The exchange of the fast substrate water in the $S_2$ state of photosystem II is limited by diffusion of bulk water through channels – implications for the water oxidation mechanism†

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The molecular oxygen we breathe is produced from water-derived oxygen species bound to the Mn$_4$CaO$_5$ cluster in photosystem II (PSII). Present research points to the central oxo-bridge O5 as the ‘slow exchanging substrate water ($W_s$)’, while, in the $S_2$ state, the terminal water ligands W2 and W3 are both discussed as the ‘fast exchanging substrate water ($W_f$)’. A critical point for the assignment of $W_f$ is whether or not its exchange with bulk water is limited by barriers in the channels leading to the Mn$_4$CaO$_5$ cluster. In this study, we measured the rates of H$_2^{16}$O/H$_2^{18}$O substrate water exchange in the $S_2$ and $S_3$ states of PSII core complexes from wild-type (WT) Synechocystis sp. PCC 6803, and from two mutants, D1-D61A and D1-E189Q, that are expected to alter water access via the Cl1/O4 channels and the O1 channel, respectively. We found that the exchange rates of $W_f$ and $W_s$ were unaffected by the E189Q mutation (O1 channel), but strongly perturbed by the D61A mutation (Cl1/O4 channel). It is concluded that all channels have restrictions limiting the isotopic equilibration of the inner water pool near the Mn$_4$CaO$_5$ cluster, and that D61 participates in one such barrier. In the D61A mutant this barrier is lowered so that $W_f$ exchange occurs more rapidly. This finding removes the main argument against Ca-bound W3 as fast substrate water in the $S_2$ state, namely the indifference of the rate of $W_f$ exchange towards Ca/Sr substitution.

Introduction

Photosynthesis performed by plants, algae and cyanobacteria is critical for life on Earth as it releases molecular oxygen into the atmosphere and stores solar energy as biomass. Utilizing sunlight, the protein complex photosystem II (PSII) generates the O$_2$ molecule (WN2).\cite{9,12,18,19} All S state transitions, with the exception of $S_1 \rightarrow S_2$, are coupled to proton release into the bulk, keeping the total charge of the cluster at 0 or +1, respectively.\cite{23} Proton release is facilitated by an intricate H-bonding network that is pivotal to the function of PSII and its earth-abundant water oxidation catalyst.\cite{17,21,24}

The water oxidation reaction is catalyzed by a metal–oxygen cluster comprising the metals manganese and calcium in a 4 : 1 stoichiometry as well as five oxo bridges (O1–O5).\cite{4,6} During the reaction cycle, the Mn$_4$CaO$_5$ cluster is stepwise oxidized by light-induced charge separations in the chlorophyll containing reaction center of PSII. Thereby, it attains four discrete reaction intermediates ($S_0$–$S_4$) and one highly reactive transient ($S_5$).\cite{7,10} The $S_1$ state is dark-stable, and the $S_2 \rightarrow S_3$ transition involves the association of a new water molecule ($W_{N1}$), yielding a Mn$_4$CaO$_6$ cluster as the last stable intermediate before O$_2$ formation.\cite{11–17} The next light-induced charge separation triggers the $S_3 \rightarrow S_4 \rightarrow S_5$ transition, which not only involves the O–O bond formation, but also O$_2$ release and the concomitant filling of the open coordination site by one of the terminal water ligands (W3 or W2) as well as the binding of a new water molecule ($W_{N2}$).\cite{9,12,18,19} All S state transitions, with the exception of $S_1 \rightarrow S_2$, are coupled to proton release into the bulk, keeping the total charge of the cluster at 0 or +1, respectively.\cite{23} Proton release is facilitated by an intricate H-bonding network that is pivotal to the function of PSII and its earth-abundant water oxidation catalyst.\cite{17,21,24}

The Mn$_4$CaO$_5$ cluster is frequently described as having a ‘chair’-like structure, with the base formed by a Mn$_3$CaO$_4$
hetero-cubane and the back by the fourth Mn ion (Mn4) that is connected to the base via the oxygen bridges O5 and O4 (Fig. 1). As there is no bond between O5 and Mn1, the structure is referred to as ‘open cubane’. Importantly, this structure binds four water molecules, two at Mn4 (W1, W2) and two at Ca (W3, W4), while all other coordination sites, except one at Mn1, are filled by five o xo-bridges, six bridging carboxylates and one histidine ligand. In the S0 state, the four Mn ions have the oxidation states Mn(III,III,IV,VII), or superoxo intermediates have been proposed; the closed cube S2 is most stable. The faster exchange at the Mn(III) induces a rapid enrichment of the sample with H2 18O by membrane inlet mass spectrometry (MIMS) allows obtaining a unique experimental signature for the two substrates: their exchange rates with bulk water. Using this approach, it was shown that the two substrates are bound differently in the S2 and S3 states. The faster exchanging substrate water is referred to as Wfl, while the slower one is denoted as WS. For the S0 and S1 states, only the exchange rates of WS were determined. However, since no water binding

![Fig. 1: Structure of the Mn4CaO5 cluster with selected ligands and water molecules in the S2 (panels A and B) and S3 (C and D) states of photosystem II (PDB: 6DHF & 6DHO). Note that the cluster has a sixth oxygen bridge labelled X in the S2 state. Panels A & C highlight the position of D61 and panels B & D that of E189 in relation to the Mn4CaO5 cluster and the O4 (blue), C11 (green) and O1 (pink) water/proton channels. Potential hydrogen bonds are shown as dashed lines, while the coordination of E189 to Ca and Mn is indicated with solid lines. The position of W20, which is not resolved in the S2- and S3-state structures, is indicated by a dashed circle. E: Cartoon of the D to A mutation (left) and the E to Q mutation (right). Color code: large black sphere – peptide backbone; red - oxygen; blue - nitrogen; purple – manganese; yellow – calcium; green – chloride; grey - methyl group. The molecular representations were generated with VMD.]

**Scheme 1** Suggested routes for insertion of WN1 and formation of the Ox hydroxo bridge during the S2 → S3 transition. Panels A and B show two proposed pathways for W3 insertion. Pathway A starts from the more stable, open cube (S2$^A$) conformation of the Mn4CaO5-cluster. W3 is inserted into the Ox site between Ca and Mn1 while WN1 replaces W3. B: The Mn4CaO5-cluster attains first the S2$^B$ conformation before W3 binds to Mn4. W3 then flips into the O5 binding site, while O5 moves into the Ox position and WN1 replenishes the original W3 coordination site at Ca. C: The pivot or carousel mechanism requires also that the cluster attains first the less stable S2$^C$ conformation. Binding of WN1 to the five-coordinate Mn4(III) induces a cascade of water/oxygen relocations allowing W1 to replace W2, W2 to flip into the O5 position, and O5 to occupy the Ox site.

**Notes:**

1. As there is no bond between O5 and Mn1, the structure is referred to as ‘open cubane’. Importantly, this structure binds four water molecules, two at Mn4 (W1, W2) and two at Ca (W3, W4), while all other coordination sites, except one at Mn1, are filled by five o xo-bridges, six bridging carboxylates and one histidine ligand.
2. In the S0 state, the four Mn ions have the oxidation states Mn(III,III,IV,VII), or superoxo intermediates have been proposed: the closed cube S$^2$ is most stable.
3. The faster exchange at the Mn(III) induces a rapid enrichment of the sample with H2 18O by membrane inlet mass spectrometry (MIMS) allows obtaining a unique experimental signature for the two substrates: their exchange rates with bulk water. Using this approach, it was shown that the two substrates are bound differently in the S2 and S3 states. The faster exchanging substrate water is referred to as Wfl, while the slower one is denoted as WS. For the S0 and S1 states, only the exchange rates of WS were determined. However, since no water binding
Three channels have been identified that lead to the Mn$_4$CaO$_5$ cluster: the O1 or ‘large’ channel, the O4 or ‘narrow’ channel, and the Cl1 or ‘broad’ channel (Fig. 1). While the O1 and Cl1 channels both split into two branches (A, B),

$$\text{S}_2 \rightarrow \text{S}_1 \rightarrow \text{S}_0$$

and secondly independent of Ca/Sr-substitution in both the S$_2$ 46 and S$_3$ 18,28,59 states. In addition, Wf exchange becomes observable first in the S$_2$ state, and then slows upon S$_3$ and S$_3$YZ state formation, making a diffusion limitation that could obscure the Ca/Sr dependence seemingly unlikely. By contrast, these two observations can be well explained with W2 as Wf by the known oxidation of Mn4 during the S$_1 \rightarrow$ S$_2$ transition and the need to involve electron back donation of Y$_2$ for Wf exchange in the S$_3$ state. Absence of a diffusion limitation is apparently further supported by molecular dynamics (MD) calculations that predict water access in the 50 ns to 100 μs time range, 60,61 i.e. orders of magnitude faster than Wf exchange (50–100 ms). 44,46,57

To probe if the fast water exchange (Wf) in the S$_2$ state is limited by diffusion through channels or by the chemical exchange process, we study here the effects of the D1-D61A and D1-E189Q mutations on the rates of substrate water exchange with bulk water in the S$_2$ and S$_3$ states.

The D61 residue is located close to Mn4 at the apex between the potential O4 and Cl1 substrate channels (Fig. 1). D61 hydrogen bonds W1 and some further waters in its surroundings. If this aspartate (D) residue is mutated to either asparagine (N) or alanine (A), O$_2$ production decreases by ~75–80%, and the S$_1 \rightarrow$ S$_2$ and S$_2 \rightarrow$ S$_3$ transitions are decelerated by factors of 2–3. 67 Meanwhile, O$_2$ release in the S$_2 \rightarrow$ S$_3$ transition is retarded 20–30 fold. 67–69 These functional effects were attributed to poor proton abstraction from the mutants, identifying this residue as an important proton relay. 68,70,71 It may be speculated that if W2 were a substrate, its exchange would be greatly affected by the D61A mutation. The S$_3$ state exchange rates were previously measured for the D61N mutant, showing 6-fold and 3-fold slower exchange rates for Wf and Ws, respectively. 72

E189 is located at the end of the O1 channel. In the S$_2$ and S$_3$ states, E189 is a ligand of Mn1, and it also weakly ligates Ca. Recently it was shown, by time-resolved X-ray crystallography, that during the S$_2 \rightarrow$ S$_3$ transition E189 detaches from Ca before Ox is inserted, and afterwards hydrogen bonds Ox (Fig. 1B and D). 15,17,21 Consequently, this glutamate residue (E189) may be
important for the insertion of Ox during the $S_2$ $\rightarrow$ $S_3$ transition, the exchange of Ox by bulk water in the $S_3$ state, and O–O bond formation. Only a handful mutations of E189 yield active PSII centers, namely isoleucine (I), lysine (K), leucine (L), glutamine (Q) and arginine (R).$^{23}$ E189Q is a conservative mutant, as it is of similar size and retains the ability to act as bidentate ligand (Fig. 1E). While the $S_2$$^{25}$ signal is not perturbed by the mutation, the oxygen evolution activity is decreased by /C2430%, indicating that some transition in the catalytic cycle does not function optimally. For the $S_3$ state, an up to 2-fold faster substrate water exchange was reported previously.$^{74}$

**Experimental procedures**

**Preparation of photosystem II core complexes**

*Synechocystis* sp. PCC 6803 strains, with a 6xHis-tag fused to the CP47 gene, expressing the psbA2-gene (WT, D1-D61A or D1-E189Q) were propagated in BG11 medium supplemented with glucose in glass carboys and grown as previously described.$^{70}$ Thylakoid membranes and core complexes were prepared as described previously.$^{70}$ The PSII core complexes were suspended in 1.2 M betaine, 10% (v/v) glycerol, 50 mM MES-NaOH (pH 6.0), 20 mM CaCl2, 5 mM MgCl2, 50 mM histidine, 1 mM EDTA, and 0.03% (w/v) n-dodecyl β-D-maltoside, and were concentrated to 0.15–0.2 mg of Chl per mL. The samples were then divided into 100 μL aliquots and flash-frozen in liquid N2. Finally, samples were stored at −80 °C.

**Time-resolved membrane-inlet mass spectrometry**

Substrate–water exchange rates were measured at 10 °C employing an isotope ratio mass spectrometer (Finnigan Delta Plus XP) featuring 7 Faraday cups ($m/z$ 32, 34, 36, 40, 44, 46 & 48) and a 165 μL rapid mixing reaction cell that was connected to the spectrometer through a stainless steel pipe that passed through a Dewar filled with liquid N2.$^{24}$ After thawing, the PSII core complexes were washed (total dilution factor: 100–1000) in 50 mM MES-NaOH pH/pD 6.5, 1 M betaine, 15 mM CaCl2, 15 mM MgCl2 using an Amicon Ultra-0.5 centrifugal filter unit and finally concentrated to 0.15–0.2 mg Chl per mL. After a saturating preflash (5 μs FWHM), the sample was dark-adapted for 1 hour at room temperature. Prior to loading in dim green light, 0.3 mM (final concentration) 2,6-dichloro-1,4-benzoquinone was added. A modified gas-tight syringe (Hamilton CR-700-50) with an air pressure driven, computer triggered piston, previously loaded under N2 atmosphere with /C2422 μL 97% H2$^{18}$O, was employed for rapid (/>6 ms) isotope enrichment to a final level of >12%.$^{57}$

Residual O2 in the H2$^{18}$O was estimated and removed from the data as described previously.$^{44}$ The measurement sequences for all samples and S states are shown in ESI Fig. S2.$^{†}$ The substrate exchange rates ($k_{f1}$, $k_{f2}$, $k_{s1}$ and $k_{s2}$) for the fast and slow substrate waters were determined by a simultaneous fit to the $m/z$ 34 and the $m/z$ 36 data (for details see ESI Text 1 and Table S1$^{†}$).

![Fig. 2](image-url) Substrate water exchange measurements in the $S_3$ state of WT- (black) and D61A- (red) PSII core complexes of *Synechocystis* sp. PCC6803. The normalized oxygen yield of a flash given after different incubation times with H2$^{18}$O in the $S_3$ state are plotted. A and C show the results for single labelled oxygen ($m/z$ 34), while panels B and D those for double labelled oxygen ($m/z$ 36). Dots represent individual measurements, while solid lines the results of kinetic fits (Table 1). The fits of the WT-PSII substrate exchange are shown as a dashed line next to the D61A-PSII data for visual comparison. The inserts show an enlarged view of the fast exchange phase in the $m/z$ 34 data. Observe differences in the time scales. The data were recorded at 10 °C, pH 6.5.
Results

The substrate water exchange rates of WT-, D61A- and E189Q-PSII core complexes from *Synechocystis* sp. PCC 6803 were studied in the S2 and S3 states of the oxygen-evolving complex at 10 °C, pH 6.5. For WT-PSII, the canonical biphasic exponential rise with a fast and slow phase was observed for the $^{16,18}$O$_2$ signal from the m/z 34 cup in the S2 and S3 states (symbols in Fig. 2A and 3A). The biphasic rise shows that the two substrate waters are bound differently to the Mn$_4$CaO$_5$ cluster in these S states. Accordingly, they are referred to as the fast, $W_f$, and slow, $W_s$, exchanging substrate waters. The corresponding rates, $k_f$ and $k_s$, obtained from the kinetic fits (solid lines) are given in Table 1. For the $^{18,18}$O$_2$ signal (m/z 36), which requires that both substrate waters exchange against H$_2^{18}$O added to the bulk water, a mono-exponential rise with the rate $k_s$ was detected (Fig. 2B and 3B). This is expected, as this process is limited by the slower exchange process. The monophasic rise of the m/z 36 signal confirms that the two kinetic phases in the m/z 34 signal do not arise from sample heterogeneity.

In the S3 state, mutation of the D1–D61 residue to alanine led to a 24- and 12-fold slowing of $W_f$ and $W_s$ exchange (Fig. 2C and D, Table 1). This slowing is one of the largest effects of a mutation or biochemical change on substrate exchange kinetics observed thus far. For example, this change is 4-fold larger than the previously reported 6- and 3-fold decelerations for the D61N mutant. Notably, the monophasic rise of the m/z 36 signal was preserved (Fig. 2D).

In the S2 state, the same mutation had the opposite effect, i.e. a strong acceleration of the exchange was found for both

| Table 1 | Exchange rates of substrate water in the S$_2$ and S$_3$ states of photosystem II core complexes isolated from wild-type (WT), D1-D61A and D1-E189Q mutants of *Synechocystis* sp. PCC 6803. The rate constants and fractions of PSII centers were obtained from global fits of the $^{16,18}$O$_2$ (m/z 34) and $^{18,18}$O$_2$ (m/z 36) data displayed as lines in Fig. 2 and 3. The data were obtained at 10 °C and pH 6.5. For additional parameters see ESI Table S1 |
|-------|-------|-------|-------|-------|-------|-------|-------|
|       | WT-PSII | D61A-PSII | E189Q-PSII |
|       | $k_f$ | $k_s$ | $k_{f1}$ | $k_{s1}$ | $k_{f2}$ | $k_{s2}$ |
| S$_3$ Fraction, % | 100 | 100 | 0 | 0 | 100 |
| Rate, s$^{-1}$ | 23.4 ± 1.4 | 0.76 ± 0.03 | 0.97 ± 0.07 | 0.064 ± 0.003 | — | — |
| Mutant/WT | — | — | — | — | 24.6 ± 1.8 | 0.76 ± 0.04 |
| S$_2$ Fraction, % | 100 | 85 | 15 | 15 | 1.07 ± 0.08 | 1.00 ± 0.07 |
| Rate, s$^{-1}$ | 84 ± 5 | 0.97 ± 0.03 | >300 | 15 ± 1 | 1.4 ± 0.9 | 0.4 ± 0.1 |
| Mutant/WT | — | — | >3.5 | 15 ± 1 | 0.017 ± 0.011 | 0.4 ± 0.1 |
|          | — | — | — | — | 0.79 ± 0.08 | 0.97 ± 0.05 |
substrates (Fig. 3C and D): 15-fold for \( W_e \) and more than 3.5-fold for \( W_o \), of which the rate could no longer be resolved with our present mixing system (Table 1).

However, detailed analysis showed that the exchange of both \( W_t \) and \( W_e \) were biphasic, and that in the smaller fraction, about 15%, the exchange of \( W_t \) and \( W_e \) occurred with rates that were slower than those of WT-PSII (Table 1). Thus, the m/z 34 data were fit with 4 kinetic phases instead of 2. This showed that in the \( S_2 \) state of D61A-PSII two stable populations of the Mn4CaO5 cluster with possibly different substrates, exchange pathways or water accessibility must exist.

To probe the effects of H-bonding and of O–H bond breaking/formation on the exchange of substrate water in the \( S_2 \) state of WT- and D61A-PSII, we performed the same experiments also in D2O (Fig. S3 and Table S2†). In general, the exchange rates of \( W_t \) and \( W_e \) were slower in D2O. \( W_t \) showed a corrected H/D isotope effect of \( \approx 1.3 \). By contrast, \( W_e \) displayed an H/D isotope effect of 1.5 [WT] to 1.9 (D61A, larger fraction) and 2.8 (D61A, smaller fraction). In D61A-PSII, the smaller phase of \( W_t \) and \( W_e \) exchange increased from 15% (H2O) to 24% (D2O) (Table S2†).

Water exchange in the \( S_2 \) and \( S_3 \) states of the D1-E189Q mutant occurred with nearly identical rates as in WT-PSII. Only the exchange of \( W_t \) was retarded by \( \approx 20\% \) in the \( S_2 \) state of the E189Q samples (Table 1; Fig. S4†). We note that a \( \approx 2\)-fold acceleration was previously observed in the \( S_1 \) state exchange rates of E189Q-PSII thylakoid membranes.74

Discussion

In this study, we observed that the mutation of D61 to alanine had a strong effect on the exchange of both substrate waters in the \( S_2 \) and \( S_3 \) states, while the mutation of E189 to glutamine had essentially no influence on either \( W_t \) or \( W_e \) exchange. As D61 is close to \( W_2 \), while E189 is near \( W_3 \) and \( O_x \), these results appear, at first glance, to favor \( W_2 \) over \( W_3 \) as fast exchanging substrate \( W_t \). However, because we previously showed that the substrate water exchange rates in PSII are strongly affected by conformational equilibria of the Mn4CaO5 cluster, and because the mutations are also located at the end points of water channels and may thereby affect the diffusion of water to the catalytic site, a more detailed analysis is required.

For example, our recent studies have shown that the exchange rate of \( W_t \) in the \( S_2 \) state depends on the equilibria between the \( S_2^A \), \( S_2^{AW} \), \( S_2^{BW} \) and \( S_2^B \) states of the Mn4CaO5 cluster (Scheme 2).46,55 This allows O5 to reach a terminal position on a MnIII ion (Mn4) and to be exchanged with bulk water. For \( W_t \) the situation is less clear as previous data allow for two options: either the \( W_t \) exchange rate also depends on conformational equilibria, or its exchange is limited by diffusion of bulk water through the channels leading to the Mn4CaO5 cluster. Knowing which exchange mechanism applies may help identifying \( W_t \) and thus for experimentally elucidating the mechanism of water oxidation.

If conformational changes determine the exchange kinetics, then the Mn4-ligated \( W_2 \) must be \( W_t \) because these equilibria only affect the exchange of \( W_2 \) and not that of the Ca-ligated W3. The absolute rate for \( W_t \) exchange, which is orders of magnitude slower than previously reported for water ligands of Ca ions and too fast for a water ligand of a Mn(IV) ion, can in this case be explained via the equilibrium between the \( S_2^A \) and \( S_2^B \) states, because in the \( S_2^B \) state Mn4 has the oxidation state Mn(III) that allows for rapid water exchange (Mn(III) is exchange-labile; Mn(IV) is exchange inert – for discussion see ref. 9, 18 and 57). Binding to Mn would also explain the insensitivity of the \( W_t \) exchange rate to Ca/Sr substitution.

If diffusion of water through channels determines the exchange kinetics, then the Ca-ligated W3 would remain an option for \( W_t \) because this limitation would explain that \( W_t \) exchange is comparatively slow for a Ca-bound water ligand and that its exchange is unaffected by Ca/Sr substitution. In this case, it would be impossible to distinguish W2 or W3 as the fast exchanging substrate in wild-type PSII in the \( S_2 \) state on the basis of substrate water exchange rates, unless some treatment shifted the equilibrium between \( S_2^A \) and \( S_2^B \) strongly towards \( S_2^A \), as this would keep W2 bound to an exchange-inert Mn(IV) ion, leading to a very slow exchange of W2.

In the following, we will first analyze if the faster water exchange in D61A-PSII is due to a shift of conformational equilibria, or if the truncation of this amino acid from aspartate to alanine increases water accessibility to the catalytic site. Subsequently, we will elucidate the consequences of this result for (i) understanding the exchange rates in the other \( S \) states and (ii) the assignment of \( W_t \). Finally, we will discuss the remaining options for the mechanism of water oxidation.

\( W_t \) exchange in the \( S_2 \) state

In the \( S_2 \) state, \( W_t \) exchanges significantly faster than in WT-PSII in the majority of D61A-PSII centers (85%; Table 1). If a shift in conformational equilibria accounts for this observation, the Mn4-bound W2 would be the most likely assignment for \( W_t \), as outlined above. In this case, the D61A mutation would induce a change in the conformational equilibria of the Mn4CaO5 cluster towards the \( S_2^B \) state (or another \( S_2^B \) state), because this allows W2 to exchange much more readily compared to WT-PSII.46 Therefore, a faster exchange of W2 in D61A-PSII would imply that the activation barrier for reaching the \( S_2^B \) state would be lower and/or the relative stability of the \( S_2^B \) state would be increased in the mutant. However, previous experimental data show that a stabilization of the HS \( S_2^B \) state can be excluded, as only the LS \( S_2^B \) multiline signal was observed in the D61A-PSII samples and its signal intensity was comparable to that of WT-PSII (see ESE-EPR spectra in ref. 75). This is supported by theoretical calculations that find the equilibrium between the \( S_2^A \) and \( S_2^B \) state unchanged or even slightly shifted in favor of the \( S_2^A \) state.73 These calculations also indicate that in D61A-PSII one proton is lost from the W1/W2/Mn4 site of the cluster.71 Such a proton loss would slow the \( W_t \) exchange. In conclusion, the direct chemical changes that can be expected to occur would either leave the water exchange the same or likely even slow the exchange of W2, the opposite to what is observed experimentally for \( W_t \) exchange. This analysis shows that a shift
of the conformational equilibrium between \(S_2^A\) and \(S_2^B\) cannot explain the present data.

On this basis, we conclude that the exchange of \(W_f\) by isotopically labelled bulk water must be slowed by a steric constraint in all the channels that supply substrate to the \(\text{Mn}_4\text{CaO}_5\) cluster in WT-PSII.\(^{37}\) The D61A mutation then appears to remove one of these diffusion barriers so that \(W_f\) exchange can occur at the experimentally observed faster rate. Indeed, barriers for water transport were described previously for all channels, and D1-D61 was identified as forming a barrier for water access together with D2-K317 and Cl1.\(^{41}\) We propose that shortening D1-D61 via the D61A mutation creates a void that is filled by one or two water molecules, which promotes faster water diffusion to the \(\text{Mn}_4\text{CaO}_5\) cluster. This idea is in line with a recent theoretical study that shows water redistributions and faster movements of water molecules in the D61A mutant.\(^{76}\)

**Model for \(W_f\) exchange via the Cl1 channel in the \(S_2\) state**

Our data strongly indicate that D61 forms a steric barrier for water access to the catalytic site that contributes to limiting the rate of \(W_f\) exchange in the \(S_2\) state. However, comparison of the measured water exchange rates to water transport rates estimated from MD simulations appears to contradict this conclusion: in WT-PSII, the rate for \(W_f\) exchange is about 80 s\(^{-1}\) (at 283 K), while barriers of 10–14 kcal mol\(^{-1}\) calculated for all channels for moving a water molecule from the bulk to the \(\text{Mn}_4\text{CaO}_5\) cluster would predict exchange rates up to a 1000-fold faster than our observation (see ESI TEXT 3\(^{37}\)).\(^{61}\) However, the two processes are not directly comparable. MD simulations of water movements always employ a force to achieve concerted or directed water movement along a certain trajectory. This force can be provided for example by inserting extra water molecules near the \(\text{Mn}_4\text{CaO}_5\) cluster, or by pulling water molecules through the channels at a constant velocity.\(^{41}\) By contrast, isotopic equilibration involves random swapping of neighboring water molecules driven by thermal energy. It thus requires many swapping events to reach isotopic equilibrium between an inner water pool and bulk water.

As D61 is located at a branching point of the O4 channel and the Cl1 channel, the faster water access may occur through either or both of these channels. The O4 pathway (channel 2 in ref. 61) has been proposed to facilitate substrate water entry\(^{36,77-80}\) because binding sites for the substrate analogues ammonia\(^{75,81-83}\) and methanol\(^{78,84-86}\) are located in the vicinity of \(\text{Mn}_4\), O4, and D1-D61. Also, the D1 residue at position 87, which is near the origin of the O4 pathway, is Ala in spinach and Asn in cyanobacteria, a fact that appears to correlate with the finding that methanol has a much larger effect on EPR signals of the \(\text{Mn}_4\text{CaO}_5\) cluster of plants than cyanobacteria.\(^{77,78}\) However,
other reports find that the O4 channel is rather narrow and possibly unsuitable for water transport and instead favor the Cl1 channel (or O1 channel) as main water access pathway.60,61,64,67

To test the validity of our conclusion we examined the expected substrate water exchange rates through the shorter (25 Å) arm of the Cl1 channel (‘channel 1’ in ref. 61). This channel is reported to have two barriers: the first is formed by the D1-E65/D1-R334/D2-E312 triad and has a barrier of 11.5 kcal mol\(^{-1}\), while the second is formed by D1-D61, D2-K317 and Cl1 and has a barrier of 7 kcal mol\(^{-1}\) in the inward direction, and about 11 kcal mol\(^{-1}\) in the outward direction (Scheme 3). Using these parameters, we constructed a model that included two significant barriers, while other waters can exchange essentially freely. Eight water molecules, including W3 (but not W1, W2 and W4), formed the inner pool. To further simulate the water channel characteristics observed in crystal structures,6,15,17,61 four water molecules were placed between the two barriers, and five crystal waters are in rapid exchange with bulk water (Scheme 3; ESI Text 3†). We achieved excellent agreement with our experimental data by assuming that the inner barrier, formed by D1-D61, D2-E317 and Cl1, has an energy of 12.8 kcal mol\(^{-1}\), and the barrier closer to the bulk formed by D1-E65, D1-P66, D1-V67 and D2-E312 has a height of 11.5 kcal mol\(^{-1}\) (Fig. S5 and Table S3†). The inner barrier is slightly higher than determined for the outward direction by MD simulations, but this value is presumably within the accuracy of the MD method. It is also possible that the barrier for swapping two water molecules is actually higher (or the frequency factor lower; see SI Text S3) than for pulling water molecules through a channel,64 as this process requires two water molecules to pass each other in a bottleneck. This simulation thus shows that our proposal of an access limitation of the fast water exchange in the S2 state is realistic.

W\(_{f}\) exchange in the S\(_{0}\), S\(_{1}\) and S\(_{2}\) states of the majority of D61A-PSII centers

In the S\(_{3}\) state, W\(_{f}\) exchange is slower than in the S\(_{2}\) state and thus no longer controlled by water access. This implies that W\(_{f}\) is now more tightly bound, in line with the suggested movement of W\(_{f}\) into the Ox or O5 positions (Scheme 1). Because in the S\(_{3}\) state all Mn ions are in oxidation state Mn(IV), the rate of the fast water exchange is limited instead by the redox equilibrium between the S\(_{3}\) \(A}\wedge W\(_{2}\) and S\(_{2}\) \(A}\wedge W\(_{2}\) states.6,46 The exchange of W\(_{f}\) in the S\(_{3}\) state most likely occurs by a reversal of the insertion pathway (Scheme 1).

The exchange of W\(_{f}\) becomes observable for the first time in the S\(_{2}\) state, which might be taken as indication of a faster exchange of W\(_{f}\) in the S\(_{0}\) and S\(_{1}\) states. This would be inconsistent with an S state independent water access barrier; that is, with a diffusion limited exchange in the S\(_{0}\), S\(_{1}\) and S\(_{2}\) states, and thus with W3 or W2 as W\(_{f}\) in these states.

W\(_{f}\) exchange in the S\(_{2}\) state of the majority of D61A-PSII centers

While improved substrate access provides a satisfying rationale for the unresolved and therefore more than 3-fold faster W\(_{f}\) exchange in D61A-PSII, it does not explain the 15-fold faster exchange of W\(_{f}\) in the dominant fraction of D61A-PSII centers. We recently observed a similar acceleration in WT-PSII at pH 8.6 and in Sr-PSII core complexes at pH 8.3.66 In this earlier study, the accelerated exchange correlated well with a stabilization of the S\(_{2}\) \(H^+\) state, indicating that at normal pH the conversion from the S\(_{2}\) \(H^+\) configuration into the S\(_{3}\) \(H^+\) configuration is limiting the rate of W\(_{f}\) exchange. We assigned the alkaline-induced S\(_{2}\) \(H^+\) state to the S\(_{2}\) \(A\wedge\) state, as this state allows an easy transition into the S\(_{2}\) \(B\wedge\) state (Scheme 2) in which O5 exchange can occur rapidly.64,66,67 As discussed above, the situation is different in the D61A mutant because the available data clearly exclude the stabilization of a S\(_{2}\) \(H^+\) form.67 However, since water exchange in the S\(_{2}\) \(B\wedge\) state is presumably very fast, and the S\(_{2}\) \(A\wedge\) to S\(_{2}\) \(B\wedge\) transition also has a comparatively low barrier,42,55 a similar acceleration of W\(_{f}\) exchange can be achieved by lowering the barrier for the rate limiting transformation of S\(_{2}\) \(A\wedge\) into the S\(_{2}\) \(B\wedge\) state.

As shown in Scheme 1, water insertion during the S\(_{2}\) \(\rightarrow\) S\(_{3}\) transition requires the deprotonation of W3. The same is true for the formation of S\(_{2}\) \(A\wedge\) from S\(_{2}\) \(A\), which likely occurs in a similar fashion to mechanism A in Scheme 1. In the S\(_{2}\) state of WT-PSII, this proton needs to be transported away from the positively charged catalytic site into the bulk phase. In D61A-PSII, W1/W2 have collectively lost one proton,23 and should thus be able to transiently act as a nearby base that accepts the W3 proton during the S\(_{2}\) \(A\wedge\) to S\(_{2}\) \(B\wedge\) formation. We propose that this lowers the energy barrier for S\(_{2}\) \(A\wedge\) formation enough to allow the observed 15-fold increase in W\(_{f}\) exchange rate. That the breakage of an OH bond is rate determining for O5 exchange in the S\(_{3}\) state is supported by the H/D isotope effect of 1.9 ± 0.2 determined for W\(_{f}\) exchange in the WT mutant (Fig. S3; Table S2†).

S\(_{2}\) state water exchange in the minority of D61A-PSII centers

We found that in about 15% of the D61A centers the exchange rates for W\(_{f}\) and W\(_{e}\) were similar to each other and to W\(_{e}\) exchange in WT-PSII (Table 1). This means that W\(_{f}\) exchange in this minority fraction was 10-fold slower than W\(_{e}\) exchange in the majority fraction, 60-fold slower than W\(_{f}\) exchange in WT-PSII, and more than 200-fold slower than W\(_{f}\) exchange in the majority fraction. By contrast, W\(_{e}\) exchange was slowed only 2–3 fold compared to WT-PSII, but nearly 40-fold relative to the majority fraction.

We see two options to explain the slow and comparatively similar rates of exchange of W\(_{f}\) and W\(_{e}\) in this fraction of the D61A-PSII. Firstly (Option 1), in these centers the D61A mutation induces a secondary structural change that restricts the water access at a different point of the channel even more than in WT-PSII. For example, if the Cl1 channel would be the
dominant substrate entry pathway, such a secondary structural change might occur at the D1-E65/D1-R334/D2-E312 triad, which was suggested previously to be another bottleneck for water transport through the C11 channel.64 As this triad provides a rather narrow path for water, a small change in protein conformation or dynamics may be enough to further restrict water passage. As the D1-D61A mutation is only 4 amino acids away from D1-E65, such an allosteric effect cannot be excluded. Secondly (Option 2), both W3 and W2 serve as Wf, but in different populations of D61A PSII centers, with one serving as Wf in the majority fraction and the other serving as Wf in the minority fraction. This idea is motivated by the similar rates of Wf in the majority fraction and the other serving as Wf in the di-be replaced state where O5 is bound in a terminal position at Mn4 and can in the S3 state would be its exchange via the S2© 14,15,17,21 The strong S2© 14,15,17,21 The strong

\[ \text{S}_2\text{AW} \rightarrow \text{S}_2\text{BYZ} \]

Thus, if it is correct that Wf is away from D1-E65, such an allosteric effect cannot be excluded.

**Absence of effects of E189Q mutation**

The analyses of recent XFEL studies favor water delivery via the O1 channel, and some of the authors suggest a gating of water access by the observed movement of E189 during the S2© 14,15,17,21

The following text describes the exchange of water in the S3 state and discusses the implications for the mechanism of water oxidation.

**Relation of water exchange and water binding**

While our data show that in D61A-PSII water exchange occurs through the O4 and/or one or both branches of the C11 channel, they do not reveal which of the channels, including the O1 channel, has the lowest barrier in WT-PSII. Additionally, it is important to note that water binding during the S2© 14,15,17,21 transition is a fundamentally different and much faster (100–400 µs) process than water exchange in the S2© 14,15,17,21 states (10–500 ms). During water binding, a nearby water attaches to an open binding site of the cluster and thereby initiates a bucket brigade of refilling vacant sites, while reaching the isotopic equilibrium with bulk water requires full equilibration of all exchangeable water molecules in the channels and around the catalytic site. Thus, our present data do not identify through which of the three water channels the substrate water is delivered in WT-PSII.

**Is the control of water access functionally important?**

It has previously been hypothesized that regulation of substrate accessibility is crucial to minimize side reactions that would lead to the production of reactive oxygen species at the Mn4CaO5 cluster.88,89 This hypothesis assumed that in intact PSII complexes only substrate water can interact in a specific way with the Mn4CaO5 cluster. Recent crystal structures have shown that the Mn4CaO5 cluster is surrounded by several additional water molecules. Nevertheless, the present data and the previous calculations by Vassiliev84 show that water access is not completely free. This somewhat regulated access likely evolved to stabilize the Mn4CaO5 cluster, and to allow for the formation of a highly specific hydrogen bonding network, which is crucial for removing protons from substrate water during the water oxidation reactions. By contrast, the access of water is fast when compared to the maximal turnover frequency of PSII, which is limited by the acceptor side reactions of PSII to about 50 O2 s−1 (20 ms)−1 while water is delivered through the channels with a time constant in the order of 100 µs.44 Interestingly, the time constant for water delivery is in the same order as that for water binding during the S2© 14,15,17,21 transition. It might thus be speculated that the restriction of water access is a compromise between excluding other redox active molecules and ions from the Mn4CaO5 cluster, while allowing fast enough water access to promote efficient S state turnover. This idea is supported by the finding that partial dehydration of PSII increases the misses specifically of the S state transitions that involve binding of water molecules.84 Similarly, addition of the water analog methanol increases the miss parameter and allows the observation of a water deprived S3 state.82,93

**Implications for the mechanism of water oxidation**

The significance of the present results is that they remove the strongest arguments against the assignment of Wf to W3 in the S2© 14,15,17,21 state, namely (i) the independence of the Wf exchange rate to
Ca/Sr substitution and (ii) the significant mismatch with reported exchange rates for water ligated to Ca.\textsuperscript{46,94}

The present data are fully consistent with O5 as slowly exchanging substrate water Ws, and W2 or W3 as fast exchanging substrate water Wf. A further distinction between W2 and W3 as Wf is not possible on the basis of substrate water exchange data alone because the rate limitation provided by the barriers in the channels obscures small perturbations such as Ca/Sr substitution that could otherwise be used to distinguish the binding sites. However, other recent experimental data favor W3 over W2 as substrate water. FTIR experiments by the groups of Noguchi and Debus have provided evidence for the involvement of W3 in water binding during the S1 → S2 transition.\textsuperscript{11,16,95} Similarly, femtosecond X-ray crystallography measurements have revealed that the largest changes in water positions during this transition occur in the O1 channel that leads to the Ca site and found no evidence for the predicted closed cube S3B-like intermediate that would be required if W2 were the fast substrate (Scheme 1).\textsuperscript{15,17} By contrast, the support for W2 is mostly based on substrate analogs like methanol or ammonia,\textsuperscript{75,77–83} which we regard as more indirect. On this basis, we propose that O5 and W3 are the two substrate water molecules under normal circumstances, but that W2 may serve as the fast exchanging substrate under some circumstances, such as in a minority of D61A PSII centers. The resulting experimentally supported ‘molecular S state cycle’ is summarized in Scheme 4.

Scheme 4  Proposed molecular Kok cycle illustrating the binding of the two substrate waters Wf and Ws in the various S states. The center shows the traditional S state scheme indicating water binding as well as proton and dioxygen release, while the outer circle depicts schematically the corresponding dominant structures of the Mn4CaO6/6 complex based on X-ray crystallography\textsuperscript{6,15,17,21} as well as calculated structural models of key intermediates during O–O bond formation.\textsuperscript{12} In dark-adapted PSII, the reaction cycle starts with the S1 state that has two Mn\textsuperscript{III} and two Mn\textsuperscript{IV} ions and in which all bridges are deprotonated.\textsuperscript{10} During the S1 → S2 transition, Mn4 is oxidized. While the S2\textsuperscript{A} state is in equilibrium with other conformations (see Scheme 2), it is proposed that W3 is inserted directly into the Ox binding site between Ca and Mn1, concomitant with Mn1 oxidation and the binding of a new water, W3, to the W3 site (dashed grey arrows; for details, see Scheme 1A).\textsuperscript{17} In S2, the dominant state is S2\textsuperscript{AW}. Upon further oxidation, the S2\textsuperscript{AW} state is formed, which is considered to be the key intermediate in the O2 release process (lag phase; not shown).\textsuperscript{102} This may be coupled to unknown rearrangements within the H-bonding network of the OEC. Only thereafter, the Mn4CaO6 cluster can be oxidized to S4. Instead of Mn oxidation, S4 state formation involves the oxidation of the fast substrate water, indicated by a black dot on W3 (in the Ox position).\textsuperscript{12} By rearranging the electrons of the chemical bonds (black half-arrows), the S4 state rapidly converts into the S4\textsuperscript{A} state, which contains a complexed peroxide. The further conversion of S4 into S4 + O2 requires the binding of one water and the release of a proton. We suggest that a pre-bound water ligand (W2 or W3) fills the empty O5 binding site,\textsuperscript{6} and that this ligand is concomitantly replaced by a new water (WN2; dashed grey arrows). In the S4 state, the O5 bridge is protonated, in line with the faster exchange of Ws and spectroscopic data.\textsuperscript{6,104,105} Oxygen atoms are labeled red, and the two substrate ‘waters’ are shown in blue. Hydrogen atoms are shown as small white spheres (protonation states based on S2 state assignment in ref. 106).
Presently no experimental data are available that allow to determine the actual O-O bond formation mechanism during the $S_3 \rightarrow S_4 \rightarrow S_0$ transition, but the present data are fully consistent with the best worked out theoretical mechanism for O-O bond formation, which involves o xo-oxyl radical coupling between oxygens in the O5 and Ox binding sites via a low-energy path paved by favorable spin pairing.\textsuperscript{12,51}

However, the recently revived idea that the formation of a peroxidic intermediate ($\lesssim$5–10\%) in the $S_4$ state is required for further oxidation to the $S_4$ state cannot be excluded on the basis of our present data (Fig. S1E and F)\textsuperscript{1,21,27,28} because the same substrates and main state conformations are involved, and such a small equilibrium population of a peroxidic intermediate would easily escape detection by, for example, femtosecond X-ray crystallography. Nevertheless, a very recent theoretical study considers a peroxidic intermediate in the S3 state as unlikely.\textsuperscript{26} By contrast, our substrate water exchange data are inconsistent with nucleophilic attack mechanisms between W3 and W2,\textsuperscript{51,97–99} and geminal coupling between W2 and O5 at Mn4 (ref. 30, 100) (for details see ESI Text 4 and Fig. S1).

Conclusions
In this study, we demonstrate that the fast water exchange in the $S_0$, $S_1$ and $S_2$ states is rate limited by specific diffusion barriers in all the channels connecting bulk water with the Mn$_4$CaO$_5$ cluster in PSII, and that the D61A mutation reduces one of these barriers so that $W_i$ exchange is accelerated. This finding removes previous arguments that appeared to exclude $W_3$ as the fast exchanging substrate water. Combining our present results with recent FTIR and XFEL data supporting the insertion of W3 into the Ox position during the $S_3 \rightarrow S_1$ transition,\textsuperscript{11,15–17,93,101} now make $W_3$ the prime candidate for $W_i$. As our previous experiments identified $O_5$ as the slow substrate water,\textsuperscript{9,10,44,46,54} this study clarifies the fate of the substrate waters during the S state cycle, and thereby limits the possible mechanisms for O-O bond formation to a few that all involve coupling between O5 and W3, while they are bound in the O5 and Ox positions of the $S_3^{aw}$ or $S_4^{aw}$ states (Scheme 4).

Data availability
All relevant data is presented in the paper and ESI.\textsuperscript{†} Raw data is available upon request by email to JM.

Author contributions
CDL, RJD and JM conceived and designed the research; CDL, CJK and PC performed the research; CDL and JM analyzed the data; CDL, RJD and JM wrote the paper with input from all authors.

Conflicts of interest
There are no conflicts to declare.

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