The Two Substrate−Water Molecules Are Already Bound to the Oxygen-Evolving Complex in the S_2 State of Photosystem II

Garth Hendry and Tom Wydrzynski*

Photobioenergetics, Research School of Biological Sciences, The Australian National University, Canberra ACT 0200, Australia

Received June 5, 2002; Revised Manuscript Received September 6, 2002

ABSTRACT: The first direct evidence which shows that both substrate−water molecules are bound to the O_2-evolving catalytic site in the S_2 state of photosystem II (PSII) is presented. Rapid ^{18}O isotope exchange measurements between $H_2{}^{18}O$ incubated in the S_2 state of PSII-enriched membrane samples and the photogenerated O_2 reveal a fast and a slow phase of exchange at *m*/*e* 34 (which measures the level of the $^{16}O^{18}O$ product). The rate constant for the slow phase of exchange ($^{34}k_1$) equals 1.9 ± 0.3 s^{-1} at 10 °C, while the fast phase of exchange is unresolved by our current experimental setup ($^{34}k_2 \geq 175$ s^{-1}). The unresolvable fast phase has left open the possibility that the second substrate−water molecule binds to the catalytic site only after the formation of the S_3 state [Hillier, W., and Wydrzynski, T. (2000) *Biochemistry 39*, 4399−4405]. However, for PSII samples depleted of the 17 and 23 kDa extrinsic proteins (Ex-depleted PSII), two completely resolvable phases of ^{18}O exchange are observed in the S_2 state of the residual activity, with the following rate constants: $^{34}k_1 = 2.6 \pm 0.3$ s^{-1} and $^{34}k_2 = 120 \pm 14$ s^{-1} at 10 °C. Upon addition of 15 mM $CaCl_2$ to Ex-depleted PSII, the O_2 evolution activity increases to ∼80% of the control level, while the two resolvable phases of exchange remain the same. In measurements of Ex-depleted PSII at *m*/*e* 36 (which measures the level of the $^{18}O^{18}O$ product), only a single phase of exchange is observed in the S_2 state, with a rate constant ($^{36}k_1 = 2.5 \pm 0.2$ s^{-1}) that is identical to the slow rate of exchange in the *m*/*e* 34 data. Taken together, these results show that the fast phase of ^{18}O exchange is specifically slowed by the removal of the 17 and 23 kDa extrinsic proteins and that the two substrate−water molecules must be bound to independent sites already in the S_2 state. In contrast, the ^{18}O exchange behavior in the S_1 state of Ex-depleted PSII is no different from what is observed for the control, with or without the addition of $CaCl_2$. Since the fast phase of exchange in the S_1 state is unresolved (i.e., $^{34}k_2 > 100$ s^{-1}), the possibility remains that the second substrate−water molecule binds to the catalytic site only after the formation of the S_2 state. The role of the 17 and 23 kDa extrinsic proteins in establishing an asymmetric dielectric environment around the substrate binding sites is discussed.

The oxidation of water to molecular oxygen (O_2) during photosynthesis is catalyzed by the oxygen evolving complex (OEC)[1] of photosystem II (PSII). The OEC contains an inorganic core consisting of four Mn ions, one Ca^{2+} ion, and possibly one Cl^- ion. The primary function of the OEC is to couple the four-electron oxidation of water to the one-electron photochemistry that occurs at the PSII reaction center chlorophyll species P_{680}. Electron transfer between the OEC and P_{680} is mediated by the redox active tyrosine residue Y_Z (Y161 of the D1 protein, *Synechocystis* numbering). To achieve the potential required to split water into molecular O_2, the OEC must successively accumulate four oxidation equivalents. These oxidation equivalents are represented by the S_n states (where $n = 0$, 1, 2, 3, or 4) (*1*). Thus, beginning in S_0, each S state transition is driven forward by the absorption of a light quantum at P_{680}. Once the S_4 state has been reached, the OEC reacts to produce O_2 and the S_0 state, as the cycle begins anew.

Important mechanistic questions remain. During which step in the S state cycle do the two substrate−water molecules bind, and how does this influence the formation of the O−O bond? In the original Kok hypothesis (*1*), it was implied that the substrate−water molecules enter the reaction sequence during the last transition ($S_3 \rightarrow [S_4] \rightarrow S_0$), immediately prior to the release of O_2. However, current mechanistic models invoke the binding of the substrate−water molecules to the OEC at the beginning of the S state cycle (*2*−*15*).

Many attempts have been made to probe the substrate−water molecules in the OEC, using proton release measurements (*7*, *16*), magnetic resonance methods (*17*−*19*), and FTIR spectroscopy (*20*, *21*). However, an unequivocal interpretation with respect to the exact nature of substrate−water binding has not been reached. The most direct information thus far has come from rapid, ^{18}O isotope exchange measurements between added $H_2{}^{18}O$ and the photogenerated O_2 using mass spectrometric techniques (*22*−*25*). The recently employed "closed chamber" system (*22*, *23*) has provided a 5000-fold improvement in the kinetic

* To whom correspondence should be addressed. Telephone: +61 2 6125 5892. Fax: +61 2 6125 8056. E-mail: TOM@rsbs.anu.edu.au.

[1] Abbreviations: Ex-depleted PSII, PSII-enriched membrane samples depleted of the 17 and 23 kDa extrinsic proteins; OEC, oxygen-evolving complex; PSII, photosystem II; MES, 4-morpholinoethanesulfonic acid; PPBQ, phenyl-*p*-benzoquinone; FTIR, Fourier transform infrared spectroscopy; Chl, chlorophyll; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

10.1021/bi026246t CCC: $22.00
Published on Web 10/11/2002

resolution over the earlier measurements (*26*, *27*) and allows ^{18}O exchange times down to ~6 ms to be determined. In the first applications of the rapid ^{18}O exchange measurements, the data obtained at *m*/*e* 34 (which measures the amount of the $^{16}O^{18}O$ product) clearly showed biphasic exchange behavior in all of the S states using thylakoid samples (*24*). Detailed analysis of the data in the S_3 state yielded two kinetically distinct exchange rate constants, reflecting the independent exchange by the substrate−water molecules at separate sites within the OEC (*23*). The data also revealed that the "slowly" exchanging substrate−water molecule is bound throughout the entire S state cycle (*24*). However, the kinetics of the "fast" exchanging substrate−water molecule could not be resolved in the S_2, S_1, or S_0 state and thus left open the possibility that the second substrate−water molecule enters the reaction sequence only after the formation of the S_3 state (*24*).

In higher plants, two extrinsic proteins with molecular masses of approximately 17 and 23 kDa are peripherally bound to PSII and are involved in regulating the O_2-evolving activity (reviewed in ref *28*). Removal of the 17 and 23 kDa extrinsic proteins (Ex-depleted PSII) by incubation in 1−2 M NaCl (*29*, *30*) largely inhibits O_2 evolution. The activity can be restored to ~80% of the untreated control by adding relatively high levels of Ca^{2+} to the sample (e.g., 10−20 mM) (*29*, *30*). It has been proposed that the 23 kDa extrinsic protein in particular promotes the high-affinity retention of Ca^{2+} within the OEC (*31*). A recent study by Vrettos et al. (*32*) showed that in Ex-depleted PSII the free energy of binding of Ca^{2+} to the OEC decreases by 2.5 kcal/mol. The authors have discussed this effect in terms of changes in the local dielectric around the Ca^{2+}-binding site.

It has also been found that Ex-depleted PSII exhibits increased sensitivity to reduction by exogenously added NH_2OH and hydroquinone, implying that in the absence of these proteins the catalytic site is more exposed to the bulk medium than in the intact system (*33*, *34*). In many enzymatic mechanisms, an important parameter in determining the reaction path is one of substrate access to the catalytic site (*35*−*37*). In photosystem II, the regulation of solvent−water access to the OEC may also be important, by preventing unwanted side reactions (such as the production of H_2O_2) (*38*). Such regulation of the solvent−water accessibility could be achieved through S state-dependent protein conformational changes (*39*) or through protein specific water channels, which may involve extrinsic proteins (*38*).

In an effort to address the role of the 17 and 23 kDa extrinsic proteins in the mechanism of O_2 evolution, we have undertaken ^{18}O isotope exchange measurements on PSII-enriched samples depleted of these proteins (Ex-depleted PSII). Previous measurements of Ex-depleted PSII in the S_3 state have shown that there is a small but significant decrease in the rate of the fast exchanging substrate−water molecule as compared with that of an intact sample (*25*). In this paper, we report the effects of the removal of the 17 and 23 kDa extrinsic proteins on ^{18}O exchange in the S_2 and S_1 states. The data provide the first direct evidence that both substrate−water molecules are bound to the OEC in the S_2 state.

EXPERIMENTAL PROCEDURES

Sample Preparations. PSII-enriched membrane fragments were isolated from fresh market spinach according to the procedure of Berthold et al. (*40*). Briefly, thylakoid membranes were solubilized in 5% Triton X-100 buffered in 30 mM MES/NaOH (pH 6.3), 15 mM NaCl, and 5 mM $MgCl_2$ with gentle stirring for 20 min in the dark at 4 °C. The PSII samples were collected by centrifugation at 36000*g* (4 °C) and were washed and resuspended in a final buffered medium containing 30 mM MES/NaOH (pH 6.3), 15 mM NaCl, 5 mM $MgCl_2$, and 400 mM sucrose. The samples were then snap frozen in liquid N_2 and stored at −80 °C until the measurements were taken.

Removal of the 17 and 23 kDa extrinsic proteins from the PSII samples was essentially performed according to the method of Kuwabara and Murata (*41*) by incubation in 1 M NaCl. At 10 min intervals during the incubation, the sample was gently passed twice through a Teflon homogenizer. Following a second 30 min treatment, the samples were washed twice and resuspended in the final buffered medium, snap frozen in liquid N_2, and stored at −80 °C until the measurements were taken.

SDS−PAGE and Protein Analysis. SDS−PAGE was performed according to the method of Laemmli (*42*). The resolving gel matrix contained a linear gradient of 10 to 17.5% acrylamide/bis solution (37.5:1, Bio-Rad) and Tris-HCl (pH 8.8) adjusted to 0.6 M. Both the stacking and resolving gel components contained 6.0 M urea. Prior to loading, the protein samples were heat-treated for 5 min at 90 °C in a denaturing buffer consisting of 0.0625 M Tris-HCl (pH 6.8), 2% (w/v) SDS, 0.1% bromophenol blue, 10% glycerol, and 5% β-mercaptoethanol. Aliquots containing 10 μg of chlorophyll (Chl) were loaded onto the gel and electrophoresed for 2 h at 100 mV. The gel was stained with Coomassie Blue R-250, and densitometry scans were made using a Bio-Rad model 1650 scanning densitometer.

O_2 Evolution Measurements. Initial steady state rates of O_2 evolution were measured at 25 °C using a Clark-type electrode (Hansatech) under continuous saturating illumination (custom-built 150 W tungsten light source, viz., >5000 μmol m^{-2} s^{-1}). A typical assay contained 10 μg of Chl/mL with 1 mM $K_3Fe(CN)_6$ and 400 μM phenyl-*p*-benzoquinone (PPBQ) as electron acceptors in the final buffered medium. Chlorophyll concentrations were determined according to the method of Porra et al. (*43*).

Mass Spectrometric Measurements. Isotopic determinations of the amount of flash-induced O_2 produced by PSII-enriched samples were made at *m*/*e* 34 and 36 using an in-line MM6 mass spectrometer (Vacuum Generation, Winsford, U.K.) as described previously (*22*, *23*). A stirred, closed chamber system was used for the rapid equilibration of 25 μL of $H_2{}^{18}O$ (98.5% enrichment, ISOTECH, Miamisberg, OH) into 160 μL of the sample. Injection of the labeled water was carried out using a Hamilton CR700-200 spring-loaded syringe triggered by a computer-actuated solenoid. Samples were activated using saturating light flashes (fwhh ~ 8 μs) provided by a battery of xenon flash lamps (FX-1163 lamp with internal reflector, 4 μF at 1 kV capacitor, EG&G, Salem, MA) through a fiber optic situated directly in front of the sample chamber window. The flash/injection protocols used to measure the extent of ^{18}O exchange in the various S states are given in Figure 1 of ref *24* and were controlled via a computer. Accurate timing intervals were established from a digital oscilloscope (Tektronix, model 350).

The ^{18}O exchange measurements were made in the presence of 1 mM $K_3Fe(CN)_6$ and 400 μM PPBQ. Samples were first preset in the S_1 state by a preflash followed by a 10 min dark period at room temperature. For each exchange measurement, electron acceptors were added to an aliquot and the aliquot was loaded into the sample chamber in the dark and degassed for 10 min at 10 °C prior to the particular flash/injection sequence that was used. For optimal signal to noise (*S*/*N*), the final sample concentration was adjusted to 0.5 mg of Chl/mL for the S_3 state measurement and to 0.2 mg of Chl/mL for the S_2 and S_1 state measurements. In the latter measurements, the short delay times (5 ms) used between the turnover flashes necessitated the use of a battery of three flash lamps in which each flash lamp was optically coupled to the sample chamber via a 3-to-1 optical fiber. To compensate for a reduction in the overall flash intensity under this arrangement, the chlorophyll concentration was lowered to ensure light saturation. After the rapid turnover flashes were given, due to the time it takes for the gas to diffuse from the sample chamber to the ionizing source within the mass spectrometer, a series of normalization flashes were applied at 0.05 Hz. To minimize S-state deactivation between the normalization flashes, measurements were taken at 10 °C.

The O_2 background introduced into the sample during the injection of $H_2{}^{18}O$ was subtracted from the amount of photogenerated O_2 by performing a preinjection under the same conditions but without illumination. To reduce the size of the O_2 background, small quantities of glucose, glucose oxidase, and catalase were added to the labeled water prior to injection without any interference with the photogenerated O_2. The signal amplitude for the third flash (Y_3) in the turnover sequence, minus the contribution from the injection (Y_{inj}) and double hits (Y_{2x}, as calculated in ref *44*), was normalized to the sum of flashes 4−7 given in the normalization sequence, to correct for small variations in sample concentration and membrane permeability between different measurements

$$Y_{3N} = \frac{Y_3 - Y_{inj} - Y_{2x}}{\sum_{n=4}^{7} Y_n} \quad (1)$$

The Y_{3N} value at each exchange time was then further normalized to the value obtained after complete exchange (i.e., 10 s) to give Y_{3C}. Finally, the data at fast exchange times (≤10 ms) was corrected for the 175 s^{-1} injection response of the sample chamber as described in ref *24*. The level of ^{18}O enrichment in the sample chamber (ϵ) was ∼12%, as determined according to ref *44*.

At *m*/*e* 34, plots of the corrected O_2 yields after the third flash, $^{34}Y_{3C}$, versus the ^{18}O exchange time in a particular S state, Δt, are clearly biphasic and were analyzed as the sum of two exponentials

$$^{34}Y_{3C} = 0.57[1 - \exp(-{}^{34}k_2 t)] + 0.43[1 - \exp(-{}^{34}k_1 t)] \quad (2)$$

where the relative contributions from the two phases follow from the isotopic distribution due to exchange at two independent sites (see ref *23*).

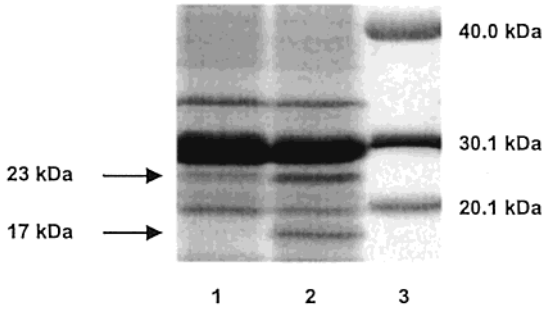

FIGURE 1: SDS−PAGE analysis of PSII-enriched membrane samples following treatment with 1 M NaCl: lane 1, Ex-depleted PSII sample; lane 2, intact PSII sample; and lane 3, molecular mass markers, as indicated.

In contrast, at *m*/*e* 36 plots of $^{36}Y_{3C}$ versus Δt exhibit only one kinetic phase and were analyzed in terms of a single exponential:

$$^{36}Y_{3C} = 1 - \exp(-{}^{36}kt) \quad (3)$$

Sigma Plot (SPSS, Chicago, IL) was used to fit the data according to eqs 2 and 3.

RESULTS

Figure 1 shows the SDS−PAGE analysis of the Ex-depleted PSII samples following treatment with 1 M NaCl. Densitometry analysis of the gel pattern indicated that the Ex-depleted PSII samples retain less than 8% of the 17 and 23 kDa extrinsic proteins and more than 90% of the 33 kDa extrinsic protein. As shown in Table 1, the removal of these proteins lowers the overall O_2-evolving activity to ∼20% of the control, which can be restored to ∼80% of the control by addtion of 15 mM $CaCl_2$.

Figure 2 shows the normalized O_2 flash yield patterns (i.e., Y_n/Y_{ss} vs flash number, *n*) for the various PSII samples measured at *m*/*e* 34 following complete isotopic equilibration with added $H_2{}^{18}O$ (i.e., at an exchange time of >10 s). The control sample reveals normal period 4 oscillations, clearly indicating that under these measuring conditions (i.e., at 10 °C with a flash repetition rate of 0.05 Hz), normal S state turnover occurs. The effective miss parameter for the control sample in this case is 10.5%, based on the Kok method of analysis. In contrast, for the Ex-depleted PSII sample, the O_2 flash yield pattern shows a much heavier damping, with an effective miss parameter of 25.5%. The increased miss parameter correlates with the reduced steady state O_2-evolving activity for the Ex-depleted samples (Table 1). As is commonly observed, the addition of 15 mM $CaCl_2$ to Ex-depleted PSII restores not only a large part of the steady state O_2-evolving activity (Table 1) but also the normal oscillation pattern (Figure 2).

To determine the influence of the 17 and 23 kDa extrinsic proteins on the binding of the substrate−water molecule to the OEC, the extent of ^{18}O exchange was measured at *m*/*e* 34 (which measures the amount of the $^{16}O^{18}O$ product) for variously treated PSII-enriched samples in the S_3, S_2, and S_1 states. The results are shown in Figure 3, where the corrected O_2 yields after the third flash in the turnover sequence ($^{34}Y_{3C}$) are plotted as a function of the $H_2{}^{18}O$ exchange times in a particular S state (Δt). For the control PSII sample, the ^{18}O exchange measurements in the S_3 state

Table 1: O_2-Evolving Activities and ^{18}O Exchange Rate Constants at *m*/*e* 34 for Variously Treated PSII-Enriched Membrane Samples at 10 °C

	O_2-evolving activity[a]	S_3 state		S_2 state		S_1 state	
		$^{34}k_1$ (s^{-1})	$^{34}k_2$ (s^{-1})	$^{34}k_1$ (s^{-1})	$^{34}k_2$ (s^{-1})	$^{34}k_1$ (s^{-1})	$^{34}k_2$ (s^{-1})
PSII membranes	862 ± 63	2.5 ± 0.2	30 ± 2	1.9 ± 0.3	≥175	0.022 ± 0.002	>100
Ex-depleted PSII membranes[b]	200 ± 6	2.4 ± 0.5	20 ± 3	2.6 ± 0.3	120 ± 14	0.021 ± 0.003	>100
Ex-depleted PSII membranes with $CaCl_2$[c]	696 ± 18	2.0 ± 0.7	18 ± 4	1.6 ± 0.2	102 ± 8	0.022 ± 0.003	>100

[a] O_2 evolution activity expressed as micromoles of O_2 per milligram of Chl per hour. [b] PSII membranes depleted of the 17 and 23 kDa extrinsic proteins (see the text for details). [c] Ex-depleted PSII membranes incubated for 2 h in 15 mM $CaCl_2$.

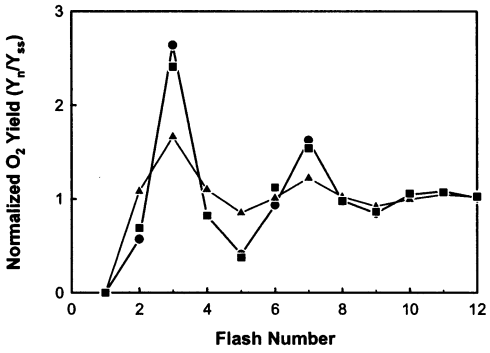

FIGURE 2: Normalized O_2 flash yield patterns determined at *m*/*e* 34 after complete isotopic equilibration with $H_2{}^{18}O$: (●) intact PSII sample, (▲) Ex-depleted PSII sample, and (■) Ex-depleted PSII sample incubated for 2 h in 15 mM $CaCl_2$. See the text for details.

exhibit strong biphasic behavior. According to eq 2, the rate constants for the slow and fast exchanging components ($^{34}k_1$ and $^{34}k_2$, respectively) were determined to be 2.5 ± 0.2 and 30 ± 2 s^{-1}, respectively (Table 1). These values compare very well with those previously reported for thylakoids (*23*) and for other PSII-enriched samples in the S_3 state (*25*).

Figure 3 also shows the first ^{18}O exchange measurements for PSII-enriched samples in the S_2 and S_1 states. The biphasic exchange behavior is maintained in both of these states as well, in which the rate constants for the slow component ($^{34}k_1$) equal 1.9 ± 0.3 s^{-1} for the S_2 state and 0.022 ± 0.002 s^{-1} for the S_1 state. These rate constants are almost identical to those reported for the slow component in the S_2 and S_1 states of thylakoids (*24*). However, as in the measurements of thylakoids, the fast component for the PSII-enriched samples remains unresolvable, where $^{34}k_2$ for the S_2 state is limited by the injection response time (~6 ms) and $^{34}k_2$ for the S_1 state is limited by the total time of the turnover flash sequence (10 ms). These data provide evidence that treatment with 5% Triton X-100 has little effect on substrate−water binding through the S state cycle and that the biphasic exchange behavior is inherent to PSII.

Although the fast phase kinetics in the S_2 state is limited by the injection response time, close examination of the data in Figure 3 reveals a *hint* of a resolvable fast component, with a number of $^{34}Y_{3C}$ points at ≤0.50 measured at exchange times of ≤15 ms. In exploring the role of the 17 and 23 kDa extrinsic proteins in the mechanism of O_2 evolution, we found initially that for Ex-depleted PSII samples in the S_3 state there is an ~30% slowing in the fast exchange component compared with the control (*25*). The same effect is clearly demonstrated in the S_3 state data shown in Figure 3, where $^{34}k_2$ decreases from 30 ± 2 to 20 ± 3 s^{-1} (Table 1). Most importantly, however, a similar trend is also observed in the S_2 state data, to the point where the fast component now becomes completely resolvable and yields a $^{34}k_2$ of 120 ± 14 s^{-1} (Table 1). Interestingly, the slow exchange component remains unaffected under these conditions. To confirm this last observation, measurements were also taken at *m*/*e* 36 (which measures the amount of the $^{18}O^{18}O$ product) for the Ex-depleted PSII in the S_2 state. The data are shown in Figure 4 and reveal only a single exchange component with a rate constant ^{36}k of 2.5 ± 0.2 s^{-1}. This value is virtually identical to $^{34}k_1$ in the S_2 state (Table 1) and confirms that the rate of $^{18}O^{18}O$ formation is limited by the substrate−water molecule undergoing the slowest exchange process. These results conclusively show that under these conditions, the second substrate−water molecule is already bound to the OEC in the S_2 state.

A similar trend could not be observed in the S_1 state of Ex-depleted PSII, where the fast exchange component remains unresolvable and is limited by the total time of the turnover flash sequence (i.e., 10 ms). There are no $^{34}Y_{3C}$ points at ≤0.50 measured at the short ^{18}O exchange times. However, like in the S_2 and S_3 states, the slow rate of exchange in the S_1 state is virtually unaffected by the removal of the 17 and 23 kDa proteins. The $^{34}k_1$ value in the S_1 state equals 0.021 ± 0.003 s^{-1} (Table 1) and is nearly identical to the corresponding rate constant obtained for the control PSII-enriched sample (Table 1) as well as for intact thylakoids (*24*).

The 17 and 23 kDa extrinsic proteins are known to modulate the binding affinity of the functional Ca^{2+} (*31*, *32*), in which the removal of these proteins leads to a slow release of the Ca^{2+}. After long incubation times (1−2 h), the resulting low O_2 evolution activity in Ex-depleted PSII samples can be restored to ~80% of the control level by the addition of millimolar concentrations of $CaCl_2$ (Table 1). Likewise, the addition of $CaCl_2$ also restores the normal O_2 flash yield pattern (Figure 2). In view of these observations, we assessed the ^{18}O exchange behavior of Ex-depleted PSII samples in the presence of 15 mM $CaCl_2$. The results are also shown in Figure 3, and the corresponding rate constants are given in Table 1. In the S_3 state. $^{34}k_1 = 2.0 \pm 0.7$ s^{-1} and $^{34}k_2 = 18 \pm 4$ s^{-1}; in the S_2 state $^{34}k_1 = 1.6 \pm 0.2$ s^{-1} and $^{34}k_2 = 102 \pm 8$ s^{-1}, and in the S_1 state, $^{34}k_1 = 0.022 \pm 0.003$ s^{-1} and $^{34}k_2 > 100$ s^{-1}. Comparison of these data with the ^{18}O exchange measurements made on Ex-depleted PSII alone shows that there is no further effect of added Ca^{2+} on the ^{18}O exchange behavior under these conditions. Thus, the specific slowing in the rate of the fast exchange component in the S_3 and S_2 states of Ex-depleted PSII samples is due only to the loss of the 17 and 23 kDa proteins.

DISCUSSION

On the basis of the time resolution of our current experimental setup (~6 ms), a measurable kinetic isotope effect will conclusively show the existence of a bound

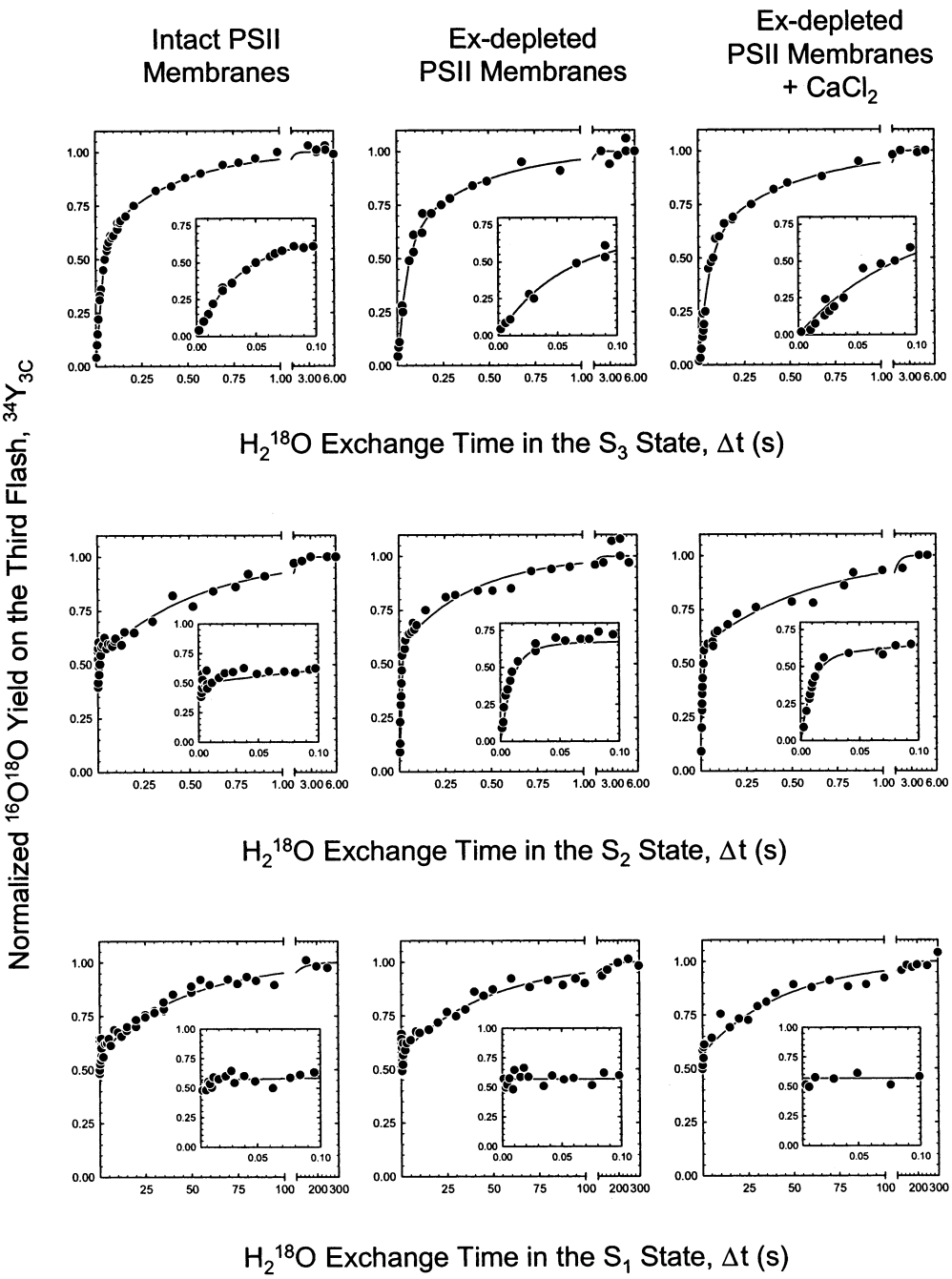

FIGURE 3: ^{18}O exchange measurements made at *m*/*e* 34 in the S_3-S_1 states for intact PSII samples, Ex-depleted PSII samples, and Ex-depleted PSII samples incubated for 2 h in 15 mM $CaCl_2$. The corrected O_2 yields on the third flash in the initial turnover sequence ($^{34}Y_{3C}$) are plotted as a function of the isotopic exchange time (Δt) in each S state at 10 °C. Solid lines are kinetic fits according to eq 2. See the text for details.

substrate−water molecule in the OEC. This is justified by the observations that O_2 release upon the $S_3 \rightarrow [S_4] \rightarrow S_0$ transition occurs in ~2 ms while the rest of the S state cycle from S_0 to S_3 takes at most an additional 1 ms (*1*). Thus, any ^{18}O exchange slower than ~3 ms will be indicative of bound water. Hence, the exact determination of the slow phase ^{18}O exchange kinetics in the S_0-S_3 states (*24*) conclusively showed that one substrate water molecule is bound to the OEC throughout the S state cycle. However, since the fast phase ^{18}O exchange kinetics in the earlier measurements could only be determined in the S_3 state, the possibility remained that the second substrate water molecule binds to the OEC only after the formation of the S_3 state (*24*).

In our effort to address the possible influence of the 17 and 23 kDa extrinsic proteins on substrate−water binding, we have extended S state-dependent ^{18}O exchange measurements to PSII-enriched membrane samples depleted of these proteins (Ex-depleted PSII). Interestingly, following the removal of these proteins, the rate constant for the fast exchanging component in the S_3 state decreases by ~30%, from 30 ± 2 to 20 ± 3 s^{-1} (Table 1), while the rate constant for the slow exchanging component remains virtually unaffected. However, most importantly, in the S_2 state there is also a slowing of the fast exchanging component, to the point where it is kinetically resolved for the first time by our current experimental setup. In this case, the rate constant ($^{34}k_2$) is found to equal 120 ± 14 s^{-1} (Table 1). As in the S_3

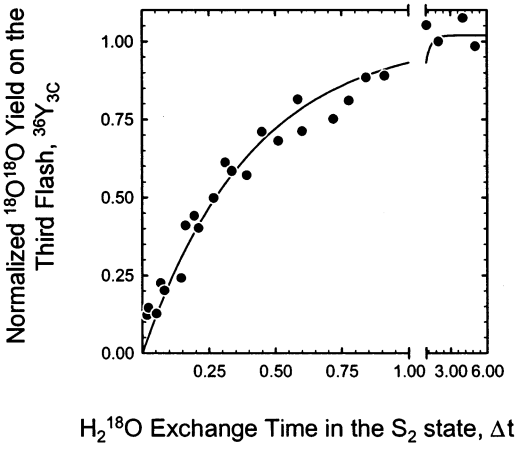

FIGURE 4: ^{18}O exchange measurements made at *m*/*e* 36 in the S_2 state for Ex-depleted PSII membranes. The corrected O_2 yields on the third flash in the initial turnover sequence ($^{36}Y_{3C}$) are plotted as a function of the isotopic exchange time (Δt) in the S_2 state at 10 °C. The solid line is a kinetic fit according to eq 3. See the text for details.

state, there is virtually no effect of protein removal on the slow exchanging component in the S_2 state (Figure 3 and Table 1).

The fact that two distinct ^{18}O exchange rates can be measured in the S_2 state of Ex-depleted PSII indicates that *both* substrate water molecules must be bound to the OEC under these conditions. Although we cannot unambiguously determine the fast phase exchange kinetics in the S_2 state for intact PSII samples, close examination of the ^{18}O exchange data reveals a *hint* of a resolvable fast exchanging component. For both PSII-enriched membrane samples (Figure 3) and thylakoids (*24*), a number of $^{34}Y_{3C}$ values of ≤ 0.50 at exchange times of ≤ 15 ms can be measured outside of the expected error. For Ex-depleted PSII in the S_3 state, there is an ~30% slowing of the fast exchanging component. If a similar trend is assumed in the S_2 state, where the fast exchanging component is at the detectable kinetic limit of 175 s^{-1} (due to the injection response), then it is likely that *both* substrate−water molecules are bound to the OEC in the S_2 state of the intact system as well.

In support of the above arguments, we note that the removal of the 17 and 23 kDa extrinsic proteins from PSII results in a much lower overall O_2-evolving activity, which can be largely restored by the addition of 15 mM $CaCl_2$ (Table 1). When the ^{18}O exchange of Ex-depleted PSII in the presence of $CaCl_2$ is assessed, the fast exchanging component in the S_2 state still remains resolvable with a $^{34}k_2$ of 102 ± 8 s^{-1}. Thus, even in this highly active sample, both substrate−water molecules must be bound to the OEC in the S_2 state and it is the loss of the 17 and 23 kDa extrinsic proteins that specifically slows the rate of fast exchange.

In contrast, no effect of the protein removal could be observed in the ^{18}O exchange behavior in the S_1 state, in which the fast phase kinetics remains completely unresolved (Figure 3 and Table 1). This last observation leaves open the possibility that the second substrate−water molecule binds to the OEC only after the formation of the S_2 state.

It is important to note that the slow phase ^{18}O exchange kinetics in the S_3-S_1 states remain virtually unaffected by the removal of the 17 and 23 kDa extrinsic proteins and that only the fast phase ^{18}O exchange kinetics in the S_3 and S_2 states are slowed. We have argued earlier that the local dielectric around the substrate binding sites can influence the ^{18}O exchange behavior (*25*). Thus, it could be that the removal of the 17 and 23 kDa proteins increases the level of exposure of the fast exchanging site to the solvent water. A consequent increase in the level of regional hydration around this site could then give rise to a local change in the dielectric (*45*, *46*) and to an altered ^{18}O exchange rate. Interestingly, there would have to be an asymmetry in the dielectric distribution within the OEC, since the slow exchanging site is unaffected by the removal of the proteins. Such a situation would strongly imply that the two substrate binding sites are located in different chemical environments.

The chemical nature of the $S_2 \rightarrow S_3$ transition is a matter of considerable debate (*2*−*15*). However, it is clear from our results that there is no change in the exchange rate of the slowly exchanging substrate-water molecule on the $S_2 \rightarrow S_3$ transition, in either the intact or Ex-depleted PSII samples (Table 1; *24*). These observations thus indicate that the slowly exchanging water is unlikely to be bound to a metal site undergoing a formal oxidation state increase, in which the oxygen ligand exchange rate would be expected to decrease by as much as a factor of $\sim 10^4$ (*47*). On the other hand, there is at best a slowing in the exchange rate of the fast exchanging water by a factor of ~5 on the $S_2 \rightarrow S_3$ transition (Table 1). Although such a decrease in the exchange rate is also unlikely to reflect a metal-centered oxidation increase, it would be compatible with a deprotonation of the substrate water (*47*) or with a rearrangement in the H-bonding at the binding site, as has been suggested previously (*2*, *4*, *5*, *8*).

Likewise, it is often implied that the two substrate−water molecules are bound to separate Mn ions differing by a formal oxidation state [e.g., Mn(III) and Mn(IV)] in the S_3 state and/or in the S_2 state (*2*, *3*, *6*, *7*, *9*, *13*, *14*). In this situation as well, we would also expect to observe a significant difference (again by as much as a factor of $\sim 10^4$) in the magnitudes of the exchange rates for the two sites (*47*). Instead, the exchange rates for the two sites differ by factors of only ~10 and ~50 in the S_3 and S_2 states, respectively (Table 1). Thus, if Mn forms the binding sites for the substrate−water molecules, we would minimally suggest that the Mn sites have similar oxidation character (*47*). Alternatively, the difference in the exchange rates for the two sites could reflect ligation by one of the substrate−water molecules to a non-manganese site such as to the functional Ca^{2+} (*5*, *8*, *12*). Indeed, a number of factors determine oxygen ligand exchange. These include not only the nature of the site, whether it is a metal, but also the oxidation level of the site, the degree of protonation and geometry of the oxygen ligand, H-bonding, and the p*K* of the reaction sphere. Clearly, the overall exchange kinetics of the bound substrate−water molecules in the OEC is a summation of these properties, all of which will need to be considered in future interpretations of the O_2-evolving mechanism.

CONCLUSION

We have presented in this communication the first direct evidence to show that both substrate water molecules are bound to the OEC in the S_2 state. The removal of the 17 and 23 kDa extrinsic proteins from PSII membranes leads

to a specific slowing of the fast exchanging substrate−water molecule to the point where its rate of exchange can be exactly determined in both the S_3 and S_2 states. On the basis of the magnitude of the change in the fast exchange rates induced by the removal of these proteins, it is likely that there is an asymmetric distribution in the dielectric around the substrate binding sites within the OEC.

ACKNOWLEDGMENT

We sincerely thank Warwick Hillier for his advice and assistance in this work.

REFERENCES

1. Kok, B., Forbush, B., and McGloin, M. (1970) *Photochem. Photobiol. 11*, 457−475.
2. Hoganson, C. W., and Babcock, G. T. (1997) *Science 277*, 1953−1956.
3. Renger, G. (2001) *Biochim. Biophys. Acta 1503*, 210−228.
4. Pecoraro, V. L., Baldwin, M. J., Cuadle, M. T., Hsieh, W.-Y., and Law, N. A. (1998) *Pure Appl. Chem. 70*, 925−929.
5. Vrettos, J. S., Limburg, J., and Brudvig, G. W. (2001) *Biochim. Biophys. Acta 1503*, 229−245.
6. Haumann, M., and Junge, W. (1999) *Biochim. Biophys. Acta 1411*, 86−91.
7. Schlodder, E., and Witt, H. T. (1999) *J. Biol. Chem. 274*, 30387−30392.
8. Siegbahn, P. E. (2000) *Inorg. Chem. 39*, 2923−2935.
9. Dau, H., Iuzzolino, L., and Dittmer, (2001) *Biochim. Biophys. Acta 1503*, 24−39.
10. Peloquin, J. M., and Britt, R. D. (2001) *Biochim. Biophys. Acta 1503*, 96−111.
11. Mino, H., and Kawamori, A. (2001) *Biochim. Biophys. Acta 1503*, 112−122.
12. Kuzek, D., and Pace, R. J. (2001) *Biochim. Biophys. Acta 1503*, 123−137.
13. Nugent, J. H. A., Rich, A. M., and Evans, M. C. W. (2001) *Biochim. Biophys. Acta 1503*, 138−146.
14. Messinger, J., Robblee, J. H., Bergmann, U., Fernandez, C., Glatzel, P., Visser, H., Cinco, R. M., McFarlane, K. L., Bellacchio, E., Pizarro, S. A., Cramer, S. P., Sauer, K., Klein, M. P., and Yachandra, V. K. (2001) *J. Am. Chem. Soc. 123*, 7804−7820.
15. Carrell, T. G., Tyryshkin, A. M., and Dismukes, G. C. (2002) *J. Biol. Inorg. Chem. 7*, 2−22.
16. Lavergne, J., and Junge, W. (1985) *Photochem. Photobiol. 41*, 183−190.
17. Hansson, Ö., Andréasson, L.-E., and Vänngård, T. (1986) *FEBS Lett. 195*, 151−154.
18. Tang, X.-S., Sivaraja, M., and Dismukes, G. C. (1993) *J. Am. Chem. Soc. 115*, 2382−2389.
19. Evans, M. C. W., Rich, A. M., and Nugent, J. H. A. (2000) *FEBS Lett. 477*, 113−117.
20. Noguchi, T., and Sugiura, M. (2000) *Biochemsitry 39*, 10943−10949.
21. Noguchi, T., and Sugiura, M. (2002) *Biochemistry 41*, 2322−2330.
22. Messinger, J., Badger, M., and Wydrzynski, T. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 3209−3213.
23. Hillier, W., Messinger, J., and Wydrzynski, T. (1998) *Biochemistry 37*, 16908−16914.
24. Hillier, W., and Wydrzynski, T. (2000) *Biochemsitry 39*, 4399−4405.
25. Hillier, W., Hendry, G., Burnap, R. L., and Wydrzynski, T. (2001) *J. Biol. Chem. 276*, 46917−46924.
26. Radmer, R., and Ollinger, O. (1986) *FEBS Lett. 195*, 285−289.
27. Bader, K. P., Thibault, P., and Schmid, G. H. (1987) *Biochim. Biophys. Acta 893*, 564−571.
28. Seidler, A. (1996) *Biochim. Biophys. Acta 1277*, 35−60.
29. Ghanotakis, D. F., Babcock, G. T., and Yocum, C. F. (1984) *FEBS Lett. 167*, 127−130.
30. Miyao, M., and Murata, N. (1984) *FEBS Lett. 168*, 118−120.
31. Ghanotakis, D. F., Topper, J. N., Babcock, G. T., and Yocum, C. F. (1984) *FEBS Lett. 170*, 169−173.
32. Vrettos, J. S., Stone, D. A., and Brudvig, G. W. (2001) *Biochemistry 40*, 7937−7945.
33. Ghanotakis, D. F., Topper, N. J., and Yocum, C. F. (1984) *Biochim. Biophys. Acta 767*, 524−531.
34. Vander Meulen, K. A., Hobson, A., and Yocum, C. F. (2002) *Biochemistry 41*, 958−966.
35. Alexander, R. S., Nair, S. K., and Christianson, D. W. (1991) *Biochemistry 30*, 11064−11072.
36. Fierke, C. A., Calderone, T. L., and Krebs, J. F. (1991) *Biochemistry 30*, 11054−11063.
37. Oprea, T. I., Hummer, G., and Garcia, E. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 2133−2138.
38. Wydrzynski, T., Hillier, W., and Messinger, J. (1991) *Physiol. Plant. 96*, 342−350.
39. Messinger, J., Wacker, U., and Renger, G. (1991) *Biochemistry 30*, 7852−7862.
40. Berthold, D. A., Babcock, G. T., and Yocum, C. F. (1984) *FEBS Lett. 134*, 231−234.
41. Kuwabara, T., and Murata, N. (1983) *Plant Cell Physiol. 24*, 741−747.
42. Laemmli, U. K. (1970) *Nature 227*, 680−685.
43. Porra, R. J., Thompson, W. A., and Kriedemann, P. E. (1989) *Biochim. Biophys. Acta 975*, 384−394.
44. Hillier, W. (1999) Ph.D. Thesis, The Australian National University, Canberra, Australia.
45. Dwyer, J. J., Gittis, A. G., Karp, D. A., Lattman, E. E., Spencer, D. S., Stites, W. E., and Garcia-Moreno, E. B. (2000) *Biophys. J. 79*, 1610−1620.
46. Pitera, J. W., Falta, M., and van Gunsteren, W. F. (2001) *Biophys. J. 80*, 2546−2555.
47. Hillier, W., and Wydrzynski, T. (2001) *Biochim. Biophys. Acta 1503*, 197−209.

BI026246T