins by salt washing [4]. Protein release by osmotic shock of membranes [20] or by extraction with acetone [3] or butanol [38] results in the partial binding of Mn to the protein. Enhanced binding of Mn to this protein is favored by inclusion of mild chemical oxidants during isolation, which apparently suppresses the reduction of Mn(III). The protein binds a maximum of 2 Mn(III) ions under these conditions [3,20].

No Mn is released initially from the membrane when these three proteins are dissociated by washing in 1 M $CaCl_2$ [21] or 2.5 M urea [22]. Loss of the 33 kDa protein is invariably accompanied by a complete loss or significant reduction of O_2 evolution activity, which can be partially reversed upon rebinding of the protein [3,23,24,42,43], additional Cl^- [22] or Ca^{2+} [25]. These results are at odds with the earlier data showing a direct correlation between O_2 evolution activity and the binding of the 33 kDa protein and Mn to the PS-II complex.

A possible answer to this contradiction has come in a recent study showing that the Mn binding site on PS-II membranes, following release of the 33 kDa protein and Ca^{2+} by washing in 1 mM $LaCl_3$, is structurally altered and not capable of photo-oxidation by the reaction center [37]. These observations have been interpreted to indicate the binding of a relatively labile pair of the four Mn ions at the interface between two protein domains, involving the amino acid residues of the peripheral 33 kDa protein and either the 47 kDa intrinsic membrane protein of the reaction center [19] or an intrinsic membrane protein of 34 kDa [39].

In an effort to resolve these seemingly contradictory results we have titrated the release of the three peripheral membrane proteins, and examined the release of manganese, the loss of O_2 rate and the loss of the S_2 multiline EPR signal. New insights on how manganese is organized within the water-oxidizing complex are revealed.

Materials and Methods

PS-II membranes were prepared as described previously [26]. They had a steady-state O_2 evolution rate (Clark type electrode) of 340 μmol per mg Chl per h. Salt-washing treatments were based on the original procedure by Kuwabara and Murata [4]. PS-II membranes retaining specific amounts of the extrinsic 33 kDa protein can be prepared by salt washing at various pH values. All salt-washing buffers contained 1 M NaCl, 0.2 M sucrose and an appropriate Good pH buffer. The buffers used were: acetate (pH 4.5); Mes (pH 5.5 and 6.5); Hepes, (pH 7.5); Tricine, (pH 8.0 and 8.5); and Ches (pH 9.0 and 9.5). All buffers were

at a final concentration of 25 mM. PS-II particles were incubated in these salt buffers at a Chl concentration of 0.5 mg/ml for 30 min on ice while shaking. Supernatants and pellets were collected after centrifugation at $48000 \times g$ for 30 min. The resultant depleted membranes were prepared for EPR measurements at a single pH by equilibration at pH 6.5 by washing twice in a buffer containing 25 mM Mes, 0.2 M sucrose, 10 mM $CaCl_2$ and either 25 or 110 mM NaCl. This washing released small quantities of additional protein which did not dissociate on the initial salt washing. Prior to EPR sample freezing 2 mM PPBQ was added as an electron acceptor.

Dark-adapted EPR samples (> 20 min, 274 K) were illuminated with a quartz-halogen lamp (1 W/cm^2) at 200 K in a dry ice/methanol bath for 3 min to produce the S_2 multiline EPR signal, or at 300 K for 3 min while cooling to 200 K in the presence of 50 μM DCMU. Heating of the EPR samples during illumination was insignificant at less than 3 K temperatures rise as monitored by measurements of both the bath and sample. Control samples were washed the same way except using a non-denaturing sucrose buffer at ph 6.5.

Proteins released by salt washing were prepared for electrophoresis by precipitation in acetone. Supernatants were diluted to a final NaCl concentration of 0.5 M and diluted with cold acetone to a final concentration of 80%. The solution was centrifuged at $48000 \times g$ for 20 min at 2°C or with a table top centrifuge for 1 h. The resulting pellet was resuspended in an electrophoresis buffer. Proteins were electrophorised by the procedure of Laemmli in the presence of 4 M urea and 0.2% LDS at 4.0°C [33].

The amount of the 33 kDa protein remaining on the membranes after salt washing can be determined by its release into the supernatant upon heat shock of the depleted membranes [34]. We have found this method to be far more reliable than estimation of the amount of protein in the pellet in cases where overlapping bands cause interference, e.g. at 31–34 kDa. For these experiments the salt-washed membranes that were prepared for EPR measurements were heat shocked at 55°C for 5 min at 1 mg Chl/ml.

Relative protein concentrations were determined by absorbance of Coomassie stained slab gel electrophoresis chromatograms with a Hewlett Packard 8450 spectrophotometer and a densitometer of local design.

EPR spectra were recorded at 11 K as previousy described [8]. The intensity of the S_2 multiline signal was obtained by summing the amplitudes of the peaks labelled −5, −6, −7, 5, 6, and 7 given in Fig. 3 after baseline subtraction. This avoids interference with the signals from chrome *b*-559 and Q^-Fe^{2+} which overlap with peaks labelled −4/−3 and 4/3, respectively.

Manganese was determined by atomic absorption using a graphite furnace, Perkin-Elmer model 305B and HGA-2000 furnace. Error analysis was conducted on the manganese and chlorophyll data in order to establish the precision of successive determinations. The reported values of Mn/RC have associated with the standard deviations of 2% and 4% for Mn and Chl determinations respectively, for replicate runs of the same sample. Averaging of data sets from three independent runs was performed on the data reported in Fig. 5. The average standard deviation from the mean of these three sets was 10%.

Results

Protein release by salt washing

Determination of the identity and extent of proteins released from PS-II membranes by salt washing at different pH values was performed by densitometry of Coomassie-stained LDS polyacrylamide gels as summarized in Figs. 1 and 2. In Fig. 1, B-I, there is nearly complete release of two proteins at M_r 17 and 23 kDa upon salt washing between pH 6.5 and 9.5, confirming earlier reports. There is 60% 17 kDa and some 23 kDa protein which remains bound at pH 4.5 and these decrease at higher pH. Progressively greater release of a 33 kDa protein is observed above pH 7. Approx. 50% of the 33 kDa band remains after salt washing at pH 8.5 and complete loss is observed at pH 9.5. These are compared to control membranes washes in non-denaturing sucrose buffer at pH 6.5 (A), and with membranes washed in 1 M $CaCl_2$ at pH 6.5 (J), a treatment known to release all three proteins. Other prominent bands associated with the PS-II core proteins at 47, 43, 34, 32, 24, 21, 20, 10 and 9 kDa and the light

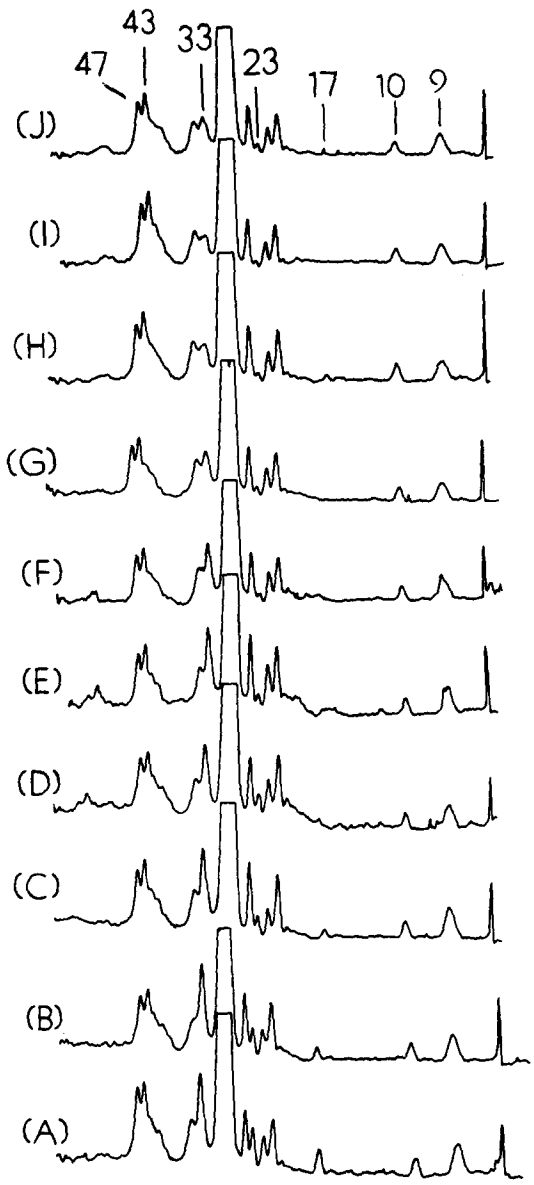

Fig. 1. LDS-PAGE densitometric traces of proteins remaining in PS II membranes following salt washing at various pHs. (A) Control, 25 mM NaCl (pH 6.5); (B) 1 M NaCl (pH 4.5); (C) 1 M NaCl, (pH 5.5); (D) 1 M NaCl, (pH 6.5); (E) 1 M NaCl, (pH 7.5); (F) 1 M NaCl (pH 8.0); (G) 1 M NaCl (pH 8.5); (H) 1 M NaCl (pH 9.0); (I) 1 M NaCl (pH 9.5); (J) 1 M $CaCl_2$ (pH 6.5).

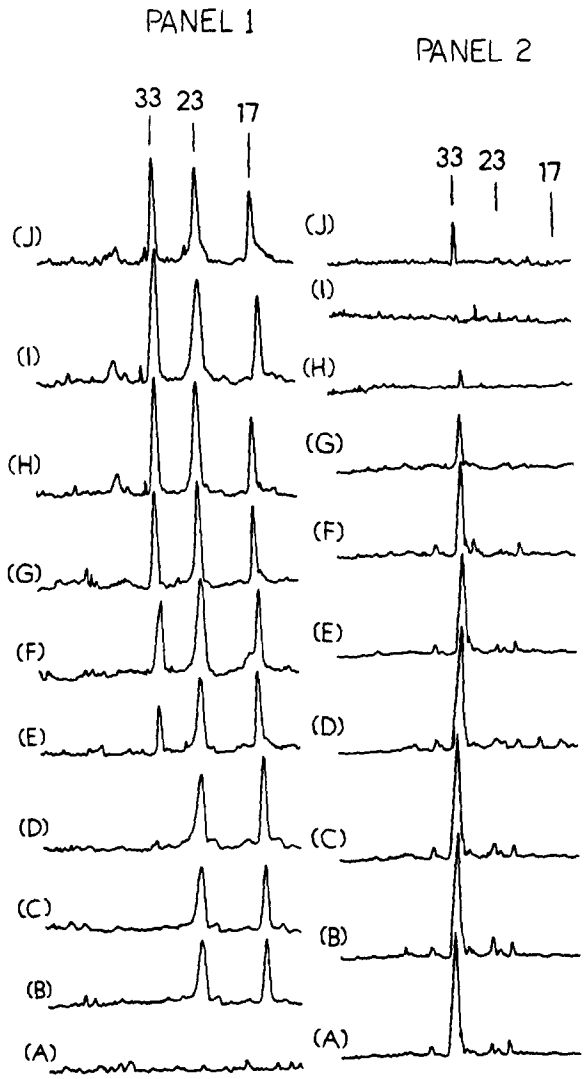

Fig. 2. Panel 1: LDS-polyacrylamide gel electrophoresis densitometric traces of proteins released from membranes by salt-washing at various pHs, as in Fig. 1. Panel 2: LDS-polyacrylamide gel electrophoresis densitometric traces of proteins released by heat shock of salt-washed PS-II membranes from Fig. 1. After salt washing, membranes were washed and resuspended twice at pH 6.5 prior to heat shocking. This removed the peripheral proteins of 23 and 17 kDa.

harvesting complex at 28–26 are resolved and are invariant under these treatments.

Quantitation of the amount of 33 kDa protein bound to the membrane is difficult using the data of Fig. 1 due to interference from neighboring bands at 32 and 34 kDa. This interference is resolved by determination of the relative amounts of protein released into the supernatants (as given in Fig. 2, panel 1). This confirms that there are only three proteins released by NaCl washing,

with nearly uniform release of the 17- and 23-kDa proteins between pH 6.5 and 9.5, and an increasing amount of the 33 kDa protein released. The residual binding of the 33 kDa protein was assayed by release upon heat shock of the depleted membranes [34]. As shown in Fig. 2, panel 2, there is a progressive increase with pH in the release of the 33 kDa protein between pH 7 and 9.5. The residual 17 and 23 kDa proteins which are bound at pH 4.5–6.5 in Figs. 1 and 2 are not observed because these are lost during the washing treatment prior to heat shocking. No significant release of the other proteins seen in Fig. 1 was observed by these treatments. The fraction of 33 kDa protein which remains bound at each pH is plotted in Fig. 4. Protein analysis conducted in this manner proved to be reproducible, while analysis of the depleted membrane was not nearly as reliable. It is interesting to note that washing in 1 M $CaCl_2$, a treatment which is claimed to quantitatively release the 33 kDa protein [22,25] leaves about 1/3 of this protein on the membrane, as can be seen by release using heat shock (Fig. 2).

The salt-washed depleted membranes were examined further to determine the extent of the $S_1 \rightarrow S_2$ reaction from the yield of the manganese S_2 multiline EPR signal, produced by illumination at 200 K, as shown in Fig. 3 for a typical control sample. In Fig. 4 the yield of this signal following salt treatment is compared to the fraction of bound 33 kDa protein, to the amount of Mn released, as

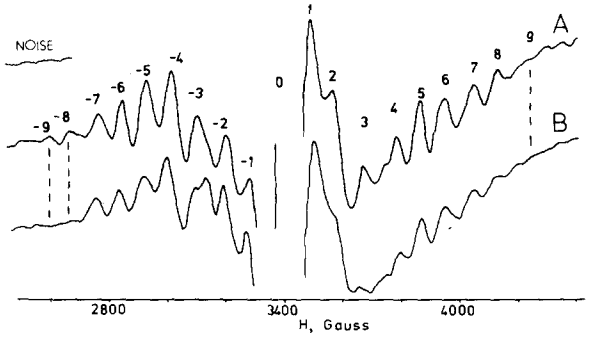

Fig. 3. EPR spectra recorded in the dark illumination of dark adapted PS II membranes at pH 6.5. The multiline signal is formed by illumination at 200 K and observed at 11 K. (A) Control, prior to salt washing (B) after washing in 1 M NaCl (pH 7.5) buffer. A numbering system for peaks is shown. The relative peak-to-noise is 0.04.

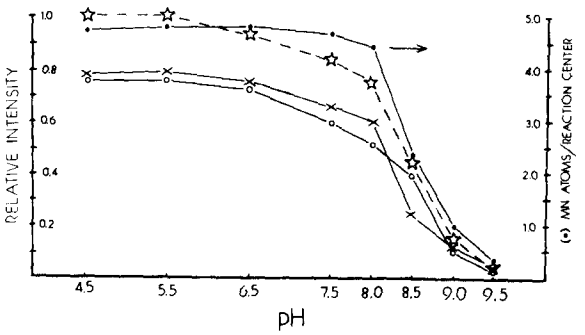

Fig. 4. Correlation between the loss of S_2 multiline EPR signal (○), binding the the 33 kDa protein (☆) (relative error, 0.04), O_2 evolution (×) [relative error, 0.07] and the amount of Mn per reaction center (●). Associated error of 0.5 atoms Mn reaction center in PS-II membranes upon salt washing at various pH values. Samples are identical to those in Figs. 1 and 2. The measurements were conducted on samples adjusted to pH 6.5 after treatment. All measurements are relative to the control samples washed in sucrose buffer at pH 6.5 without salt. The protein data are an average of three experiments involving quantiation by heat shock as in Fig. 3. The multiline intensities and manganese content are the average of two data sets. Optimum O_2 evolution rates were assayed at pH 6.5 and 235 mM Cl^-/5 mM Ca^{+2}.

determined by atomic absorption of the supernatants and to the O_2 evolution rate. It shows that nearly complete release of the 17 and 23 kDa proteins by salt washing is accomplished by a 75% retention in the yield of the multiline signal relative to the control. This loss was smaller for incubation times less than 30 min and at higher Cl^- concentrations. The decrease of the multiline signal parallels the release of the 33 kDa, both approachng zero at pH 9.5. On the other hand, starting with membranes containing close to the minimal Mn content (4–5 Mn/PS II) capable of sustaining high activity, there is almost no Mn release, except above pH 7.5. At pH 5.5–7.5 there is no release of Mn, while at pH 4.5 and 8.0 there is less than 0.2–0.3 Mn released per PS-II. 90% of the Mn, about 4 Mn/PS II, is released by pH 9.5. This contrasts with about 20% release of the 33 kDa protein with increasing pH between pH 6.5 and 8.0 where no significant release of Mn occurs. This Mn is, however, not photo-oxidized as seen by the loss of the multiline signal. At pH 8.5 and above the release of Mn and protein parallel each other.

The losses in protein and multiline signal are accompanied by a parallel loss in the O_2 evolution rate. Three different Cl^-/Ca^{2+} concentrations were surveyed in the assay for O_2 rate in order to optimize activity (data not shown). As appreciably higher Cl^- requirement for O_2 evolution in membranes depleted of the 17- and 23-kDa proteins was confirmed [16–18,28,29]. About 80% restoration of the O_2-evolution rate was achieved with concentrations of 5 mM $CaCl_2$ and 235 mM NaCl. The data given in Fig. 4 indicate that electron transfer, as monitored by O_2 evolution, follows the content of 33 kDa protein rather than Mn content in salt washed membranes. This again supports the claim that the Mn which remains bound following release of the 33 kDa is not capable of oxidizing water to oxygen.

Table I gives the same results for samples washed in 1 M $CaCl_2$ at pH 6.5. Here we see that, as found previously by others, there is appreciable retention of Mn on the membrane, about 3.7 Mn/PS-II. However, the yield of the multiline signal parallels the amount of 33 kDa protein remaining on the membrane, about 1/3 of the control, and not the Mn content. The optional O_2 evolution rate which could be observed was also about 40% of the control. Apparently, the bound Mn without the 33 kDa protein is not photo-oxidizable. This is compared to a sample that is salt-washed at pH 8.5 and has comparable yields of the S_2 signal and 33 kDa protein, but only 2.2 Mn/PS II. We find no evidence that this protein can be replaced by Ca^{2+} or Cl^- in the formation of the S_2 multiline signal or O_2 evolution, in contrast to O_2 evolution [22,25].

We felt it was important to learn if the loss of the S_2 multiline signal upon salt washing might be due to a change in the conditions necessary for generation of the signal, rather than due to loss of an essential cofactor for the manganese site. We tested two parameters: the Cl^- concentration in the assay medium and the illumination temperature. We found little further improvement in the amplitude of the multiline signal, in membranes washed at pH 6.5 or above, upon increasing the concentration of Cl^- in the assay medium. The yield increased only from 75% to 80% of the control for the optimum samples (pH 6.5) upon increasing the salt concentration from 25 mM to 110 mM. Therefore, the loss of multiline signal above pH 6.5 cannot be accounted for by an unsatisfied Cl^- requirement. The yield of the multiline signal in membranes depleted below pH 6.5 actually decreased when the Cl^- concentration was increased from 25 mM to 110 mM, indicating an antagonistic influence of Cl^- below about pH 6.

The results on the temperature dependence of the formation of the multiline signal in salt-treated membranes by illumination at 200 K and 300 K (50 μM + DCMU) showed that a lower yield is observed at 300 K for all pH/salt extractions. Thus an increased activation energy is not the source of the reduction in the signal relative to control membranes (data not shown). It may be possible to reduce further the 20% loss of the S_2 multiline signal by using shorter times for salt incubation and centrifugation than the 30 min intervals we adopted for both.

TABLE I

CORRELATION OF RELATIVE S_2 MULTILINE SIGNAL YIELD, OXYGEN EVOLUTION, MANGANESE AND 33 kDa PROTEINS IN PS-II MEMBRANES TREATED WITH 1 M $CaCl_2$, pH 6.5 OR 1 M NaCl, pH = 8.5.

Treatment	Relative S_2 yield	Relative 33 kDa bond	Relative Oxygen evolution	Manganese reaction center
$CaCl_2$ (pH 6.5)	0.37	0.35	0.39	3.7
NaCl (pH 8.5)	0.39	0.43	0.24	2.2
Control	1.0	1.0	1.0	4.7

The g = 4.1 signal

We also searched for the light-induced $g = 4.1$ EPR signal attributed to an oxidized donor, possibly Mn(IV) in PS II membranes [35,36]. This signal did not form in salt-treated samples illuminated at 200 K or in control samples (data not shown).

Discussion

Protein determination

The results in Figs. 1 and 2 illustrate that accurate determination of the relative extent of 33 kDa protein bound to the membrane is greatly aided by release of the protein from the mem-

brane by the heat shock method [34]. Once in solution interference from non-soluble proteins is eliminated. When combined with a titration method for protein release this offers a quantitative assay for the presence of this protein.

Salt washing

The 20–25% reduction in the amplitude of the multiline signal which accompanies the release of the 23- and 17-kDa proteins at pH 4.5–5.5 (Fig. 4) suggests a role for these proteins in maintaining efficient photooxidation of the S_2 state of the O_2-evolving complex by the reaction center, but not an obligatory role. We cannot rule out the possibility that the loss of one or both of these proteins upon salt washing is unrelated to the reduced quantum yield for the S_2 multiline signal. In contrast, the large loss in O_2 evolution rate upon dissociation of the 23 kDa protein [28] when combined with the present results, indicate that it must be required for physiological activity involving the formation of S_3 or S_4. These results agree with the general findings in Ref. 30 showing that salt treatment to release the 17- and 23-kDa proteins abolishes the S_2 signal and O_2 evolution, but reversibly, upon dialysis to remove excess salt. An earlier report [41] has found that removal of the 17- and 23 kDa-proteins by salt washing blocks or greatly suppresses the advancement of the water oxidizing complex to the S_2 state, as seen by the multiline yield. This discrepancy is probably attributable to the absence of sufficient Cl^- (1 mM) and Ca(II) (none) in their assay medium. This underscores the ease with which this signal is lost if conditions are not optimized.

The spectrum of the S_2 multiline signal which forms in salt-washed membranes has fewer lines compared to the minimum of 19 lines which form in the control membranes at 200 K. Fig. 3 shows that the two low field peaks, -8 and -9, disappear upon salt washing at pH 7.5. This signal is referred to as the '16-line' S_2 multiline signal. '16-line' EPR spectra analogous to this signal are observed for synthetic binuclear manganese complexes in the mixed-valence state Mn_2 (III, IV) [14]. The S_2 multiline signal in untreated membranes converts also from a '19-line' form, achieved by illumination at 200 K, to a '16-line' form produced by multiple turn-overs at 300 K [1,14]. It has been attributed to an altered coupling between the manganese ions.

The results of Fig. 4 show that it is possible to dissociate about 20% of the 33 kDa protein without loss of Mn from the membrane between pH 6.5 and 8.0. Consequently, this protein is not essential for the binding of Mn to the membrane, but it is essential for photo-oxidation of Mn (S_2 multiline signal) and the evolution of O_2 (Fig. 4). These results are in agreement with previous results showing conditions under which the three peripheral proteins may be partially, dissociated prior to significant Mn release [21,22,30]. The multiline signal and O_2 evolution are restored upon rebinding of the 33 kDa protein, provided manganese has not been lost from the protein depleted membrane [42,43].

The Mn which remains on the membrane following partial dissociation of the 33 kDa protein is more susceptible to dissociation than in intact PS-II membranes, suggesting a direct influence of this protein on Mn binding [19,43,44]. Previous results [20] show binding of two Mn(III) ions to the 33 kDa protein in the presence of oxidants can be understood in terms of the current results. Manganese binding to PS-II membranes appears to occur at the interface between the 33 kDa protein and an integral membrane protein of the reaction center core, so that it may be carried with either the dissociated protein or with the depleted membrane depending upon the conditions used for extraction. Similar conclusions were reached in studies of Ca(II) exchange for La(III) [37]. This other protein has been suggested to be either the 47 kDa reaction center core protein [19], or possibly an intrinsic membrane protein of 34 kDa [39]. Another less likely possibility is that the 33 kDa protein, while stabilizing the binding of manganese, is not directly involved in manganese coordination; inferring that the observed isolation of a manganese protein complex by use of chemical oxidants [20,38] is a curious artifact. The capability of this manganese protein to restore lost O_2 evolution activity to depleted membranes [3] favors the former interpretation.

Manganese binding

A comparison of the observed binding of Mn to the membrane with theoretrical predictions for

cooperative binding is given in Fig. 5. The theoretical curves are generated using the Hill formulation for the fraction of bound sites, Y [40]:

$$\bar{Y} = \frac{\left(\frac{[OH^-]}{K}\right)^{\alpha}}{1+\left(\frac{[OH^-]}{K}\right)^{\alpha}} \quad (1)$$

Two curves are shown for Hill parameters of $\alpha = 1$ and 2. The case of $\alpha = 1$ corresponds to non-cooperative binding. The experimental data are clearly in better agreement with a cooperative release of the initial 2 or 3 Mn ions by alkaline salt washing. Therefore, at least 2 and possibly all 4 Mn ions may be grouped together into a common binding site. This conclusion was evident already from the spectroscopic interpretation of the S_2 multiline EPR signal which is best understood by models involving the electronic interaction of four Mn ions [1,9,13,14]. Combined with the present biochemical data these results indicate that these four Mn ions are organized into two groups comprised of 2 or 3 Mn ions that are cooperatively released and another 2 or 1 Mn ions that bind at a site which is characterized by release which is thermodynamically non-cooperative. These results do not preclude the possibility that all four Mn ions are bound to a common site.

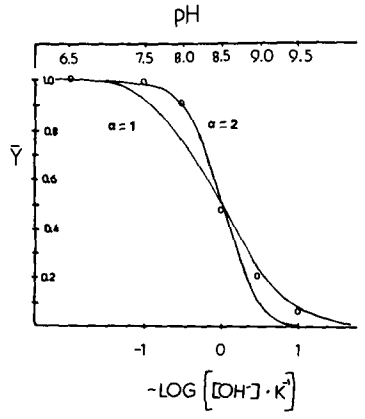

Fig. 5. Comparison of the Mn binding data taken from Fig. 4 with an empirical model for cooperative binding: see Eqn. 1. Curves are plotted for the Hill coefficients of $\alpha = 1$ and $\alpha = 2$. The data represent averages from three independent determinations each subject to a precision error of 0.04.

$CaCl_2$ washing

Previous studies have found that PS-II membranes which have been completely stripped of the 17-, 23- and 33-kDa proteins by washing in 1 M $CaCl_2$ retain Mn which is functional in the reconstitution of partial O_2 evolution activity by addition of Ca(II) [25] or excess Cl^- [22]. While we see retention of Mn binding, we have found no evidence supporting the claim that this Mn, without the 33 kDa protein, is functional in O_2 evolution or S_2 multiline formation with or without added Ca(II) or Cl^- (Fig. 4 and Table I). The answer to this difference may lie in the results in Table I, showing that this treatment actually leaves about 1/3 of the 33 kDa protein on the membrane. This is easily missed if protein determination by gel electrophoresis is conducted using the depleted membrane for which interference from bands at 34- and 32 kDa occurs (Fig. 1). Therefore, the partial O_2 rate recovery seen in previous work may reflect a Cl^- or Ca(II) dependent recoupling of undissociated 33 kDa protein to the O_2-evolving complex.

Osmotic release

Previously, we observed that release of the 33 kDa protein by osmotic shock also releases 2 or 3 Mn atoms from thylakoid membranes [20] and 2 Mn atoms from PS-II membranes [3]. Furthermore, a strict correlation was observed between the extent of release of the 33 kDa protein and the loss of the multiline signal and O_2 evolution from PS-II membranes by osmotic shock [3]. These results agree well with the results from alkaline salt washing, and confirm how general the results are with different methods for protein dissociation. The release of only 2 of the 4 Mn ions by the milder osmotic treatment indicates that the 33 kDa protein stabilizes preferentially 2 of the 4 Mn ions that can be released by alkaline salt washing.

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