Mutation Val235Ala Weakens Binding of the 33-kDa Manganese Stabilizing Protein of Photosystem II to One of Two Sites†

Scott D. Betts,‡,§ Jeannine R. Ross,§ Eran Pichersky,§ and Charles F. Yocum*,§,||

Departments of Biology and Chemistry, The University of Michigan, Ann Arbor, Michigan 48109-1048

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ABSTRACT: The 33-kDa protein of the photosynthetic O_2-evolving complex, also known as manganese stabilizing protein, contributes to the structural stability of the photosystem II tetranuclear Mn cluster and stimulates the water-oxidizing activity of this cluster. Quantification of extrinsic polypeptides in photosystem II has yielded data that support stoichiometries of either one or two copies of each protein per photosystem II reaction center. We recently described the cold-sensitive assembly of a mutant 33-kDa protein with a single amino acid replacement (Val235Ala) [Betts, S. D., Ross, J. R., Pichersky, E., & Yocum, C. F. (1996) *Biochemistry 35*, 6302−6307]. We have extended the characterization of this mutation. When photosystem II membranes depleted of the 33 kDa extrinsic protein are exposed to mixtures of wild type and Val235Ala manganese stabilizing protein, binding of the wild type protein is strongly preferred. If, however, protein containing the Val235Ala mutation is first bound to photosystem II only half of this protein (about 1 mol/mol of photosystem II reaction centers) is susceptible to displacement by the wild type protein, even after multiple exposures to the latter. These results support the conclusion that 2 mol of manganese stabilizing protein are bound per reaction center. Our data show as well that the mutant 33-kDa protein competes with the wild type protein for at least one of two binding sites on photosystem II and that the mutant protein binds tightly to only one of two sites. These results demonstrate that the two binding sites on photosystem II for the 33-kDa protein have different properties with respect to recognition and binding of this protein.

The water-oxidizing activity of photosystem II (PSII)[1] generates the reducing equivalents required for oxygenic photosynthesis. Photosystem II is a multi-subunit pigment−protein complex found in the thylakoid membranes of chloroplasts and is organized functionally into two subcomplexes: a core complex and the O_2-evolving complex. The core complex includes at least six integral membrane subunits, which together bind pigments, quinones, redox-active tyrosine residues, and inorganic cofactors (Mn, Ca^{2+}, and Cl^-). Three peripheral proteins are associated with the core complex where it protrudes from the lumenal side of the thylakoid membrane. This lumenal portion of PSII, called the O_2-evolving complex (OEC), includes the active site of water oxidation shielded by the three extrinsic polypeptides. The oxidation of two water molecules to dioxygen is catalyzed by a metal cluster comprised of four manganese atoms. The two smaller OEC proteins (17 and 23 kDa) regulate retention of the Cl^- and Ca^{2+} atoms (Ghanotakis et al., 1984a; Miyao & Murata, 1984b) that are essential activators of O_2 evolution. The 33-kDa manganese-stabilizing protein (MSP) stabilizes the tetranuclear manganese cluster under physiological salt conditions (Bricker, 1992; Miyao & Murata, 1984c; Ono & Inoue, 1983), and it accelerates O_2 evolution activity (Miyao et al., 1987; Ono & Inoue, 1986).

While the role of MSP in regulating and stabilizing O_2 evolution activity is well-established, less is known about the structure of MSP and of its binding site or sites in PSII. Different methods for quantification of MSP in PSII have produced data to indicate stoichiometries of either one or two molecules per PSII complex (Cammarata et al., 1984; Enami et al., 1992; Millner et al., 1987; Miyao & Murata, 1989; Murata et al., 1984; Xu & Bricker, 1992; Leuschner & Bricker, 1996). Similarly, different methods for identifying structural interactions between MSP and PSII core subunits have demonstrated close association between MSP and any one of three different PSII core subunits (Bricker et al., 1988; Eisenberg-Domovich et al., 1995; Enami et al., 1991; Isogai et al., 1985; Yamamoto & Akasaka, 1995). Leuschner and Bricker (1996) have investigated binding of MSP to PSII preparations containing 4, 2, or 0 Mn atoms. Their data show that two separate sites can be defined for MSP binding to PSII, irrespective of Mn content, on the basis of differing dissociation constants for the two sites.

We recently described a mutant of MSP with a single amino acid substitution (V235A) that exhibits cold-sensitive assembly into the OEC (Betts et al., 1996b). The mutation inhibited assembly of MSP into PSII at 4 °C but subsequent incubation at 22 °C alleviated the assembly defect. Results from circular dichroism spectroscopy revealed that the Val

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* Address correspondence to this author.

‡ Present address: Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139

§ Department of Biology.

|| Department of Chemistry.

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[1] Abbreviations: Bis-Tris, bis[2-hydroxyethyl]iminotris[hydroxymethyl]-methane; BSA, bovine serum albumin; Chl, chlorophyll; DTT, dithiothreitol; MES, 2-[*N*-morpholino]ethanesulfonic acid; MSP, manganese-stabilizing protein; OEC, O_2-evolving complex; PAGE, polyacrylamide gel electrophoresis; pET, plasmid for expression with T7 RNA polymerase; preMSP, psbO gene product and precursor polypeptide of MSP; psbO, cDNA clone encoding preMSP; PS, photosystem; SDS, sodium dodecyl sulfate; TMA^+, tetramethylammonium; Tris, tris-[hydroxymethyl]aminomethane.

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to Ala substitution causes a reduction in the secondary structure content of MSP at both the restrictive (4 °C) and permissive (22 °C) temperatures. Here we report results from detailed binding studies of both wild type MSP and [V235A]MSP which indicate that the structural destabilization induced by the cold-sensitive mutation persists following assembly and affects the interaction between PSII and a subpopulation of bound mutant MSP. These results are consistent with a structural model in which two molecules of MSP occupy nonidentical binding sites on PSII.

MATERIALS AND METHODS

Purification of PSII Membranes. Photosystem II membranes were isolated from thylakoids by detergent extraction according to the method of Berthold et al. (1981) with three modifications (Ghanotakis & Babcock, 1983; Ghanotakis et al., 1984a,b). The 17- and 23-kDa extrinsic subunits were released from PSII membranes by incubation in 2 M NaCl (Betts et al., 1994); this "salt-washed" PSII preparation retains native MSP and was used as the control in reconstitution experiments. Native MSP was extracted from salt-washed PSII membranes by incubation in 2.6 M urea/200 mM NaCl (Betts et al., 1994), yielding "urea-washed" PSII membranes.

Expression and Purification of Recombinant MSP. The bacterial expression of psbO cDNA clones encoding the precursor proteins of spinach MSP and *Arabidopsis* MSP was described previously (Betts et al., 1994, 1996a). Inclusion bodies containing mature MSP were purified from *E. coli* lysates essentially as described in Betts et al. (1994). Inclusion bodies were solubilized in urea, and MSP was renatured by dialysis and purified by anion-exchange chromatography according to Betts et al. (1996a).

Reconstitution of the OEC with Recombinant MSP. Recombinant MSP was mixed with urea-washed PSII membranes and incubated at room temperature (22 °C) for 1 h in the dark. The reconstitution mixture included 50 mM MES-NaOH (pH 6), 0.4 M sucrose, 20 mM $CaCl_2$, 60 mM NaCl, 100 μg of BSA/mL, 2% betaine (w/v), and MSP. Photosystem II membranes were present at 0.2 mg of Chl/mL (1 μM PSII reaction center complexes based on 250 Chl/PSII). All components used in the reconstitution were equilibrated to 22 °C before mixing. Previous reconstitution experiments have shown that spinach wild type MSP and *Arabidopsis* wild type MSP are equally effective at restoring O_2 evolution activity to urea-washed spinach PSII (Betts et al., 1995b).

Analysis of Reconstituted PSII Membranes by SDS−PAGE and Densitometry. The amount of MSP assembled into PSII was determined by laser densitometry of dried polyacrylamide gels. Unbound MSP was removed from reconstitution mixtures by centrifugation (12000*g*, 10 min, 4 °C). Bound MSP was pelleted with PSII, and pellets were washed once in 2 vol of 0.4 M sucrose, 50 mM MES-TMAOH (pH 6), 60 mM TMACl, and 20 mM $CaCl_2$.

Washed reconstituted PSII samples were analyzed by SDS−PAGE (10% acrylamide/4.7 M urea) according to the method of Piccioni et al. (1982) using the Neville buffer system. Polyacrylamide gels were stained with Coomassie Brilliant Blue R-250 or with silver and then analyzed by monitoring absorbance at 663 nm with an LKB 2222-010 UltroScan XL laser densitometer. The relative amounts of MSP present in reconstituted PSII samples were determined by integration of MSP absorbance peaks using GelScan XL software.

For quantitative SDS−PAGE of reconstituted PSII samples, the concentration of MSP standards was determined by the Lowry assay using BSA as a standard (Lowry et al., 1951). Concentrations of MSP standards were also determined based on absorbance at 276 nm using the millimolar extinction coefficient of 16 determined for native spinach MSP (Xu & Bricker, 1992). The concentrations of MSP samples, based on absorbance at 276 nm, were 15−17% lower than the corresponding Lowry values for all MSP preparations used. Lowry values were used because this method is less sensitive to potential changes in extinction coefficient caused by amino acid sequence changes and will permit more direct comparisons of mutational effects on MSP structure and function as additional mutants, including deletion and insertion mutants, are generated and analyzed.

The calculated molecular weights of 26.5 kDa for spinach MSP and 26.7 kDa for *Arabidopsis* MSP were used to convert concentration estimates from units of mg/mL to μM. The amounts of bound spinach wild type MSP, [V235A]-MSP, and *Arabidopsis* MSP were estimated using standards of the corresponding protein analyzed on the same gel. This method avoided possible differences in Coomassie binding among the three proteins. The amount of native spinach MSP present in salt-washed PSII samples was estimated using recombinant wild type spinach MSP as a standard. A linear relationship between Coomassie-staining intensity and the amount of standard protein loaded was observed in all cases. The results of this analysis showed that control salt-washed PSII membranes retained 1.7−1.9 mol of native MSP/mol of PSII; the loss of about 15% of MSP (assuming 2 mol/mol of PSII) is a consequence of extraction of small amounts of MSP concurrent with release of the 23 and 17 kDa proteins by the high-salt treatment used to extract the latter proteins (data not shown).

Other Methods. Chlorophyll was assayed in 80% acetone (v/v) according to Arnon (1949). The concentration of PSII was based on Chl concentrations and a stoichiometry of 250 Chl per PSII complex (Berthold et al., 1981).

RESULTS

[V235A]MSP and Arabidopsis MSP Compete for 1−2 Binding Sites on Spinach PSII. An analysis of the competition between [V235A]MSP and *Arabidopsis* MSP for binding sites on PSII was carried out at 22 °C, the permissive temperature for assembly into the OEC of the mutant protein (Betts et al., 1996b). Wild type *Arabidopsis* MSP was used because it has a greater electrophoretic mobility than spinach MSP but is indistinguishable from wild type spinach MSP in binding and functional analyses (Betts et al., 1995b); maximum separation of spinach and *Arabidopsis* MSP on SDS−PAGE gels is desirable because this facilitates analysis of the corresponding densitometric peaks after Coomassie staining. Figure 1 shows the results of an experiment in which increasing amounts of an equimolar mixture of [V235A]MSP and *Arabidopsis* MSP were incubated with urea-washed (MSP-depleted) PSII at 22 °C. As the figure shows, the *Arabidopsis* protein is preferentially bound to sites on spinach PSII as the total MSP concentration increases above 2 mol/mol of PSII reaction centers. This is particularly evident in

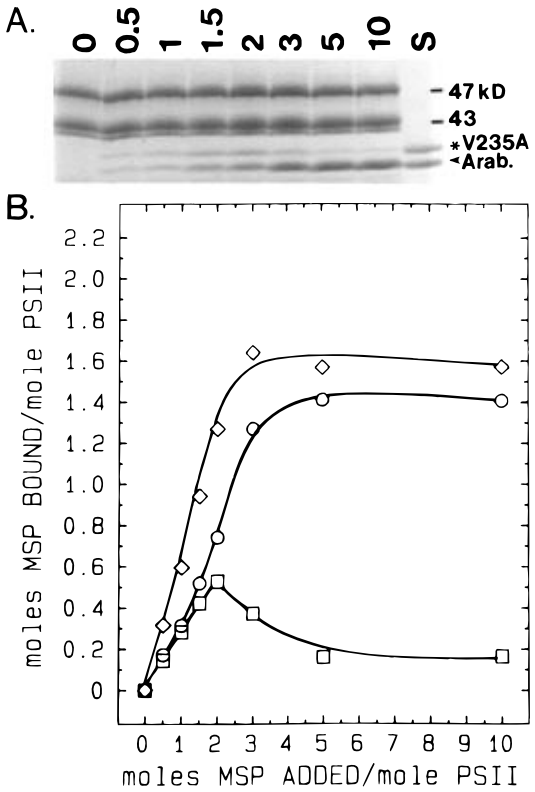

FIGURE 1: Reconstitution of PSII with an equimolar mixture of [V235A]MSP and *Arabidopsis* MSP. (A) Reconstituted and washed PSII samples were analyzed by SDS−PAGE, and proteins were stained with Coomassie Blue. Numbers above lanes (0−10) indicate the total moles of MSP added per mol of PSII (e.g., 2 indicates that 1 mol each of [V235A]MSP and *Arabidopsis* MSP were added per mol of PSII). A sample of the MSP mixture used for the reconstitutions was included as a standard (S). The amount of the 1:1 mixture of MSP in lane S corresponds to the amount added to the PSII sample shown in lane 2 before centrifugation, washing, and resuspension. (B) Densitometric analysis of Coomassie-stained MSP in A. Squares, [V235A]MSP; circles, *Arabidopsis* MSP; diamonds, sum of [V235A]MSP and *Arabidopsis* MSP. Integrated areas for MSP gel bands in lanes 0−10 were divided by the integrated areas for the corresponding bands in lane S (equal to 1 mol each MSP per mol of PSII) to generate the values plotted on the *y*-axis.

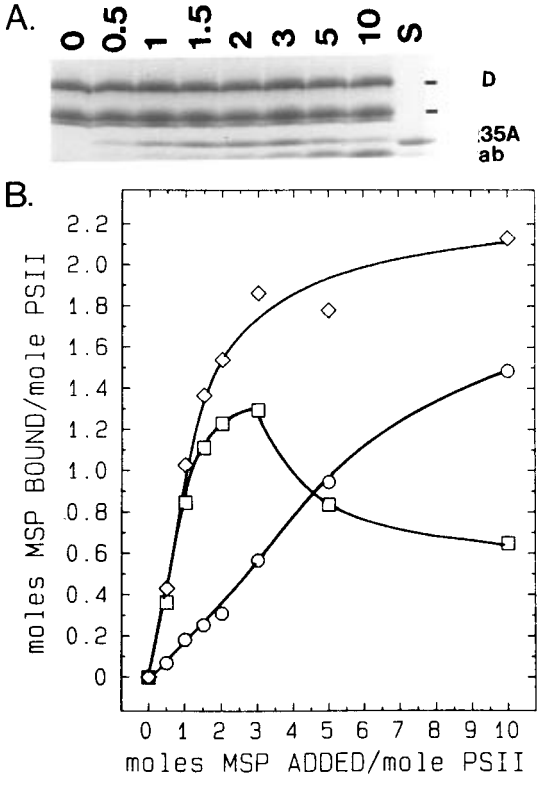

FIGURE 2: Reconstitution of PSII with a 4:1 mixture of [V235A]-MSP and *Arabidopsis* MSP. (A) SDS−PAGE analysis. Numbers above lanes (0−10) indicate the total moles of MSP added per mol of PSII (e.g., 5 indicates that 4 mol of [V235A]MSP and 1 mol of *Arabidopsis* MSP were added per mol of PSII). A sample of the MSP mixture used for the reconstitutions was included as a standard (S). The amount of the 4:1 mixture of MSP in lane S corresponds to the amount added to the PSII sample shown in lane 2 before centrifugation, washing, and resuspension. (B) Densitometric analysis of Coomassie-stained MSP in A. Squares, [V235A]MSP; circles, *Arabidopsis* MSP; diamonds, sum of [V235A]MSP and *Arabidopsis* MSP. Integrated areas for MSP gel bands in lanes 0−10 were divided by the integrated areas for the corresponding band in lane S (equal to 1.6 mol of [V235A]MSP + 0.4 mol of *Arabidopsis* MSP per mol of PSII) to generate the values plotted on the *y*-axis.

the protein quantifications shown in Figure 1B. Substitution of Ala for Val235 thus causes an assembly defect that is retained even at 22 °C, a result consistent with the decrease in secondary structure content of [V235A]MSP measured by circular dichroism spectroscopy at 25 °C (Betts et al., 1996b). The data in Figure 1 show that the amount of both proteins bound to PSII reached a maximum value in the sample reconstituted with a total of 3 mol of added MSP (1.5 mol of each protein added) per mole of PSII, and also that PSII samples exposed to saturating concentrations of MSP, followed by washing to remove non-specifically bound protein, retained 1.6 mol of total MSP per mole of PSII (Figure 1B, diamonds). Both results are consistent with a proposed stoichiometry of more than one MSP per PSII reaction center. The stoichiometry of 1.6 MSP per PSII should represent a minimum value due to partial sample loss during the wash and resuspension steps (see "Quantification of MSP in Reconstituted PSII" below for a more detailed analysis of MSP stoichiometry avoiding complications due to possible sample loss). When 5 and 10 equiv of the equimolar MSP mixture were added per PSII, the *Arabidopsis* protein diminished [V235A]MSP binding to a level of about 10% of the total MSP bound. This result demonstrates direct competition between the two proteins for MSP binding sites on PSII.

A second experiment was conducted in which the ratio of [V235A]MSP to *Arabidopsis* MSP was increased to 4:1. Although these conditions would strongly favor binding of [V235A]MSP, the *Arabidopsis* protein outcompeted the mutant spinach protein for binding sites on PSII (Figure 2A); binding of the wild type protein reached a maximum level at 10 mol of total MSP/mol of PSII, which, after washing, is equivalent to about 1.5 mol of wild type MSP/mol of PSII (diamonds; Figure 2B). This stoichiometry was determined using PSII samples incubated with saturating amounts of MSP and then washed to remove unspecifically bound protein; the actual value determined by densitometry was 1.8−2.1 mol of total MSP per mole (diamonds of Figure 2B). As with the data of Figure 1, these results support a stoichiometry of two MSP per PSII complex. While *Arabidopsis* MSP differs from wild type spinach MSP at 42 out of 247 amino acid residues, Val is present at the position corresponding to V235 in spinach. By contrast, [V235A]MSP differs from the wild type spinach protein by only two methyl groups. Thus, these competitive binding experiments emphasize the importance of Val235 in the assembly of MSP into PSII.

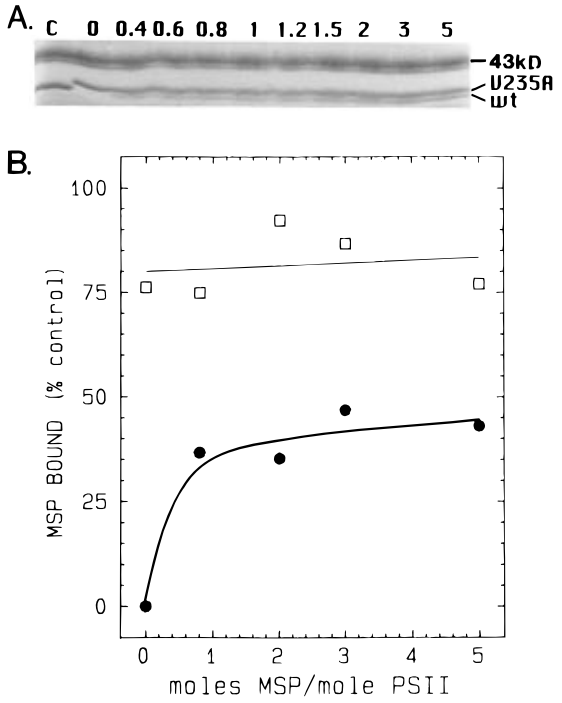

FIGURE 3: Reconstitution of PSII-[V235A] with increasing amounts of wild type MSP. Urea-washed PSII was reconstituted twice with [V235A]MSP (3 mol/mole of PSII) as described in Materials and Methods. The resulting washed PSII-[V235A]MSP preparation was then reconstituted with wild type MSP. (A) SDS−PAGE analysis. C, control salt-washed PSII membranes; 0−5, moles of wild type MSP added per mol of PSII-[V235A]MSP. (B) Densitometric analysis of gel in A. Squares, [V235A]MSP retained; triangles, wild-type MSP bound.

[V235A]MSP Binds Tightly to a Subpopulation of MSP Binding Sites. The ability of [V235A]MSP to saturate MSP binding sites in spinach PSII was tested in the following experiment. Urea-washed PSII membranes were reconstituted in two cycles, each of which employed an excess of [V235A]MSP. Then, the resulting PSII-[V235A]MSP sample was exposed to increasing amounts of wild type spinach MSP under the same reconstitution conditions. The amount of PSII-bound wild type MSP corresponds to the fraction of MSP binding sites that were vacant in the original sample of PSII-[V235A]MSP and/or sites from which the mutant protein was displaced during incubation with wild type protein. The results are shown in Figure 3. While the amount of bound [V235A]MSP remained constant (80% compared to the Coomassie staining intensity of native MSP in salt-washed PSII), the amount of bound wild type MSP increased to a steady value of about 33% based on the same standard. Because the amount of bound [V235A]MSP remained constant in all samples, independent of the amount of wild type protein added, we conclude that approximately 33% of MSP binding sites were vacant in the [V235A]-saturated samples. The result further suggests two possibilities with regard to the original sample of PSII-[V235A]MSP: either both sites for MSP binding were only about 67% saturated or one site was saturated with the mutant protein and the second site was only 35−40% occupied. The latter assumption requires that the mutant protein have a high affinity for one site and a low affinity for the other.

The stability of the binding interaction between [V235A]-MSP and PSII was tested by subjecting samples of PSII-[V235A]MSP to several consecutive exposures to either wild type spinach or *Arabidopsis* MSP. When PSII-[V235A]MSP was exposed twice to either wild type spinach or *Arabidopsis* MSP, bound wild type protein was easily detectable by visual inspection of silver-stained polyacrylamide gels (Figure 4B and D, lanes 1 and 2). Relatively small changes were observed in the amount of [V235A]MSP retained by PSII and also in the amount of wild type MSP bound as a consequence of subsequent cycles of reconstitution with the wild type protein (Figure 4B and D, lanes 3−7). In control experiments, PSII samples reconstituted with either wild type spinach MSP or *Arabidopsis* MSP were next exposed to [V235A]MSP. Binding of [V235A]MSP to PSII in these experiments was not visually detectable until at least three or more reconstitution cycles were completed (Figure 4A and C). These observations confirmed the demonstration in Figure 3 of significant numbers of vacant binding sites in samples of PSII saturated with [V235A]MSP. The control experiments, on the other hand, show that PSII reconstituted under the same conditions with either wild type spinach MSP or *Arabidopsis* MSP does not have a significant population of vacant MSP binding sites.

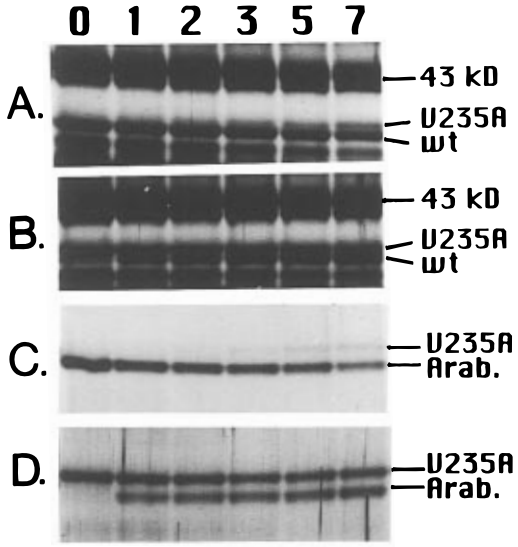

FIGURE 4: Identification of vacant MSP binding sites on PSII-[V235A]MSP. (A) PSII-[wild type]MSP + [V235A]MSP. (B) PSII-[V235A]MSP + wild-type MSP. (C) PSII-[*Arabidopsis*]MSP + [V235A]MSP. (D) PSII-[V235A]MSP + *Arabidopsis* MSP. Urea-washed PSII membranes were reconstituted with wild type MSP (3 mol per mol of PSII), *Arabidopsis* MSP (3/PSII), or [V235A]MSP (5/PSII); the reconstitutions were repeated once for wild-type and for *Arabidopsis* MSP and twice for [V235A]MSP to ensure that MSP binding sites were saturated. Samples were then subjected to seven reconstitution cycles. PSII-[V235A]MSP was reconstituted with wild type spinach MSP or *Arabidopsis* MSP at a ratio of two MSP to 1 PSII. In the reciprocal experiments, PSII-[wild type]MSP and PSII-[*Arabidopsis*]MSP were reconstituted with [V235A]MSP at a ratio of three mutant MSP to one PSII. Each reconstitution cycle involved 15 min incubation at 22 °C, centrifugation, and washing (see Materials and Methods). Washed samples taken after each reconstitution cycle were analyzed directly (A and B) or treated further. Partial sample loss is evident with each reconstitution/wash step as decreasing staining intensity of the intrinsic 43 kDa PSII core protein. To avoid the possible overlap of *Arabidopsis* MSP and the PSII core protein of similar size, samples from experiments using *Arabidopsis* MSP were incubated in 3 M urea to release MSP; supernatant fractions were then analyzed (C and D). Proteins were visualized by silver staining. Lanes 0−7, number of reconstitution cycles.

The relative staining intensities of *Arabidopsis* MSP and [V235A]MSP present in individual gel lanes in Figure 4C

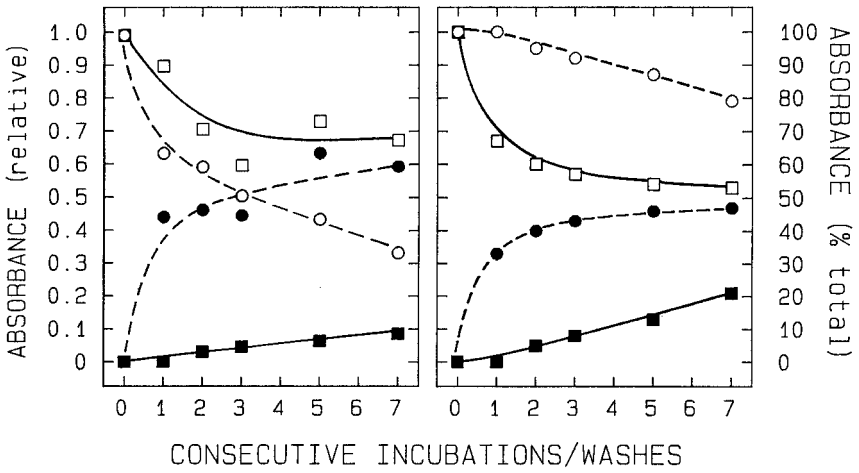

FIGURE 5: Reconstitution of PSII-[V235A]MSP with wild type MSP: densitometric analysis of [V235A]MSP and *Arabidopsis* MSP in gels shown in Figure 4C and D. (A) Relative staining intensities of MSP in gel bands were normalized based on the A663 of the gel band in Figure 4C (*Arabidopsis* MSP) and 4D ([V235A]MSP). Open circles, *Arabidopsis* MSP retained by PSII (Figure 4C); solid squares, [V235A]MSP bound to PSII-[*Arabidopsis*]MSP (Figure 4C); Open squares, [V235A]MSP retained by PSII (Figure 4C); solid circles, *Arabidopsis* MSP bound to PSII-[V235A]MSP. (B) The data in A were replotted to correct for sample loss due to washing and to show the ratio of the two proteins in each gel lane. 100% corresponds to the total A663 of the two MSP gel bands for each lane. Symbols same as in A.

and D are plotted in Figure 5A. Only trace amounts of [V235A]MSP bound to samples of PSII-[*Arabidopsis*]MSP after two reconstitution cycles (solid squares). In contrast, a sample of PSII-[V235A]MSP exposed to one reconstitution cycle with *Arabidopsis* MSP (solid circles) retained about 30% of this wild type protein after just one reconstitution cycle, as evidenced by the silver staining intensity. This is the same result as shown in Figure 3, where spinach wild type MSP constituted about one-third of the total MSP Coomassie staining intensity in saturated PSII samples. The relative staining intensity of the *Arabidopsis* protein increased to 40% after two reconstitution cycles and then approached a steady value of about 50% after additional rounds of reconstitution (Figure 5B).

Quantification of MSP in Reconstituted PSII. Chlorophyll concentrations of photosystem II samples taken at the beginning and end of the seven reconstitution cycles (Figure 4) were measured, and 18 μg of Chl of each sample was loaded on an SDS gel for quantitative analysis of MSP. The amount (μg) of each MSP present was estimated on the basis of the standard curves of samples run on the same gel. Each standard curve was generated from three internal standards of either wild-type MSP, [V235A]MSP, or *Arabidopsis* MSP (not shown). Stoichiometries are reported in Table 1. As noted in Materials and Methods, control salt-washed PSII membranes retained 1.7−1.9 mol of native MSP per mol of PSII (Table 1). Photosystem II samples reconstituted with either wild type protein bound 1.8 mol of MSP per mol of PSII after seven reconstitution cycles with [V235A]MSP (Table 1). In contrast, the stoichiometry of [V235A]MSP in PSII decreased from maximum values of 1.3 and 1.6 per PSII before the first reconstitution cycle with wild type protein to, in both cases, 1.1 equiv per PSII after the seventh cycle (Table 1), respectively. To summarize, PSII samples reconstituted with wild type protein and then subjected to seven reconstitution/wash cycles with [V235A]MSP retained nearly two copies of either wild type spinach MSP or *Arabidopsis* MSP. Photosystem II samples reconstituted with [V235A]MSP and subjected to seven reconstitution/wash cycles with wild type protein retained only one copy of [V235A]MSP.

Table 1: Stoichiometry of MSP in Reconstituted PSII

PSII preparation	1st MSP added	2nd MSP added	amount of bound MSP/PSII[a]	
			1st MSP	2nd MSP
salt-washed	none	none	1.8 (native)	−
urea-washed	wt	none	1.6[b] (wt)	−
	wt	V235A	1.8[c] (wt)	0.6 (V235A)
	Arab.	none	1.9[b] (*Arab.*)	−
	Arab.	V235A	1.8[c] (*Arab.*)	1.2 (V235A)
	V235A	none	1.3[b] (V235A)	−
	V235A	wt	1.1[c] (V235A)	1.8 (wt)
	V235A	none	1.6[b] (V235A)	−
	V235A	*Arab.*	1.1[c] (V235A)	1.1 (*Arab.*)

[a] The values reported here were obtained using MSP concentrations estimated from Lowry assays. See Materials and Methods for comparison of MSP concentrations obtained by the colorimetric assay and by UV absorbance measurements at 276 nm. [b] MSP present before reconstitution cycles. [c] MSP retained after seven reconstitution cycles.

The net gain of MSP estimated from all four reconstitution/wash experiments shown in Figure 4 is presented in Table 1. The total MSP retained at the end of these experiments was between 2.2 and 3.0 mol per mol of PSII. We cannot exclude the possibility of a third, weak binding site for MSP that becomes saturated only after several reconstitution cycles. However, because binding of 3 mol of MSP per mol of PSII was only observed under extreme conditions where PSII membranes were repeatedly exposed to high concentrations of MSP, it is probable that the association between PSII and the third copy of MSP is an in vitro artifact. For example, repeated reconstitution and washing cycles with MSP removes residual detergent from PSII membranes, and this might favor non-specific association between MSP and PSII. Consequently, we have emphasized the number of MSP *retained* following seven cycles of reconstitution and washing (see previous paragraph) and not the total MSP bound.

DISCUSSION

[V235A]MSP Binds Tightly to Only One of Two MSP Binding Sites. The reconstitution data presented here and in Betts et al. (1996a,b) are consistent with proposals

(Cammarata et al., 1984; Millner et al., 1987; Xu & Bricker, 1992) of a stoichiometry of two MSP per PSII. In competition experiments, *Arabidopsis* MSP almost completely prevented binding of [V235A]MSP to PSII, consistent with direct competition between the two proteins for the same two binding sites on PSII. The stoichiometry of [V235A]-MSP in reconstituted PSII was reduced to a stable value of 1.1 per PSII following two to three reconstitution/wash cycles employing wild type protein. In control experiments, 1.8 mol of wild type spinach or *Arabidopsis* MSP were retained per mol of PSII following seven consecutive cycles. These results are consistent with a model in which [V235A]MSP can bind stably to only one of two MSP binding sites on PSII. We concluded previously that the V235A mutation destabilized a structural feature in MSP, possibly a β-sheet, which normally lowers the activation energy barrier for assembly of MSP into PSII (Betts et al., 1996b). The results presented here indicate that the same structural feature may be required for stable binding of MSP to only one of its two sites on PSII. Furthermore, the [V235A]MSP binding data suggest that the two PSII binding sites for MSP differ either in structure or in amino acid sequence or in both. One PSII MSP binding site is unable to bind [V235A]MSP with high affinity, whereas a second binding site can accommodate this mutation provided assembly of the altered protein is allowed to proceed in the absence of wild type protein.

We would emphasize that our data cannot distinguish between outright high-affinity binding of [V235A]MSP to one of two PSII sites or the displacement of mutant protein from one site by wild type protein which then stabilizes binding of the remaining copy of the mutant protein at a second site. In this scenario, binding of wild type protein to one site would strengthen binding of the mutant protein, already present, at the other site. This would require a cooperativity between binding sites, and such cooperativity is indicated by the results of Leuschner and Bricker (1996).

Mechanism of [V235A]MSP Binding to PSII. The data of Figures 1 and 2 show that the native solution conformation of MSP confers on the protein a structure which is preferentially assembled into PSII binding sites, even in the presence of as much as a 4-fold excess of the mutant [V235A]MSP. It is clear from these experiments that even if the mutant protein attaches to a PSII binding site, this interaction is non-productive in the presence of protein bearing the wild type solution conformation. A very different situation is observed, however, if mutant protein is allowed to bind to PSII sites in the absence of the wild type species. The data of Figures 3−5 show that once bound to one of two PSII sites, [V235A]MSP cannot be displaced by extensive exposure of the reconstituted O_2-evolving complex to wild type protein. Since [V235A]MSP can assemble with high affinity in the absence of wild type protein, it seems likely that assembly of the mutant protein proceeds in a two-step process. The first step consists of attachment of MSP to its binding site on PSII, and the second step involves a structural rearrangement of the protein that results in a high-affinity interaction with the membrane-associated binding site. This rearrangement can only occur after attachment to the PSII complex, as evidenced by the ability of wild type protein to interfere with [V235A]MSP assembly, and can only occur at one of the two MSP binding sites on PSII.

Structural Implications for the Oxygen-Evolving Complex. The existence of non-identical MSP binding sites may be a consequence of the pseudo-2-fold symmetry within the core complex of PSII. The three-dimensional crystal structure of the photosynthetic reaction center from *Rhodopseudomonas viridis* revealed that the L and M subunits are positioned symmetrically with respect to each other (Deisenhofer et al., 1985). The core complex of PSII includes six subunits which can be grouped into three pairs on the basis of shared structural and functional properties. The two largest subunits of the PSII core complex, CP47 (47 kDa) and CP43 (43 kDa), bind antennae Chl *a* molecules (Bricker, 1990), while the smallest pair of subunits are the α (9 kDa) and β (4.5 kDa) subunits of cyt b_{559}. The remaining pair of subunits is the homologous polypeptides called D1 (32 kDa) and D2 (34 kDa), proposed by Michel and Deisenhofer (1988) to be arranged with the same approximate 2-fold symmetry that exists between L and M.

Recently, Xu and Bricker (1992), on the basis of the structural and functional pairing of PSII core subunits, suggested that all of the protein components of the PSII core complex might also be arranged with approximate pseudo-2-fold symmetry. The observation that two molecules of each extrinsic polypeptide are present in PSII suggested that the hypothesized 2-fold symmetry of the core complex extends to the OEC as well (Xu & Bricker, 1992). The possibility that the entire PSII complex is arranged with pseudo-2-fold symmetry has interesting implications for the structure and location of the tetranuclear Mn cluster. Biochemical observations indicate that the four PSII Mn atoms can be grouped into pairs on the basis of similarities in structural and chemical properties: two of four Mn are lost from PSII following extraction of MSP (Kuwabara et al., 1985; Miyao & Murata, 1984a; Ono & Inoue, 1984); and two of four Mn are more susceptible to reduction by hydroquinone (Mei & Yocum, 1992). Results from spectroscopic analyses of the Mn cluster also favor a symmetrical dimer-of-dimers arrangement of the PSII Mn cluster (Yachandra et al., 1993). The likelihood of structural symmetry within the OEC suggests the possibility that the pairs of extrinsic polypeptides are organized with respect to a symmetry axis that passes between Mn dimers. A symmetrical model for the OEC, including four Mn and two copies each of the three extrinsic polypeptides, was originally proposed by Cammarata and colleagues (1984).

Cross-linking and chemical modification studies indicate that MSP interacts not only with CP47, as mentioned above, but also with its structural and functional counterpart, CP43 (Enami et al., 1989; Frankel & Bricker, 1992, 1995; Hayashi et al., 1993; Isogai et al., 1985; Yamamoto & Akasaka, 1995). Based on the argument for approximate C_2 symmetry in both the core complex and the OEC, we suggest that the second copy of MSP binds to CP43 (site 2). A model of the OEC in which one copy of MSP binds to CP47 and the second copy to CP43 is consistent with results from cross-linking and protein modification studies as well as with the results described here. Binding sites for the same protein on related but nonidentical subunits would require different sets of protein−protein interactions. In this context, the V235A mutation would destabilize a structure in MSP required for a protein−protein interaction specific to one of its two PSII binding sites.

[V235A]MSP restores O_2 evolution activity to the same extent as wild type MSP (Betts et al., 1996b). In view of the results presented here, we conclude that the mutant

protein is able to effectively restore O_2 evolution activity by binding efficiently to only one site in PSII. A small but significant difference was reported in the amount of added MSP required to restore maximum rates of O_2 evolution (1.2−1.6 mol/mol of PSII) and the amount required to saturate binding or stabilize O_2 evolution activity under intense illumination (at least 2 mol/mol of PSII) (Betts et al., 1995a). This difference was interpreted as evidence that the two molecules of MSP in PSII serve separate functions, and it was proposed that one molecule regulates O_2 evolution while the other simply provides structural support. The results presented in this report demonstrate that the two binding sites for MSP are not identical and support a model of the OEC in which MSP bound at the two sites serves different functions. It seems reasonable to propose that the sites at which [V235A]MSP binds with high affinity is the site that regulates O_2 evolution, although this remains to be conclusively demonstrated.

This model of one regulatory MSP and one structural MSP in the OEC implies an analogy with known structure−function relationships within the bacterial photosynthetic reaction center. Although the electron transfer cofactors associated with the L and M subunits are identical and arranged with approximate C_2 symmetry, only the cofactors associated with the L subunit actually transfer electrons (Heller et al., 1995). The corresponding and identical cofactors on the M subunit serve no apparent function other than providing structural support. By analogy, one copy of MSP bound to CP47 may be sufficient to accelerate O_2 evolution, while the second copy of MSP, bound to CP43, stabilizes this activity by occupying the complementary position in the structure. A logical extension of this hypothesis is that only two of the four Mn in PSII catalyze water oxidation while the other two Mn atoms provide complementary structural support.

REFERENCES

Arnon, D. I. (1949) *Plant Physiol. 24*, 1−15.

Berthold, D. A., Babcock, G. T., & Yocum, C. F. (1981) *FEBS Lett. 134*, 231−234.

Betts, S. D., Hachigian, T. M., Pichersky, E., & Yocum, C. F. (1994) *Plant Mol. Biol. 26*, 117−130.

Betts, S. D., Ross, J. R., Pichersky, E., & Yocum, C. F. (1995a) in *Photosynthesis: From Light to Biosphere* (Mathis, P., Ed.) Vol. 2, pp 381−384, Kluwer Academic Publishers, Dordrecht.

Betts, S. D., Ross, J. R., Pichersky, E., & Yocum, C. F. (1995b) in *Photosynthesis: From Light to Biosphere* (Mathis, P., Ed.) Vol. 2, pp 385−388, Kluwer Academic Publishers, Dordrecht.

Betts, S. D., Ross, J. R., Hall, K. U., Pichersky, E., & Yocum, C. F. (1996a) *Biochim. Biophys. Acta 1274*, 135−142.

Betts, S. D., Ross, J. R., Pichersky, E., & Yocum, C. F. (1996b) *Biochemistry 35*, 6302−6307.

Bricker, T. M. (1990) *Photosynth. Res. 24*, 1−13.

Bricker, T. M. (1992) *Biochemistry 31*, 4623−4628.

Cammarata, K., Tamura, N., Sayre, R., & Cheniae, G. (1984) in *Advances in Photosynthesis Research* (Sybesma, C., Ed.) Vol. 1, pp 311−320, Martinus Nijhoff/Dr. W. Junk Publishers, The Hague.

Deisenhofer, J., Epp, O., Miki, K., Huber, R., & Michel, H. (1985) *Nature (London) 318*, 618−624.

Eisenberg-Domovich, Y., Oelmuller, R., Herrmann, R. G., & Ohad, I. (1995) *J. Biol. Chem. 270*, 30181−30186.

Enami, I., Miyaoka, T., Mochizuki, Y., Shen, J.-R., Satoh, K., & Katoh, S. (1989) *Biochim. Biophys. Acta 973*, 35−40.

Enami, I., Kaneko, M., Kitamura, N., Koike, H., Sonoike, K., Inoue, Y., & Katoh, S. (1991) *Biochim. Biophys. Acta* 1060, 224−232.

Enami, I., Ohta, S., Mitsuhashi, S., Takahashi, S., Ikeuchi, M., & Katoh, S. (1992) *Plant Cell Physiol. 33*, 291−297.

Frankel, L. K., & Bricker, T. M. (1992) *Biochemistry 31*, 11059−11064.

Frankel, L. K., & Bricker, T. M. (1995) *Biochemistry 34*, 7492−7497.

Ghanotakis, D. F., & Babcock, G. T. (1983) *FEBS Lett. 153* (1), 231−234.

Ghanotakis, D. F., Babcock, G. T., & Yocum, C. F. (1984a) *FEBS Lett. 167*, 127−130.

Ghanotakis, D. F., Babcock, G. T., & Yocum, C. F. (1984b) *Biochim. Biophys. Acta 765*, 388−398.

Hayashi, H., Fujimura, Y., Mohanty, P. S., & Murata, N. (1993) *Photosyn. Res. 36*, 35−42.

Heller, B. A., Holten, D., & Kirmaier, C. (1995) *Science 269*, 940−945.

Isogai, I., Yamamoto, Y., & Nishimura, M. (1985) *FEBS Lett. 187*, 240−244.

Kuwabara, T., Miyao, M., Murata, T., & Murata, N. (1985) *Biochim. Biophys. Acta 806*, 283−289.

Leuschner, C., & Bricker, T. M. (1996) *Biochemistry 35*, 4551−4557.

Lowry, O. H., Rosebough, N. J., Farr, A., & Randall, R. L. (1951) *J. Biol. Chem. 193*, 265−275.

Mei, R., & Yocum, C. F. (1992) *Biochemistry 31*, 8449−8454.

Michel, H., & Deisenhofer, J. (1988) *Biochemistry 27*, 1−7.

Millner, P. A., Gogel, G., & Barber, J. (1987) *Photosynth. Res. 13*, 185−198.

Miyao, M., & Murata, N. (1984a) *Biochim. Biophys. Acta 765*, 253−257.

Miyao, M., & Murata, N. (1984b) *FEBS Lett. 168*, 118−120.

Miyao, M., & Murata, N. (1984c) *FEBS Lett. 170*, 350−354.

Miyao, M., & Murata, N. (1989) *Biochim. Biophys. Acta 977*, 315−321.

Miyao, M., Murata, N., Lavorel, J. Maison-Peteri, B., Boussac, A., & Etienne, A.-L. (1987) *Biochim. Biophys. Acta 890*, 151−159.

Murata, N., Miyao, M., Omata, T., Matsunami, H., & Kuwabara, T. (1984) *Biochim. Biophys. Acta 765*, 363−369.

Odom, W. R., & Bricker, T. M. (1992) *Biochemistry 31*, 5616−5620.

Ono, T.-A., & Inoue, Y. (1983) *FEBS Lett. 164*, 255−259.

Ono, T.-A., & Inoue, Y. (1984) *FEBS Lett. 168*, 281−286.

Ono, T.-A., & Inoue, Y. (1986) *Biochim. Biophys. Acta 850*, 380−389.

Piccioni, R., Bellemare, G., & Chua, N.-H. (1982) in *Methods in Chloroplast Molecular Biology* (Edelman, M., Hallick, R. B., & Chua, N.-H., Eds.) pp 985−1014, Elsevier Biomedical Press, Amsterdam.

Saner, R., Nickel, S., & Coolness, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463−5467.

Studier, F. W., Rosenberg, A. H., Dunn, J. J., & Dubendorff, J. W. (1990) *Methods Enzymol. 185*, 60−89.

Tyagi, A., Hermans, J., Steppuhn, J., Jansson, Ch., Vater, F., & Herrmann, R. G. (1987) *Mol. Gen. Genet. 207*, 288−293.

Xu, Q., & Bricker, T. M. (1992) *J. Biol. Chem. 267*, 25816−25821.

Yachandra, V. K., DeRose, V. J., Latimer, M. J., Mukerji, I., Sauer, K., & Klein, M. P. (1993) *Science 260*, 675−679.

Yamamoto, Y., & Akasaka, T. (1995) *Biochemistry 34*, 9038−9045.

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