Interaction of the 33 kDa Extrinsic Protein with Photosystem II: Rebinding of the 33 kDa Extrinsic Protein to Photosystem II Membranes Which Contain Four, Two, or Zero Manganese per Photosystem II Reaction Center†

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ABSTRACT: The 33 kDa extrinsic protein of photosystem II acts to enhance oxygen evolution and to stabilize the manganese cluster at low chloride concentrations. Due to controversies concerning the stoichiometry of this protein [Miyao, M., & Murata, N. (1989) *Biochim. Biophys. Acta 977*, 315−321, *versus* Xu, Q., & Bricker, T. M. (1992) *J. Biol. Chem. 267*, 25816−25821] we have examined the rebinding of this protein to PS II membrane preparations which contain four, two, or zero manganese per photosystem II reaction center. After rebinding, immunoquantification of the 33 kDa extrinsic protein demonstrated that each of these photosystem II membrane preparations strongly bound two copies of the 33 kDa extrinsic protein per photosystem II reaction center. The first and second stoichiometric binding constants (K_{a1} and K_{a2}) for the binding of the 33 kDa protein to PS II centers containing four manganese were 0.42 and 0.67 nM^{-1}, respectively. Disruption of the manganese cluster either by removal of the chloride-sensitive manganese or extraction of the manganese cluster by alkaline Tris led to a 5−6-fold decrease in K_{a1} and about a 3-fold decrease in K_{a2}. In all cases the binding of the two copies of the 33 kDa extrinsic protein exhibited positive cooperativity with Hill coefficients ranging from 1.6 to 2.2. These findings demonstrate that damage to the manganese cluster alters the binding affinity of the 33 kDa extrinsic protein to photosystem II but does not alter the molecularity of the binding reaction.

The light-driven oxidation of water to molecular oxygen and the reduction of plastoquinone to plastoquinol is catalyzed by Photosystem II (PS II).[1] PS II is a multisubunit thylakoid membrane protein complex which contains both intrinsic and extrinsic components. Seven major intrinsic proteins, CP 47, CP 43, D1, D2, the α and β subunits of cytochrome b_{559}, and the *psb*I gene product, are associated with this photosystem. A number of other low molecular mass intrinsic proteins of unknown function are also present. In higher plants, three extrinsic proteins with molecular masses of 33, 24, and 17 kDa are associated with this oxygen-evolving complex. The 24 and 17 kDa components appear to play a role in regulation of calcium and chloride, which are cofactors in oxygen evolution (Ghanotakis & Yocum, 1990). Removal of the 24 and 17 kDa extrinsic proteins markedly decreases the oxygen evolution capacity of PS II vesicles (Akerlund et al., 1982) and PS II membranes (Kuwabara & Murata, 1982a,b). Rebinding of these proteins brings about the recovery of oxygen evolution activity. The 17 and 24 kDa extrinsic proteins are absent in cyanobacteria (Stewart et al., 1985) although other proteins appear to perform analogous functions (Shen et al., 1992).

The 33 kDa extrinsic protein is present in all oxygenic organisms, and its presence is required for binding the 24 and 17 kDa extrinsic proteins (Miyao & Murata, 1989a,b; Kavelaki & Ghanotakis, 1991). It is unclear, however, if the 33 kDa extrinsic protein provides a binding site for the 24 and 17 kDa proteins or if the binding of the 33 kDa protein to PS II induces conformational changes in other PS II components which provide the actual binding site(s) (Yamamoto, 1988). Although this protein is not absolutely required for oxygen evolution (Burnap, 1991; Bricker, 1992), it is required for high rates of oxygen evolution at physiological inorganic cofactor concentrations. The 33 kDa extrinsic protein is closely associated with CP 47 (Bricker, 1990) and perhaps the D1 and D2 reaction center proteins (Mei et al., 1989). Bricker et al. reported that the 33 kDa extrinsic protein protects CP 47 from tryptic attack (Bricker & Frankel, 1987) and modification with NHS-biotin (Bricker et al., 1988; Frankel & Bricker, 1992). Cross-linking experiments performed on PS II membranes with the water-soluble carbodiimide (EDC) indicated that the 33 kDa extrinsic protein and CP 47 interact via a salt bridge (Bricker et al., 1988; Enami et al., 1991; Odom & Bricker, 1992).

In addition to these proteins a number of inorganic cofactors are associated with PS II. These include manganese, chloride, and calcium. A tetrameric manganese cluster is responsible for the accumulation of oxidizing equivalents for water oxidation. This cluster is bound to the intrinsic proteins by ligands which appear to be primarily provided by the D1 protein (Boerner et al., 1992; Chu et al., 1994; Ikeuchi et al., 1988; Nixon & Diner, 1992; Nixon et al., 1992; Seibert et al., 1989). Heterogeneity within this manganese is well documented. In the absence of the 33 kDa extrinsic protein, PS II membranes exposed to low chloride conditions

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[1] Abbreviations: Chl, chlorophyll; EDC, 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide; MES, 2-(*N*-morpholino)ethanesulfonic acid; NHS-biotin, *N*-hydroxysuccinimidobiotin; PS II, photosystem II; Tris, tris(hydroxymethyl)aminomethane.

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(<100 mM) lose two of the four manganese associated with the tetrameric cluster (Ono & Inoue, 1984). At these low chloride concentrations, the chloride-sensitive manganese become paramagnetically uncoupled from the manganese cluster and are lost (Mavankal et al., 1986). This loss can be prevented by the rebinding of the 33 kDa extrinsic protein onto membranes from which this protein had been removed (Ono & Inoue, 1985). It is unclear if the effect of chloride on the stability of the manganese cluster is direct or indirect. Chloride has been suggested to serve as a bridging ligand within the manganese cluster (Sandusky & Yocum, 1982) or to serve in a charge-screening role (Homann, 1986). Recent EXAFS evidence suggests that one chloride may be present as a terminal ligand within the manganese cluster (Yachandra et al., 1993). Calcium also appears to be associated with the manganese cluster (Boussac et al., 1985; Latimer et al., 1995), although its function is unclear.

Different approaches have been described for the removal of the extrinsic proteins from PS II membranes. Treatment of PS II membranes with 1.0 M NaCl releases the 17 and 24 kDa proteins. Treatment with alkaline Tris releases all three extrinsic proteins and three to four of the manganese associated with the tetrameric manganese cluster (Yamamoto et al., 1981; Kuwabara & Murata, 1982a,b; Franzen & Anderson, 1984). Treatments of 1.0 M $CaCl_2$ (Ono & Inoue, 1989) or NaCl−urea (Miyao & Murata, 1984) release the 33 kDa protein without destroying the tetrameric manganese cluster. PS II membranes depleted of the 33 kDa extrinsic protein by either of these treatments lose two manganese and the ability to evolve oxygen at low chloride concentrations (<100 mM). On the basis of these results the 33 kDa extrinsic protein is considered to be a manganese-stabilizing protein.

While the extrinsic 33, 24, and 17 kDa proteins appear to be present in equimolar amounts (Murata et al., 1984; Anderson et al., 1984), their stoichiometry remains controversial. Previous studies reported one to three molecules of each extrinsic protein per PS II center. (Murata et al., 1984; Millner et al., 1987; Andersson et al., 1984; Enami et al., 1991). Recently, results from our laboratory (Xu & Bricker, 1992) indicated the presence of two 33 kDa extrinsic proteins per PS II reaction center in both PS II membranes and oxygen-evolving PS II core complexes.

Here we present data examining the rebinding of the 33 kDa extrinsic protein to PS II membrane preparations containing four, two, or zero manganese per PS II reaction center. We find that in all instances two copies of the 33 kDa protein bind to these preparations and that their binding appears to be cooperative. Additionally, the removal of the chloride-sensitive manganese leads to a lowering of the apparent binding constants for both copies of the 33 kDa extrinsic protein. Removal of the chloride-insensitive manganese, however, does not further affect these apparent binding constants.

MATERIALS AND METHODS

Chloroplasts were isolated from market spinach as described previously (Bricker et al., 1985). Oxygen-evolving PS II membranes were isolated as described by Berthold et al. (1981) with modifications described by Ghanotakis and Babcock (1983). Typical preparations had a Chl *a*/*b* ratio of 1.8−2.1. Chlorophyll concentration was determined according to Arnon (1949).

Freshly prepared PS II membranes were treated to remove proteins and/or manganese. PS II membranes which contained four manganese per reaction center were prepared by resuspension of PS II membranes in 2.6 M urea, 200 mM NaCl, 300 mM sucrose, 10 mM $MgCl_2$, and 50 mM Mes-NaOH, pH 6.0, and incubation for 1 h at 4 °C. These membranes, which were depleted of the 33 kDa protein, were then washed three times with 100 mM NaCl, 300 mM sucrose, 10 mM $MgCl_2$, and 50 mM Mes-NaOH, pH 6.0 (incubation buffer), and resuspended at a Chl concentration of 3−5 mg/mL. PS II membranes which contained two manganese per reaction center were prepared by suspending PS II membranes that had been depleted of the 33 kDa extrinsic protein by urea−NaCl washing (above) in a buffer containing 300 mM sucrose, 5 mM NaCl, 10 mM $MgCl_2$, and 50 mM Mes-NaOH, pH 6.0. After incubation, which released the chloride-sensitive manganese, the membranes were washed three times with incubation buffer and resuspended at a Chl concentration of 3−5 mg/mL. PS II membranes that contained zero to one manganese per reaction center were prepared by treatment with 1 M Tris, pH 9.2, 300 mM sucrose, 10 mM $MgCl_2$, 15 mM NaCl, followed by washing in incubation buffer. In this paper these membranes will be referred to as zero-manganese-containing PS II membranes. In all cases, PS II membranes were suspended at a Chl concentration of 1 mg/mL in the appropriate buffer during treatment using a glass homogenizer and incubated twice for 1 h in the dark at 4 °C.

The 33 kDa extrinsic protein was purified as previously described (Xu & Bricker, 1992). Aliquots of the different PS II membrane preparations were resuspended to a final Chl concentration of 30 μg/mL (117 nM PS II reaction centers) in incubation buffer. Purified 33 kDa extrinsic protein at concentrations between 0 and 1400 nM was added and incubated for 1 h at 4 °C in the dark. The reconstituted membranes were collected at 40 000*g* and washed twice with incubation buffer to remove nonspecifically bound 33 kDa extrinsic protein.

The amount of rebound 33 kDa extrinsic protein was determined immunologically essentially as described by Xu and Bricker (1992). 5 μg Chl samples of the reconstituted membranes were electrophoresed in a 12.5%−20% LDS polyacrylamide gradient gel. For calibration of a standard curve, known quantities of the purified 33 kDa extrinsic protein were added to each 5 μg Chl sample of 33 kDa extrinsic protein-depleted PS II membranes. These protein standards were applied to the same polyacrylamide gel. For "Western" blotting the separated proteins were transferred onto polyvinylidine difluoride membranes (Immobilon P, Millipore Corp.) followed by immunodetection (Bricker et al., 1988). The anti-33 kDa extrinsic protein antibody which was produced in rabbits by standard techniques (Harlow & Lane, 1988) was kindly provided by Dr. B. A. Barry. The blots were probed with IgG anti-rabbit peroxidase conjugate and color developed with 4-chloro-1-naphthol. Quantitative analysis of the Western blots was performed with Quantiscan software (Biosoft, Inc.).

The binding constants of the 33 kDa extrinsic protein were determined by the method of Klotz (1990). A graphical method was used to determine the first binding constant. This was followed by an algebraic calculation of the second binding constant. The determination of the first stoichiometric binding constant was on the basis of the following

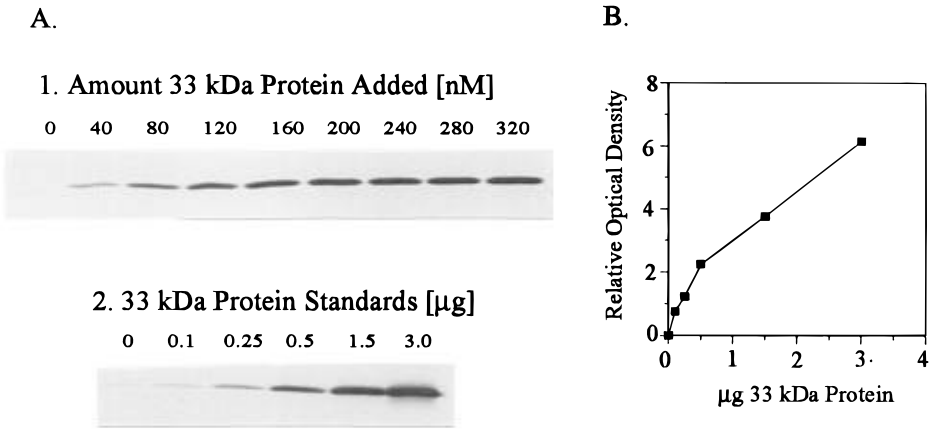

FIGURE 1: Analysis of the binding of the 33 kDa extrinsic protein to PS II membranes. A1: Western blot of samples from PS II membranes containing four manganese per PS II reaction center which were incubated with the given concentrations of the 33 kDa extrinsic protein (0−320 nM). A2: Purified 33 kDa extrinsic protein (with 33 kDa protein-depleted membranes, see Materials and Methods) applied at the indicated concentrations as standards. The amount of bound 33 kDa protein in each PS II membrane preparation was calculated from the calibration curve B, which had been analyzed via the Quantiscan 2 program (Biosoft, Inc.).

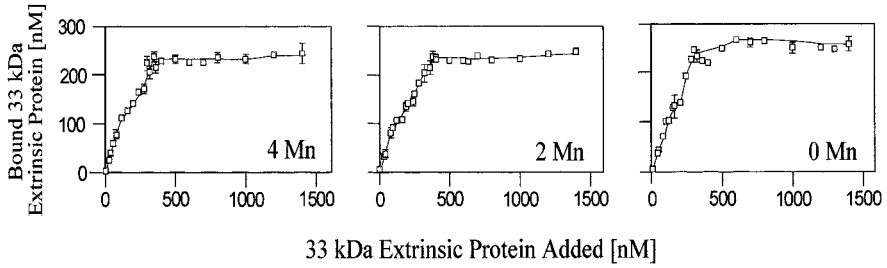

FIGURE 2: Direct plots of the rebinding of the 33 kDa extrinsic protein to PS II membranes containing four, two, or zero manganese per PS II reaction center. Individual plots are labeled in the lower right hand corner. Error bars are shown.

equation: $\lim_{[S_f]\to 0} S_b/E_t \times S_f = K_{a1}$ in which S_b is the concentration of bound ligand, E_t the receptor concentration, S_f the amount of free ligand, and K_{a1} the first stoichiometric binding constant. Generally the first stoichiometric binding constant can be determined with a precision of about 20% using this method. We then calculated the second binding constant (K_{a2}) from an algebraic expression based on the first binding constant, K_{a1}. It describes an equilibrium of substrate to receptor, which is in the same order of magnitude until saturation is reached (Klotz, 1990). At values higher than saturation, the amount of free substrate increases markedly and therefore the value for K_{a2} shifts to lower values, respectively yielding higher dissociation constants. The following equation was used for calculating the second binding constant.

$$K_{a2} = \frac{S_b/E_t + [S_b/E_t - 1] \times K_{a1}[S_f]}{2 - S_b/E_t \times K_{a1}[S_f]^2}$$

S_b is the bound ligand, S_f is the free ligand, and E_t is the receptor concentration.

RESULTS

Figure 1 shows a typical Western blot used to immunoquantitate the rebinding of the 33 kDa extrinsic protein to PS II membranes. In order to analyze the amounts of 33 kDa extrinsic protein bound to the different types of PS II membranes, protein samples and standards were always analyzed from the same gels. It should be noted that in this gel system the 33 kDa extrinsic protein and the D2 protein comigrate. The standard protein lanes, therefore, contain both known amounts of the 33 kDa extrinsic protein and the 5 μg Chl sample of 33 kDa protein-depleted PS II membranes (Xu & Bricker, 1992). The relative optical densities of immunodecorated 33 kDa extrinsic protein standards and the immunodecorated protein present on the PS II membranes were determined by scanning densitometry. Figure 1B illustrates this quantification for the Western blot shown in Figure 1A.

Figure 2 shows a direct plot of the amount of the 33 kDa extrinsic protein bound to PS II membranes containing four, two, and zero manganese per PS II reaction center with increasing 33 kDa extrinsic protein concentrations. In all cases a saturation level was reached. This was verified by examining the semilogarithmic plots of these results (data not shown). For four-manganese-containing PS II membranes the saturation level was reached at a concentration of 360 nM of added 33 kDa extrinsic protein, while the two- and zero-manganese-containing PS II membranes became saturated at a concentration of 500 nM added 33 kDa extrinsic protein. This indicates that the 33 kDa protein does not bind as tightly to PS II membranes depleted of manganese as it does to membranes with an intact manganese cluster. The stoichiometry of bound 33 kDa extrinsic protein per PS II center was calculated to be 2.0−2.1 mol of the 33 kDa extrinsic protein per mol of PS II centers for all three types of PS II membranes (Table 1). These data are in agreement with results from Xu and Bricker (1992). In order to exclude the possibility that weakly bound 33 kDa extrinsic protein was released during the washing procedure, incubation mixtures were kept on ice after the first incubation buffer

Table 1: Summary of the Binding Characteristics of the 33 kDa Extrinsic Protein to Photosystem II Membranes Containing Four, Two, or Zero Manganese

type of PS II membrane	binding constant (nM^{-1})	dissociation constant (nM)	Hill coefficient	stoichiometry[a]
4 Mn	$K_{a1} = 0.42 \pm 0.03$ $K_{a2} = 0.67 \pm 0.20$	$K_{s1} = 2.40 \pm 0.20$ $K_{s2} = 1.49 \pm 0.80$	1.7	2.01 ± 0.08
2 Mn	$K_{a1} = 0.09 \pm 0.00$ $K_{a2} = 0.22 \pm 0.07$	$K_{s1} = 11.1 \pm 0.20$ $K_{s2} = 4.50 \pm 1.43$	1.6	2.03 ± 0.06
0 Mn	$K_{a1} = 0.07 \pm 0.00$ $K_{a2} = 0.25 \pm 0.08$	$K_{s1} = 14.28 \pm 0.10$ $K_{s2} = 4.00 \pm 1.28$	2.2	2.14 ± 0.13

[a] Mol of 33 kDa extrinsic protein/mol of PS II reaction center.

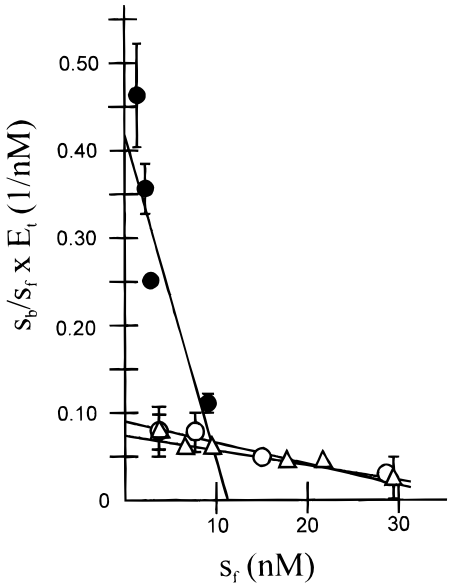

FIGURE 3: Plot of $S_b/S_f \times E_t$ vs S_f. The *Y*-intercept of this plot represents the first stoichiometric binding constant (K_{a1}) for the binding of the 33 kDa protein to the different PS II preparations. Please note that the values for K_{a1} determined by this method are accurate to ±20% (Klotz, 1970). Filled circles, membranes containing four manganese per PS II; open circles, PS II membranes containing two manganese per PS II; and open triangles, PS II membranes containing zero manganese per PS II. Error bars are shown.

wash for 0, 30, 60, and 90 min. No release of bound 33 kDa extrinsic protein was detected (data not shown), indicating that the two copies are tightly bound to the PS II membrane preparations. Our results significantly differ from those obtained by Miyao and Murata (1989a,b) and Enami et al. (1991) who found an equimolar ratio of 33 kDa extrinsic protein to PS II.

Figure 3 shows the plot which was used to estimate the K_{a1} of PS II membranes containing four, two, or zero manganese. The first stoichiometric binding constant was 0.42 nM^{-1} for four-manganese-containing PS II membranes, 0.09 nM^{-1} for two-manganese-containing PS II membranes, and 0.07 nM^{-1} for zero-manganese-containing PS II membranes (Table 1). These binding constants indicate that there is a 5-fold decrease in K_{a1} upon release of the chloride-sensitive manganese. Only a slight further decrease in the K_{a1} was observed upon release of the chloride-insensitive manganese.

The second stoichiometric binding constant (K_{a2}) for each membrane type was then determined algebraically (see Materials and Methods). The K_{a2} for PS II membranes containing four manganese was calculated to be 0.67 nM^{-1}, and membranes containing two or zero manganese exhibited K_{a2} values of 0.22 and 0.25 nM^{-1}, respectively (Table 1). It should be noted that the binding constants which we have determined in this communication almost undoubtedly underestimate the true values. This is due to the necessity of maintaining high chloride concentrations (100 mM) to prevent the loss of the chloride-sensitive manganese in the four-manganese-containing samples. High chloride concentrations will interfere electrostatically with the binding of the 33 kDa protein leading to lower estimates of K_{a1} and K_{a2}.

In all of the PS II membrane types the values observed for the second binding constant are higher than those observed for the first binding constant. This phenomenon is known as positive cooperativity which can be evaluated numerically by use of the Hill plot. Figure 3 shows the Hill plots from data obtained for the 33 kDa extrinsic protein binding to four-, two-, and zero-manganese-containing PS II membranes. In every case a linear relationship was observed with Hill coefficients ranging from 1.6 to 2.2.

DISCUSSION

Stoichiometry of Binding. A summary of our results is shown in Table 1. In agreement with Xu and Bricker (1992), these clearly indicate the presence of two strongly bound copies of the 33 kDa extrinsic protein per PS II reaction center regardless of the number of manganese associated with the reaction center. These findings support the hypothesis of Millner et al. (1987), who identified homodimers of the 24 and 17 kDa extrinsic proteins in cross-linking experiments. Since both of these proteins were present in stoichiometry of 1:1 (Murata et al., 1984) with the 33 kDa extrinsic protein, they hypothesized the existence of two copies of the 33 kDa extrinsic protein per PS II. Additionally, Yamamoto et al. (1987) had developed a high-efficiency purification procedure for the 33 kDa extrinsic protein from PS II membranes. These authors obtained 20 mg of the 33 kDa extrinsic protein from a 100 mg Chl sample of PS II membranes. PS II membranes contain 250−270 Chl per PS II center (Berthold et al., 1981; Babcock et al., 1983; Ghanotakis et al., 1984; Xu & Bricker, 1992). These values yield 1.9−2.0 copies of the 33 kDa extrinsic protein per PS II reaction center. Recently, Betts et al. (1994) have demonstrated that two copies of the 33 kDa extrinsic protein were required to fully reconstitute oxygen evolution in PS II membranes depleted of this protein.

Our results differ significantly from those obtained by Murata et al. (1984), Enami et al. (1991), and Andersson and Styring (1991), who presented data indicating that there was one molecule of the 33 kDa extrinsic protein per PS II center. Murata et al. (1984) used purified 33 kDa extrinsic protein for calibration of polyacrylamide gels and analyzed

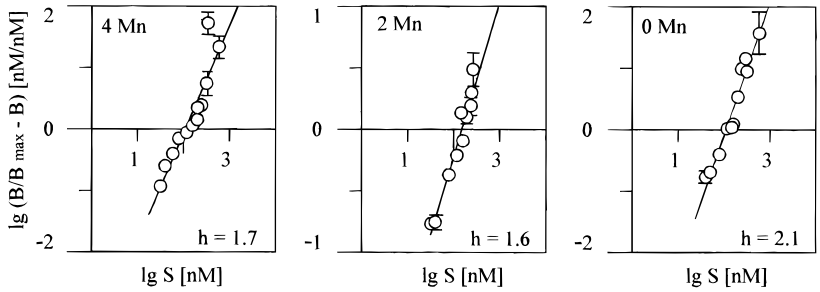

FIGURE 4: Hill plot analysis for PS II membranes containing four, two, and zero manganese per PS II reaction center. *B* is the amount of bound 33 kDa extrinsic protein; B_{max} is the maximal amount of bound 33 kDa extrinsic protein; *S* is the concentration of ligand added; *h* is the Hill coefficient. Individual plots are labeled in the upper left hand corner. Error bars are shown.

the amounts of 33 kDa extrinsic protein by scanning densitometry after Coomassie Blue staining. Since the D1 and D2 proteins migrate in the vicinity of the 33 kDa extrinsic protein on polyacrylamide gels, these protein bands may overlap. Under these conditions an accurate estimation of the amount of 33 kDa protein present may be difficult. Additionally, at low protein concentrations the hydrophilic 33 kDa extrinsic protein does not appear to exhibit quantitative staining (Camm et al., 1987). An underestimation of the amount of bound 33 kDa extrinsic protein could also have been caused by the use of an uncorrected extinction coefficient for the 33 kDa extrinsic protein. An early estimate of this extinction coefficient (20 mM^{-1} cm^{-1}; Kuwabara & Murata, 1984) assumed that this protein had a molecular mass of 33 kDa, the mass which it exhibits on polyacrylamide gels. The actual mass, however, is 26.7 kDa as determined by protein sequencing. This mass yields an extinction coefficient of 16 mM^{-1} cm^{-1} (Oh-oka et al., 1986). Other difficulties with the analysis presented by Murata et al. (1984) have been discussed in more detail previously (Xu & Bricker, 1992).

Enami et al. (1991) analyzed the stoichiometry in cross-linking experiments of 33 kDa extrinsic protein to CP 47 and estimated a 1:1 stoichiometry. This result was based on the simultaneous loss of antibody reactivity against both proteins at their native molecular masses and the appearance of antibody reactivity of both proteins at a higher molecular mass with increasing concentrations of the protein cross-linking reagent EDC. These authors assumed that their polyclonal reagents would react equivalently with the uncross-linked target antigens and the cross-linked complex. They present no evidence, however, that this is the case. Indeed, examination of their Western blots [see Figure 6 in Enami et al. (1991)] indicates that the cross-linked product (labeled A) reacts relatively poorly with their polyclonal antisera directed against CP 47 and the 33 kDa extrinsic protein. It should also be pointed out that their molecular weight estimate of the cross-linked product (80 kDa) is also subject to question. The estimation of the molecular masses of cross-linked complexes is not straightforward since variations in the geometry of cross-linking can lead to variations in the apparent molecular mass. This effect is exacerbated at the high EDC concentration used in this study. In experiments which we performed at significantly lower EDC concentrations (Odom & Bricker, 1992), two cross-linked products were observed at 95 and 110 kDa. These molecular masses are consistent with a CP 47 to 33 kDa extrinsic protein stoichiometry of 1:2.

Andersson and Styring (1991) proposed that one copy of 33 kDa extrinsic protein is bound to the PS II membranes whereas the other is free in the lumenal space. This hypothesis is not consistent with our results, since we have shown that the two copies of the 33 kDa extrinsic protein are tightly bound with no significant loss of rebound 33 kDa extrinsic protein being detected during washing. Additionally, we had earlier demonstrated that an oxygen-evolving reaction center complex also contained two copies of the 33 kDa extrinsic protein per PS II reaction center (Xu & Bricker, 1992).

Manganese Cluster Effects on the Apparent Binding Constants of the 33 kDa Protein. As shown in Figure 2 and as summarized in Table 1, damage or removal of the manganese cluster has a profound effect on the apparent binding constants of the 33 kDa protein to PS II. Removal of the chloride-sensitive manganese by low chloride treatment lowered the K_{a1} 5-fold. A slight additional decrease was observed upon alkaline Tris treatment, which removes three to four of the bound manganese. Decreases in the K_{a2} upon release of the chloride-sensitive manganese was also observed.

Although other investigators have observed decreases in the binding of the 33 kDa protein upon perturbation of the manganese cluster, they were not able to associate these effects specifically with release of the chloride-sensitive manganese pool. Miyao and Murata (1989a,b) estimated the binding constants of the extrinsic proteins to PS II. They observed a binding constant of 0.083 nM^{-1} for binding of the single 33 kDa protein which they observed to PS II preparations containing four manganese and 0.011 nM^{-1} for preparations containing zero manganese. Kavaleki and Ghanotakis (1991) qualitatively examined the interaction of the extrinsic proteins with the intrinsic components of PS II. These authors examined the release of the extrinsic proteins by various salt treatments either with or without the extraction of three to four bound manganese by hydroquinone or hydroxylamine treatment. They concluded that the strength of the interaction of the extrinsic 33 kDa protein (and the 24 and 17 kDa species) with PS II was modulated by the presence of an intact manganese cluster.

We speculate that the release of the chloride-sensitive manganese induces a conformational change in the protein(s) responsible for the binding of the 33 kDa component to PS II. What proteins might be involved in such a conformational change? An obvious candidate would be CP 47. A variety of lines of evidence have indicated that the 33 kDa extrinsic protein is closely associated with this intrinsic chlorophyll-protein (Frankel & Bricker, 1989, 1992; Odom & Bricker, 1992). However, although a conformational change involving the release of the chloride-insensitive manganese has been associated with CP 47 (Frankel &

Bricker, 1989), no conformational change associated with the release of the chloride-sensitive manganese has ever been detected in this or other PS II proteins. It should be noted that in the cyanobacterium *Synechocystis* 6803 we have produced a site-directed alteration $^{448}R \Rightarrow {}^{448}G$ in CP 47 yielding the mutant R448G (Putnam-Evans & Bricker, 1992). This mutant exhibits an almost complete loss of photoautotrophic growth and PS II assembly when grown at limiting chloride concentrations. These results suggest that CP 47 is involved in the chloride requirement for the photosystem. It is possible that the alteration at ^{448}R may influence the stability of the chloride-sensitive pool of manganese within PS II.

Other proteins, in particular D1, have also been implicated in the binding of the 33 kDa extrinsic protein to PS II (Mei et al., 1989). This protein provides the majority of putative ligands to the manganese cluster (Boerner et al., 1992; Chu et al., 1994; Ikeuchi et al., 1988; Nixon & Diner, 1992; Nixon et al., 1992; Seibert et al., 1989). No direct observation of any conformational changes in D1 associated with the release of the manganese, however, has been reported. Finally, it is possible that the 33 kDa protein provides some replaceable ligands to the manganese cluster. Upon removal of the 33 kDa extrinsic protein (by methods which maintain low rates of oxygen evolution; Bricker, 1992), water, chloride, or amino acid residues on other proteins could provide substitute ligands to the manganese. If the manganese cluster was damaged by removal of the chloride-sensitive manganese, then one might observe a decreased strength of binding of the 33 kDa extrinsic protein to PS II.

Cooperativity in the Binding of the Two Copies of the 33 kDa Protein to PS II. The Hill plots shown in Figure 3 demonstrate that the binding of the two copies of 33 kDa extrinsic protein was cooperative ($h = 1.6-2.1$), regardless of the number of manganese associated with PS II. Within the range of error exhibited by this data, we feel that these values are essentially indistinguishable and represent moderate to strong cooperativity in binding of the 33 kDa extrinsic protein on all three types of PS II membranes. The presence of cooperative binding suggests that conformational changes occur upon binding of the first 33 kDa extrinsic protein which enhance the binding of the second. In other words, the two 33 kDa extrinsic protein molecules interact with each other either directly or indirectly (Bohinsky, 1987). These conformational changes are distinct from those hypothesized to occur upon the release of the chloride-insensitive manganese. We were somewhat surprised that cooperativity persisted in a background of the rather significant changes which we observed in K_{a1} (5−6-fold). One hypothesis that could explain these results is that it is possible that supplementary binding sites for the second 33 kDa protein exist on the first bound 33 kDa component. Thus, regardless of the strength of binding of the first subunit, an enhanced binding domain is assembled for the second leading to a cooperative interaction between these two subunits and PS II.

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