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The *psbo1* Mutant of *Arabidopsis* Cannot Efficiently Use Calcium in Support of Oxygen Evolution by Photosystem II*

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The *Arabidopsis thaliana* mutant *psbo1* contains a point mutation in the *psbO-1* gene (At5g66570) leading to the loss of expression of the PsbO-1 protein and overexpression of the PsbO-2 protein (Murakami, R., Ifuku, K., Takabayashi, A., Shikanai, T., Endo, T., and Sato, F. (2002) *FEBS Lett.* 523, 138–142). Previous characterization of fluorescence induction and decay kinetics by our laboratory documented defects on both the oxidizing and reducing sides of Photosystem II. Additionally, anomalous flash oxygen yield patterns indicated that the mutant contains a defective oxygen-evolving complex that appears to exhibit anomalously long-lived S_2 and S_3 oxidation states (Liu, H., Frankel, L. K., and Bricker, T. M. (2007) *Biochemistry* 46, 7607–7613). In this study, we have documented that the S_2 and S_3 states in *psbo1* thylakoids decay very slowly. The total flash oxygen yield of the *psbo1* mutant was also significantly reduced, as was its stability. Incubation of *psbo1* thylakoids at high NaCl concentrations did not increase the rate of S_2 and S_3 state decay. The oxygen-evolving complexes of the mutant did, however, exhibit somewhat enhanced stability following this treatment. Incubation with $CaCl_2$ had a significantly more dramatic effect. Under this condition, both the S_2 and S_3 states of the mutant decayed at nearly the same rate as the wild type, and the total oxygen yield and its stability following $CaCl_2$ treatment were indistinguishable from that of the wild type. These results strongly suggest that the principal defect in the *psbo1* mutant is an inability to effectively utilize the calcium associated with Photosystem II. We hypothesize that the PsbO-2 protein cannot effectively sequester calcium at the oxygen-evolving site.

Photosystem II (PS II)[2] functions as a light-driven, water-plastoquinone oxidoreductase. In higher plants and cyanobacteria at least six intrinsic proteins appear to be required for O_2 evolution (1–3). These are CP47, CP43, D1, D2, and the α and β subunits of cytochrome b_{559}. Deletion of these subunits uniformly results in the loss of PS II function and assembly (4). Additionally, in higher plants, three extrinsic proteins, with apparent molecular masses of 33 kDa (PsbO), 24 kDa (PsbP), and 16 kDa (PsbQ), are also required for maximal rates of O_2 evolution at physiological inorganic cofactor concentrations. Of these three proteins, the PsbO protein appears to play a central role in the stabilization of the manganese cluster, is essential for efficient and stable O_2 evolution, and is required, along with PsbP, for photoautotrophic growth and PS II assembly in higher plants propagated under normal growth conditions (5, 6).

In *Arabidopsis thaliana*, two genes that encode PsbO (*psbO-1*, At5g66570 and *psbO-2*, At3g50820) are normally expressed, yielding two different PsbO proteins (PsbO-1 and PsbO-2, respectively). A highly fluorescent mutant, *psbo1*, was recently identified in which a stop codon has been introduced in the *psbO-1* gene by ethane methylsulfonate mutagenesis at amino acid residue 74 of the mature PsbO protein ($Gln^{74} \rightarrow$ Stop), which leads to the loss of this component (7). The mutant exhibits a lower variable fluorescence yield (F_V/F_M), lower rates of steady state O_2 evolution, and retarded growth. It was demonstrated that PsbO-1 is the major isoform in the wild type under normal growth conditions and that in the *psbo1* mutant the PsbO-2 protein is up-regulated in a semicompensatory manner. The mechanism that leads to the increased expression of the PsbO-2 protein in the *psbo1* mutant is unclear at this time. Domain-swapping analysis followed by *in vitro* reconstitution experiments were interpreted as indicating that two amino acid differences between the PsbO-1 and PsbO-2 components, $Val^{186} \rightarrow$ Ser and $Leu^{246} \rightarrow$ Ile, could explain the functional differences between the two PsbO proteins (8). It was concluded that inherent functional defects of this component are responsible for the phenotype observed in the mutant *psbo1*. Further characterization by our laboratory (9) indicated that the *psbo1* mutant possesses significant functional defects on both the reducing and oxidizing sides of the photosystem. During fluorescence induction, the *psbo1* mutant exhibited an enhanced O-to-P transition. Additionally, the J-to-I transition accounted for a $<2\%$ rise of the total fluorescence yield, whereas in the wild type, this transition accounted for $>30\%$ of the total fluorescence yield. Analysis of the flash-induced fluorescence rise in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea indicated that in the wild type the ratio of PS II_α to PS II_β reaction centers is $\sim$1.2, whereas in the mutant, the ratio is $\sim$0.3. Fluorescence decay kinetics in the absence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea indicated that electron transfer to Q_B was significantly altered in the mutant. Fluorescence decay kinetics in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea indicated that the charge recombination between Q_A^- and the higher S states of the oxygen-evolving

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[2] The abbreviations used are: PS II, Photosystem II; S_x states, oxidation states of the oxygen-evolving complex; Tricine, *N*-tris(hydroxymethyl)-methylglycine.

complex was retarded. Interestingly, flash oxygen yield analysis indicated that, after 5 min of dark incubation, a higher proportion of PS II reaction centers were in the S_2 and S_3 states. In the wild type, 6 ± 8% of the oxygen-evolving PS II reaction centers were in the higher S states, whereas in the mutant, this value was 28 ± 8%. These latter two observations appeared to indicate that the higher S states were more stable in the *psbo1* mutant than in the wild type.

In this study, we performed a detailed analysis of the S_2 and S_3 state lifetimes in *psbo1* and wild type thylakoid membranes. We also examined the total flash oxygen yield and its stability during incubation at room temperature. Our findings indicate that both the S_2 and S_3 states decay extremely slowly in *psbo1*. The oxygen-evolving complex in the *psbo1* mutant is also quite unstable and rapidly loses its ability to evolve oxygen. Incubation of *psbo1* thylakoids at a high NaCl concentration did not increase the rate of S_2 and S_3 state decay or the total oxygen yield, but the stability of the oxygen-evolving complex was somewhat enhanced. Incubation with a moderate concentration of $CaCl_2$ had a significantly more dramatic effect. Under this condition, the S_2 and S_3 states of the mutant decayed nearly as rapidly as in the wild type. Additionally, the total flash oxygen yield and its stability in *psbo1* following $CaCl_2$ treatment were indistinguishable from the wild type. Steady state oxygen evolution experiments in large measure support these findings. These results strongly suggest that the principal defect in the *psbo1* mutant is an inability to effectively utilize the calcium cofactor associated with PS II. We hypothesize that the *Arabidopsis* PsbO-2 protein cannot effectively sequester this ion in the vicinity of the oxygen-evolving site.

EXPERIMENTAL PROCEDURES

Plant Materials—Seeds of wild type *A. thaliana* (var. *Landsberg erecta*) and the mutant *psbo1* were germinated on potting mixture and grown at 19–22 °C under 50–80 μmol photons m^{-2} s^{-1} white light with an 8-h light/16-h dark diurnal cycle. This suppressed flowering and maximized the yield of leaf material. Only leaves of mature plants were used in these experiments.

Flash Oxygen Yield Experiments—Thylakoids were isolated by grinding leaves in a blender with a buffer containing 0.45 M sorbitol, 10 mM EDTA, 0.1% bovine serum albumin, 1% polyvinylpyrrolidone, and 20 mM Tricine-NaOH, pH 8.4. The homogenate was filtered through two layers of cheesecloth and one layer of Miracloth (Calbiochemical Co.), and the thylakoids were pelleted by centrifugation at 4 °C at 1500 × *g*. The membranes were then washed twice with 0.3 M sorbitol, 5 mM $MgCl_2$, and 20 mM Tricine-NaOH, pH 7.6. The membranes were finally pelleted and applied to the platinum electrode as a thin paste. For treatment with 150 mM NaCl or 20 mM $CaCl_2$, these salts were included in the wash buffer. In the case of $CaCl_2$ treatment, the washed membranes were resuspended in 1 ml of wash buffer plus 20 mM $CaCl_2$ and brought to 50 μM ionomycin (a calcium ionophore). After incubation for 5 min at 4 °C, the treated thylakoids were pelleted and applied to the platinum electrode as described above. The flash oxygen yield measurements were performed on a bare platinum electrode (Artesian Scientific Co., Urbana, IL). The samples were incubated briefly (30 s to 1 min) under fluorescent room light (~5 μmol photons m^{-2} s^{-1}) prior to the onset of the experiment to allow the equilibration of the S states and then dark-incubated for only 20 s (during the electrode polarization). The electrode was polarized at 0.73 V for 20 s, and a series of 16 saturating flashes was supplied by an integrated, computer-controlled xenon flash lamp (20 μs). The polarization was turned off, and the same sample was then incubated in the dark at room temperature for various lengths of time followed by additional flash series. The data were analyzed using a four-state, homogeneous model (10). Five- and six-state models that incorporated either an S_{-1} state or S_{-1} and S_{-2} states, respectively, uniformly failed to fit the data acquired from either wild type or mutant thylakoids. Prior to fitting, the raw oxygen yield data were normalized to the average oxygen yield obtained on flashes 13–16. It is important to note that standard methods for measuring S state lifetimes (11, 12) could not be used in this study. The fact that the oxygen evolution of the mutant thylakoid membranes is very unstable in the absence of added calcium (see below), coupled with the fact that the S_2 and S_3 states are quite persistent during dark incubation (9), precludes the use of the long dark incubation periods normally used in such experiments. We consider our measurements to be semiquantitative and satisfactory for our comparative studies (but not sufficiently accurate for more detailed bioenergetic studies, such as those performed in Ref. 11).

Steady State Oxygen Evolution—For these experiments, thylakoids were isolated as described above and resuspended in a small volume of wash buffer. Chlorophyll was measured by the method of Arnon (13). Oxygen evolution was measured polarographically in a Hansatech oxygen electrode at 22 °C in the wash buffer supplemented with 0.5 mM potassium ferricyanide, 0.5 mM dichlorobenzoquinone, and 10 mM ammonium chloride. The chlorophyll concentration was 10 μg/ml, and the light intensity was 2000 μmol photons m^{-2} s^{-1}. In some experiments, 150 mM NaCl or 20 mM $CaCl_2$ was added directly to the reaction mixture. In the $CaCl_2$ experiments, the thylakoid membranes were pretreated with 50 μM ionomycin for 5 min in the dark.

RESULTS AND DISCUSSION

Fig. 1 shows examples of typical flash oxygen yield experiments performed for the wild type (Fig. 1*A*) and *psbo1* mutant (Fig. 1*B*) under control cofactor conditions (10 mM chloride with no added calcium). Before the onset of these experiments, the samples were incubated in room light (5 μmol photons m^{-2} s^{-1}) to allow mixing of the S states. The first flash series was performed after 20 s of dark incubation, which was required to allow polarization of the electrode. As expected, both wild type and mutant thylakoids exhibited large oxygen yields on the first and second flashes, indicating that a large proportion of PS II reaction centers (42–45%) were in the $S_2 + S_3$ states. Additional flash series were then performed after increasing intervals of dark incubation of the same sample. In the wild type (Fig. 1*A*), the oxygen yield observed on the first and second flashes decreased rapidly with increasing lengths of dark incubation. This indicates that the proportion of PS II reaction centers in the S_2 and S_3 states decreased rapidly with increasing length of

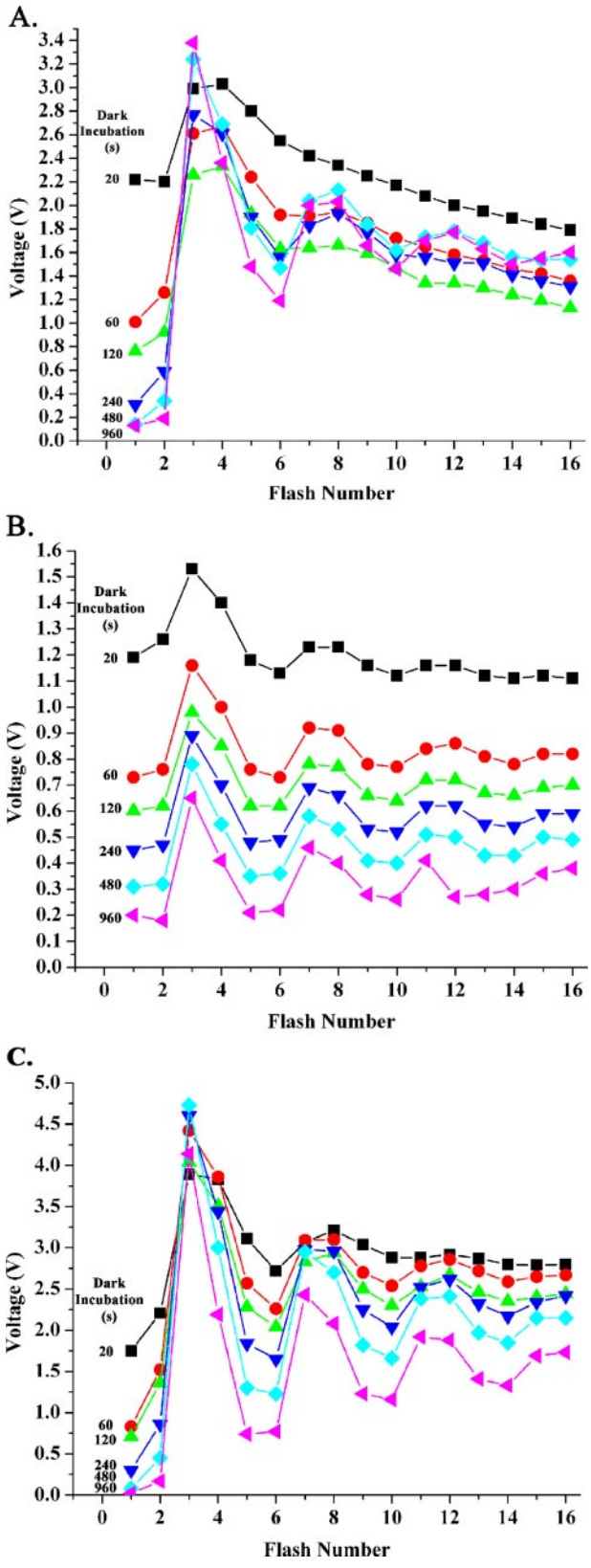

FIGURE 1. **Raw data illustrating typical flash oxygen yield curves obtained from the wild type (*A*), the *psbo1* mutant (*B*), and the *psbo1* mutant + 20 mM $CaCl_2$ + 50 μM ionomycin (*C*).** A single sample was used to collect the data in each graph. The length of dark incubation between each flash series is shown. In *A* and *B*, the samples contained 10 mM chloride with no added calcium. In *C*, the sample contained 20 mM $CaCl_2$ + 50 μM ionomycin. The dark incubation time for each 16-flash series is color-coded as follows: 20 s, *black squares*; 60 s, *red circles*; 120 s, *green triangles*; 240 s, *blue triangles*; 480 s, *aqua diamonds*; and 960 s, *magenta triangles*. Please note that the flash experiments shown in *B* are not offset. The addition of 20 mM $CaCl_2$ + 50 μM ionomycin to wild type thylakoids had no apparent effect on the flash oxygen yield pattern and is not shown.

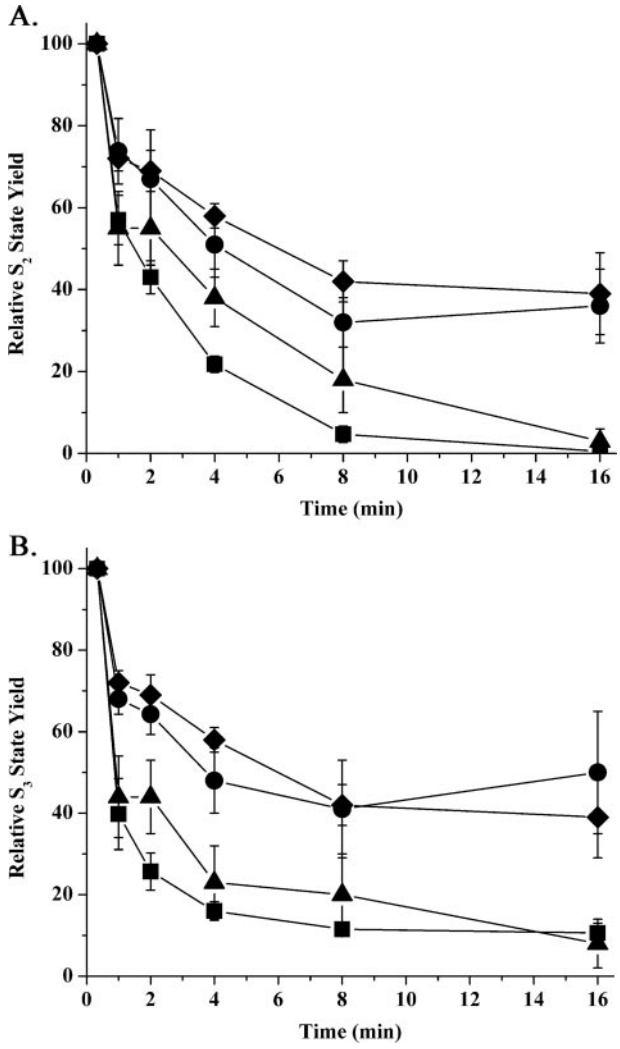

FIGURE 2. **S_2 (*A*) and S_3 (*B*) state lifetime decay curves for wild type *Arabidopsis* and *psbo1* mutant thylakoids under different ionic conditions.** The *time axis* indicates time of dark incubation at 24 °C. ■, wild type decay curve under control ionic conditions (10 mM chloride with no added calcium); ●, *psbo1* control; ◆, *psbo1* + 150 mM NaCl; ▲, *psbo1* + 20 mM $CaCl_2$ + 50 μM ionomycin (n = 4–13; *error bars* are ±1 S.E.). In some instances, the *symbols* are larger than the *error bars*. Treatment of wild type thylakoids with either 150 mM NaCl or 20 mM $CaCl_2$ + 50 μM ionomycin had little effect on the S state lifetimes or other parameters examined in this study and are not shown.

dark incubation. After 960 s of dark incubation, <3% of the reaction centers were in the S_2 + S_3 states. In the mutant, a significantly different pattern was observed. Whereas the overall flash oxygen yield dropped substantially during the course of the experiment (see below), the proportion of reaction centers in the S_2 + S_3 states remained quite high (18%), even at the longest period of dark incubation. This indicates that the S_2 and S_3 states in the mutant are very long-lived, confirming our earlier observations (9).

Fig. 2 explicitly illustrates the decay of the S_2 and S_3 states of the wild type and *psbo1* mutant under a variety of ionic condi-

tions. Qualitatively, under the control conditions of 10 mM chloride and no added calcium, the S_2 and S_3 states decayed rapidly in the wild type but very slowly in the *psbo1* mutant. The addition of 150 mM NaCl did not accelerate the rate of decay of these S states in the mutant. The addition of 20 mM $CaCl_2$, however, nearly fully restored the S state decay characteristics in the mutant (a typical flash oxygen yield experiment for the mutant in the presence of 20 mM $CaCl_2$ is shown in Fig. 1*C*). The addition of 150 mM NaCl or 20 mM $CaCl_2$ had little effect on the decay rates of the S_2 and S_3 states of the wild type (data not shown).

In spinach, which possesses only a single PsbO protein isoform, chemical removal of this component from PS II membranes leads to increased lifetimes for the S_2 and S_3 states (14) and a marked slowing of the $S_3 \rightarrow [S_4] \rightarrow S_0$ transition (14, 15). These preparations also exhibit a large loss (75%) of steady state oxygen evolution capability, with high concentrations of calcium and chloride being required to observe even this low activity (2, 16). Additionally, in these experiments, the PsbO protein appears to lower the calcium requirement for oxygen evolution by at least a factor of 2 (2). Similar results were obtained for the cyanobacterium *Synechocystis* 6803 in mutants from which the single PsbO protein present had been genetically deleted (3, 17). Whereas these mutants can grow photoautotrophically in standard BG-11 medium (albeit more slowly and with lower oxygen evolution rates), they cannot grow in either calcium-depleted (18) or chloride-depleted (19) medium. These findings indicate that, *in vivo*, the PsbO protein modulates the utilization of calcium in support of normal oxygen-evolving activity. Flash oxygen yield measurements on the *Synechocystis* Δ*psbO* mutant also indicated that the S_2 and S_3 states are stabilized and that the $S_3 \rightarrow [S_4] \rightarrow S_0$ transition is strongly retarded (3). Mutants containing alterations in other PS II components that cannot bind the PsbO protein normally exhibit similar characteristics (Ref. 21; for an in-depth review, see Ref. 22). Our results indicate that the PsbO-2 protein that is present in the *psbo1* mutant is defective in supporting normal operation of the oxygen-evolving complex. Our *in vitro* characterization of mutant thylakoids indicates that loss of the PsbO-1 protein results in a modified oxygen-evolving complex, which exhibits S state characteristics similar to those obtained by the chemical removal of the PsbO component from higher plant oxygen-evolving membranes and to cyanobacterial cells from which the PsbO protein had been removed genetically. It should be emphasized here, however, that the PsbO-2 protein is, at least, partially functional. The *psbo1* mutant grows photoautotrophically (albeit slowly) and can set seed. If the PsbO-2 protein were fully nonfunctional, we would expect the *psbo1* mutant to be unable to grow photoautotrophically, as we had observed previously in *Arabidopsis* that contained RNA interference designed to suppress the expression of both PsbO-1 and PsbO-2 simultaneously (5).

Fig. 3*A* illustrates the total flash oxygen yield of wild type and *psbo1* thylakoids. This was estimated by summing the oxygen yields obtained on the first four flashes after a 20-s dark incubation period for the wild type and mutant under various treatment regimes. The oxygen yield for the wild type was essentially constant for untreated, NaCl-treated, and $CaCl_2$-treated thylakoids. However, the *psbo1* thylakoids exhibited a significantly different pattern. Under control and elevated NaCl conditions, the mutant exhibited oxygen yields ~50% of that observed in the wild type. However, treatment with $CaCl_2$ increased the total oxygen yield to near wild type values. This result was quite surprising. Previously, Murakami *et al.* (7) reported that the *psbo1* mutant accumulated fewer PS II reaction centers (~75% of the wild type for mature leaves) as measured by immunoquantification of the D1 protein. Consequently, it would be expected that the maximal observable total oxygen yield of the mutant would be 75% of wild type values. However, such an analysis does not take into account the large variability that Murakami *et al.* (7) observed in their measurements of D1. Our observation of near wild type total oxygen yield in the mutant is

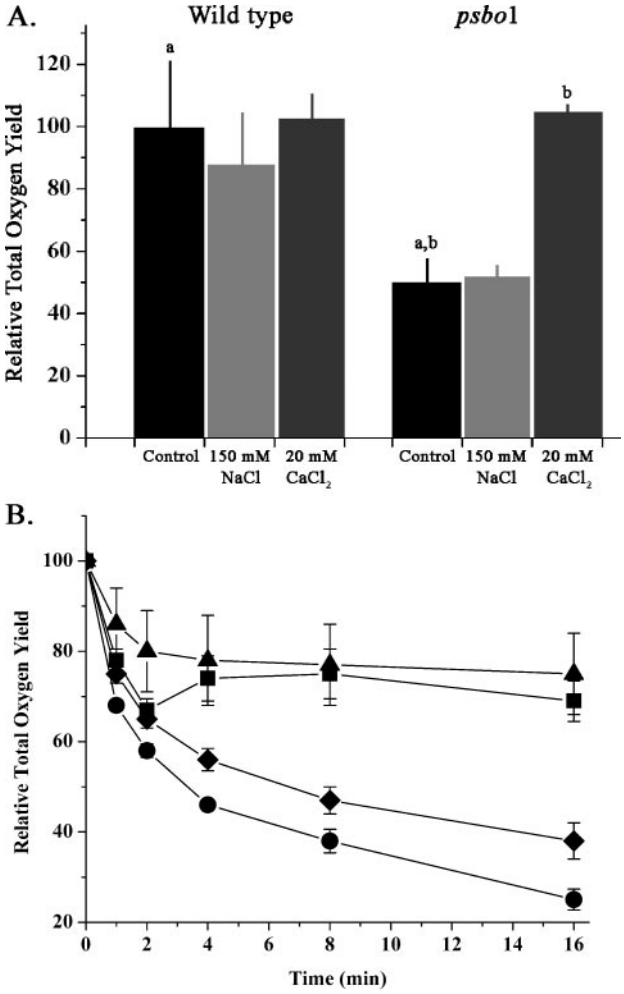

FIGURE 3. **Analysis of total flash oxygen yield.** *A*, histogram illustrating the total flash oxygen yield for wild type *Arabidopsis* and *psbo1* mutant thylakoids under different ionic conditions. The $CaCl_2$ treatments also contained 50 μM ionomycin. The total oxygen yield is the sum of the oxygen yields observed on the first four saturating flashes. For the wild type, this value was 10.8 ± 2.6 V, and for *psbo1*, this was 5.3 ± 1.1 V under control ionic conditions ($n = 4–13$; *error bars* indicate ±1 S.E.). ^{a,b}, $p < 0.05$ using Student's *t* test (one-tailed). *B*, stability of the total flash oxygen yield during dark incubation at 24 °C under different ionic conditions. The *time axis* indicates the length of dark incubation. ■, wild type decay curve under control ionic conditions; ●, *psbo1* control; ◆, *psbo1* + 150 mM NaCl; ▲, *psbo1* + 20 mM $CaCl_2$ + 50 μM ionomycin ($n = 4–13$, *error bars* ±1 S.E.). In some instances, the *symbols* are larger than the *error bars*. Treatment of wild type thylakoids with either 150 mM NaCl or 20 mM $CaCl_2$ had little effect on the stability of the total oxygen yield or other parameters examined in this study and are not shown.

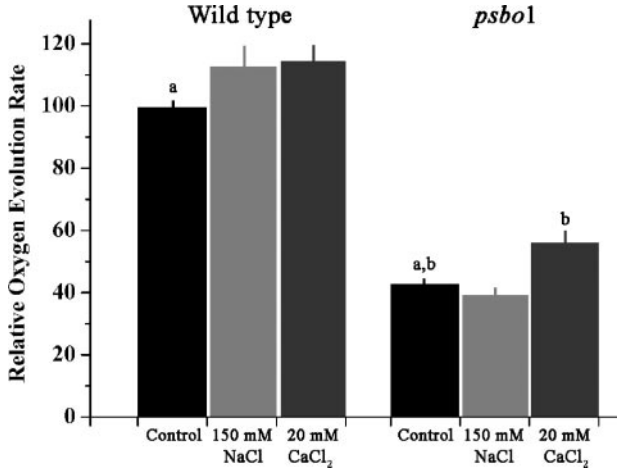

FIGURE 4. **Comparison of the steady state oxygen evolution rates of wild type and *psbo1* mutant thylakoids.** The $CaCl_2$ treatments also contained 50 μM ionomycin. The wild type control oxygen evolution rate was 162 ± 12 μmol oxygen mg chlorophyll^{-1} h^{-1}, whereas the *psbo1* control oxygen evolution rate was 72 ± 9 μmol oxygen mg chlorophyll^{-1} h^{-1} (n = 9–12; *error bars* ± 1 S.E.). ^{a,b}, $p < 0.05$ using Student's t test (one-tailed).

fully consistent with Murakami *et al.* (7), given the level of variability that they report.

In Fig. 3*B,* the stability of the total flash oxygen yield of wild type and *psbo1* thylakoids at room temperature was examined. The total oxygen yield of wild type thylakoids was quite stable, with ~70% of the total oxygen yield being retained after 16 min of incubation. The *psbo1* thylakoids were, however, very unstable, retaining only 20% of their oxygen yield over the same time period. Treatment of the *psbo1* thylakoids with 150 mM NaCl marginally increased the stability of the oxygen yield. However, treatment of the mutant thylakoids with 20 mM $CaCl_2$ dramatically increased the stability of the total flash oxygen yield such that it was indistinguishable from wild type thylakoids.

The increase in the total flash oxygen yield observed after $CaCl_2$ treatment of the mutant thylakoids is, in large measure, also reflected in steady state oxygen evolution measurements (Fig. 4). No significant differences were observed in the oxygen evolution rate of wild type thylakoids upon the addition of either NaCl or $CaCl_2$. However, addition of $CaCl_2$ to mutant thylakoids significantly increased the oxygen evolution rate by ~30%. These results indicate that the defect in the *psbo1* mutant affects the ability of the mutant to effectively utilize calcium in support of oxygen evolution, both with respect to total flash oxygen yield and the oxygen evolution rates measured under steady state conditions. Interestingly, the addition of high concentrations of chloride did not appear to restore the total flash oxygen yield of *psbo1*, nor did it improve the steady state oxygen evolution rates of this mutant.

Earlier studies on the *psbo1* mutant indicated that the absence of PsbO-1 led to a decrease in PS II variable fluorescence yield (F_V/F_M) to between 70 and 90% of wild type levels (8). Additionally, as noted above, an apparent decrease in assembled PS II reaction centers was observed (monitored by a decrease in the amount of the D1 protein). Our observation that the addition of $CaCl_2$ to mutant thylakoids restored the total oxygen yield to near wild type levels may indicate that the lower quantum yield observed *in vivo* (8) was principally due to PS II reaction centers that were defective in the sequestration of calcium. It should be noted that whereas in our steady state oxygen evolution measurements the *psbo1* rates were significantly increased, they did not achieve wild type levels. This highlights a major difference between flash oxygen yield measurements and steady state experiments. During a steady state experiment, the samples are exposed to saturating light intensities for at least 1 min, whereas during a flash oxygen yield experiment, the sample is exposed to saturating light for <0.5 ms. We hypothesize that the lower level of activity restoration observed in the steady state experiment possibly indicates an increased rate of photoinactivation in the mutant that is not fully complemented by increased calcium concentrations.

Our observations demonstrate that the *Arabidopsis psbo1* mutant thylakoids exhibit long-lived S_2 and S_3 states, lower total oxygen yields, lower stability of the oxygen-evolving complex, and depressed steady state rates of oxygen evolution. These results are very similar to those obtained by the chemical removal of the PsbO protein from higher plant PS II membranes and to those obtained from genetic deletion of the single *psbO* gene present in *Synechocystis*. Our results indicate that the PsbO-2 protein, the only PsbO protein present in the *psbo1* mutant, is defective in supporting normal operation of the oxygen-evolving complex, even though this protein appears to bind normally to PS II membranes (8). Additionally, the majority of the defects we observed in the *psbo1* mutant thylakoids can be nearly fully reversed by calcium in the presence of moderate chloride concentrations. These findings indicate that the PsbO-2 protein is defective in the utilization of these cofactors in support of normal oxygen evolution activity. Consequently, comparisons between the PsbO-1 and PsbO-2 proteins should yield important insights into the function of the competent PsbO-1 protein. PsbO-2 is very similar to PsbO-1, containing only 11 amino acid substitutions (7, 23), the majority of which are conservative replacements. Murakami *et al.* (8) generated *Escherichia coli*-expressed chimeric PsbO-1/PsbO-2 proteins and evaluated their ability to reconstitute steady state oxygen-evolving activity in NaCl/urea-washed spinach PS II membranes. They concluded that the observed defects in steady state oxygen evolution rates were due to amino acid substitutions in the C-terminal domain of PsbO-2, specifically the $Val^{186} \rightarrow$ Ser and/or $Leu^{246} \rightarrow$ Ile substitutions. If their observations are correct, then these residues are responsible either directly or indirectly for the loss of the ability of PsbO-2 to utilize calcium. It should be noted, however, that these steady state oxygen evolution experiments were apparently performed at high calcium and chloride concentrations during the oxygen evolution assays (due to the absence of the PsbP and PsbQ components). Although these authors did not specify the ionic cofactor concentrations used during their assays, they did tangentially cite Betts *et al.* (24), who included 20 mM Ca^{2+} and 100 mM Cl^- in their assay buffer. It is probable that, under conditions of elevated calcium and chloride, the full extent of the defects associated with only PsbO-2-containing reaction centers would not have been observed (8). Consequently, it is possible that other residues that are present in PsbO-1 but replaced in PsbO-2 may be important with respect to the maintenance of calcium at the active site.

What is the nature of the defect in the PsbO-2 protein? Our observations indicate that the PsbO-2 protein cannot maintain normal levels of calcium in the vicinity of the oxygen-evolving site. Previously, we suggested that the PsbO protein, in concert with the large extrinsic loops of CP47 and CP43, formed a "sequestered domain" for maintaining high concentrations of chloride in the vicinity of the oxygen-evolving site (25). Experimental support for this suggestion was recently provided (26). Our current results indicate that a fully functional PsbO protein is also required for the normal sequestration of calcium at the oxygen-evolving site (for an alternative viewpoint, however, see Ref. 27). One possible hypothesis consistent with the *psbo1* phenotype would be that the PsbO-2 protein cannot support a stable, high affinity structural association of PsbP and PsbQ with PS II. It has been shown that the PsbO protein is required for binding of the PsbP component and that PsbP is required for binding of the PsbQ protein (28, 29). More recently, we have identified carboxylates on the PsbO protein that are required for PsbP binding (30). Decreased binding affinity of these two components would lead to lowered affinity for both calcium and chloride at the oxygen-evolving site (for reviews see Refs. 20 and 22). It is also possible that the PsbP and PsbQ proteins can structurally associate normally with PsbO-2-containing PS II reaction centers but that the functional interaction between either PsbO-2 and PsbP/PsbQ or PsbO-2 and intrinsic membrane protein components of the photosystem is compromised. Finally, it is possible that the PsbO-2 protein alone cannot support normal assembly of PS II and that the defective photosystem that does assemble cannot effectively sequester calcium at the active site. Our earlier studies (5) indicated that the PsbO component is required for normal assembly of PS II. We did not, however, examine the relative efficiencies of PsbO-1 *versus* PsbO-2 in support of normal photosystem assembly. Experimental differentiation among these and other hypotheses is ongoing.

CONCLUSIONS

The *psbo1* mutant exhibits significant defects in its ability to evolve oxygen. Steady state oxygen evolution rates are depressed, total flash oxygen yield is significantly lower, oxygen yeild stability is markedly decreased, and the dark decay of both the S_2 and S_3 states is significantly retarded. The majority of these defects are nearly fully reversed by moderate concentrations of $CaCl_2$. These results indicate that the PsbO-2 protein, the only PsbO protein present in the *psbo1* mutant, leads to a defect in the ability of PS II to utilize calcium in support of oxygen evolution.

REFERENCES

1. Murata, N., Mijao, M., Omata, T., Matsunami, H., and Kuwabara, T. (1984) *Biochim. Biophys. Acta* **765,** 363–369
2. Bricker, T. M. (1992) *Biochemistry* **31,** 4623–4628
3. Burnap, R., Shen, J. R., Jursinic, P. A., Inoue, Y., and Sherman, L. A. (1992) *Biochemistry* **31,** 7404–7410
4. Bricker, T. M., and Ghanotakis, D. F. (1996) in *Oxygenic Photosynthesis: The Light Reactions* (Ort, D. R., and Yocum, C. F., eds) pp. 113–136, Kluwer Academic Publishers, Dordrecht, The Netherlands
5. Yi, X., McChargue, M., Laborde, S. M., Frankel, L. K., and Bricker, T. M. (2005) *J. Biol. Chem.* **280,** 16170–16174
6. Yi, X., Hargett, S., Liu, H., Frankel, L. K., and Bricker, T. M. (2007) *J. Biol. Chem.* **282,** 24833–24841
7. Murakami, R., Ifuku, K., Takabayashi, A., Shikanai, T., Endo, T., and Sato, F. (2002) *FEBS Lett.* **523,** 138–142
8. Murakami, R., Ifuku, K., Takabayashi, A., Shikanai, T., Endo, T., and Sato, F. (2005) *FEBS J.* **272,** 2165–2175
9. Liu, H., Frankel, L. K., and Bricker, T. M. (2007) *Biochemistry* **46,** 7607–7613
10. Meunier, P. C. (1993) *Photosynth. Res.* **36,** 111–118
11. Messinger, J., Schroder, W. P., and Renger, G. (1993) *Biochemistry* **32,** 7658–7668
12. Bricker, T. M., Lowrance, J., Sutton, H., and Frankel, L. K. (2001) *Biochemistry* **40,** 11483–11489
13. Arnon, D. I. (1949) *Plant Physiol.* **24,** 1–15
14. Miyao, M., Murata, M., Lavorel, J., Maison-Petri, B., Boussac, A., and Etienne, A.-L. (1987) *Biochim. Biophys. Acta* **890,** 151–159
15. Ono, T.-A., and Inoue, Y. (1985) *Biochim. Biophys. Acta* **806,** 331–340
16. Miyao, M., and Murata, N. (1984) *FEBS Lett.* **170,** 350–354
17. Vass, I., Cool, K. M., Zsuzsanra, D., Mayes, S. R., and Barber, J. (1992) *Biochim. Biophys. Acta* **1102,** 195–201
18. Philbrick, J. B., Diner, B. A., and Zilinskas, B. A. (1991) *J. Biol. Chem.* **266,** 13370–13376
19. Engels, D. H., Lott, A., Schmid, G. H., and Pistorious, E. K. (1994) *Photosynth. Res.* **42,** 227–244
20. Miqyass, M., van Gorkom, H. J., and Yocum, C. F. (2007) *Photosynth. Res.* **92,** 275–287
21. Putnam-Evans, C., Wu, J., Burnap, R., Whitmarsh, J., and Bricker, T. M. (1996) *Biochemistry* **35,** 4046–4053
22. Popelkova, H., and Yocum, C. F. (2007) *Photosynth. Res.* **93,** 111–121
23. Bricker, T. M., and Burnap, R. L. (2005) in *Photosystem II: The Water/Plastoquinone Oxido-Reductase of Photosynthesis* (Wydrzynski, T., and Satoh, K., eds) pp. 95–120, Springer, Dordrecht, the Netherlands
24. Betts, S. D., Ross, J. R., Pichersky, E., and Yocum, C. F. (1997) *Biochemistry* **36,** 4047–4053
25. Bricker, T. M., and Frankel, L. K. (1998) *Photosynth. Res.* **56,** 157–173
26. Popelkova, H., Betts, S. D., Lydakis Simantiris, N., Im, M. M., Swenson, E., and Yocum, C. F. (2006) *Biochemistry* **45,** 3107–3115
27. Seidler, A. (1996) *Biochim. Biophys. Acta* **1277,** 35–60
28. Andersson, B., Larsson, C., Jansson, C., Ljungberg, U., and Akerlund, H.-E. (1984) *Biochim. Biophys. Acta* **766,** 21–26
29. Kavelaki, K., and Ghanotakis, D. F. (1991) *Photosynth. Res.* **29,** 149–155
30. Bricker, T. M., and Frankel, L. K. (2003) *Biochemistry* **42,** 2056–2061