Substrate–water exchange in photosystem II is arrested before dioxygen formation

Håkan Nilsson¹, Fabrice Rappaport², Alain Boussac³ & Johannes Messinger¹

Light-driven oxidation of water into dioxygen, catalysed by the oxygen-evolving complex (OEC) in photosystem II, is essential for life on Earth and provides the blueprint for devices for producing fuel from sunlight. Although the structure of the OEC is known at atomic level for its dark-stable state, the mechanism by which water is oxidized remains unsettled. Important mechanistic information was gained in the past two decades by mass spectrometric studies of the H₂¹⁸O/H₂¹⁶O substrate–water exchange in the four (semi) stable redox states of the OEC. However, until now such data were not attainable in the transient states formed immediately before the O–O bond formation. Using modified photosystem II complexes displaying up to 40-fold slower O₂ production rates, we show here that in the transient S₃Y₂ state the substrate–water exchange is dramatically slowed as compared with the earlier S states. This further constrains the possible sites for substrate–water binding in photosystem II.

¹Department of Chemistry, Kemiskt Biologiskt Centrum (KBC), Umeå University, Linnaeus väg 6, 901 87 Umeå, Sweden. ²Institut de Biologie Physico-Chimique, UMR 7141 CNRS and Université Pierre et Marie Curie, 13 rue Pierre et Marie Curie, 75005 Paris, France. ³iBiTec-S, CNRS UMR 8221, CEA Saclay, 91191 Gif-sur-Yvette, France. Correspondence and requests for materials should be addressed to A.B. (email: alain.boussac@cea.fr) or to J.M. (email: johannes.messinger@chem.umu.se).
Photosynthesis provides the driving force for most life on Earth by converting sunlight into chemical energy. Cyanobacteria, algae, and higher plants couple two photosystems in series to exploit water as the electron and proton source for the synthesis of carbohydrates from CO₂. In the process they replenish the atmosphere with the dioxygen we live on. The complex four-electron four-proton chemistry of water oxidation is catalysed in photosystem II (PSII) by an inorganic cluster containing the earth-abundant metals Mn and Ca, which are bridged by five oxygen. The structure of this Mn₄CaO₅ cluster, which together with its ligands forms the oxygen-evolving complex (OEC), is now known at the atomic scale in its dark-stable state. Density functional theory-based refinements have provided OEC structures (Fig. 1a) that can rationalize the vast majority of the available spectroscopic data.

Water oxidation to dioxygen is energetically driven by light-induced charge separations within the reaction centre of PSII. These occur via the chlorophyll-containing photo-oxidant P₆₈₀/P₆₈₀⁺ and the primary electron acceptor pheophytin. The OEC and P₆₈₀ are connected via the redox-active tyrosine residue 161 of the D1 protein. In this way the OEC steps in response to short light flashes almost in synchrony through the four (semi)-stable oxidation states S₀Y₉, S₁Y₉, S₂Y₉ and S₃Y₉ (Fig. 1b), where the subscript signifies the number of stored oxidizing equivalents, and the plus sign indicates an extra charge caused by the lack of proton release during the transition from the dark-stable S₀Y₉ state to the S₁Y₉ state. These (semi)-stable intermediates of water oxidation can be trapped with high yield and are thereby readily accessible for biophysical investigation. Although it is largely agreed that the S₃ state transitions between S₀Y₉ and S₃Y₉ involve Mn-centred oxidation of the Mn₄CaO₅ cluster., some experimental results suggest ligand (oxo-bridge) participation and/or a structural change during the S₂⁺Y₉ transition. The dark-stable S₁Y₉ state is generally considered to have the formal oxidation states Mn₄(III,III,IV,IV) (high oxidation state scenario), but also the lower valent Mn₄(II,III,III,IV)/Mn₄(III,III,III,III) options are bridged by five oxygen.

Water oxidation starts only after the fourth oxidizing equivalent has been accumulated in the OEC, that is, once the transient S₃⁺Y₉ state is reached (Fig. 1c). It is widely agreed that this dioxygen-forming reaction sequence starts with a proton release during the S₃⁺Y₉ → S₄⁺Y₉ transition, followed by the formation of a Ca- or Mn-bound oxyl radical or of a Mn⁻⁻oxyl group due to oxidation of the cluster by Y₉⁺. Although the following reaction steps towards O₂ must include the formation of a bound peroxodic intermediate, dioxygen formation and release, and the rebinding of substrate water (Fig. 1c), the exact nature of the chemistry involved remains unsettled, as these transient states have so far largely eluded biophysical investigations.

Figure 1 | The water-oxidizing complex in PSII and its reaction sequence. (a) Density functional theory-based model of the Mn₄CaO₅ cluster in the ‘open cube’ (S₂ EPR multiline) configuration together with its water derived ligands W₁–W₄. The model is inserted into the 1.9Å crystal structure of photosystem II. The majority of the available spectroscopic data and the protonation state of the substrate oxygen) and a Mn⁻⁻oxy radical. Alternatively, the O–O bond may be formed via radical coupling between either a Ca-oxyl radical and an Mn-bound oxyl radical (Fig. 2c) or between an Mn-oxyl radical and an Mn-oxo (bridge) (Fig. 2d).

Substrate–water exchange experiments, which monitor the rate of incorporation of isotopically labelled bulk water into the substrate sites of the OEC, have allowed determining the relative binding characteristics of the two substrate–water molecules in all (meta)-stable SᵢY₉ states under various conditions, and have
thereby provided unique insight into the mechanism of photosynthetic water oxidation\textsuperscript{24–31,37–40}. In these experiments, PSII samples are preset to the desired $S_{3YZ}$ state by light flashes, and are then rapidly mixed with $H_2^{18}O$. After desired incubation times they are further advanced by flashes to produce dioxygen. The isotopic composition of the product $O_2$ ($^{16,16}O_2$, $^{16,18}O_2$, $^{18,18}O_2$) is monitored by membrane-inlet mass spectrometry\textsuperscript{51}, and the substrate–water exchange rates are calculated from the $H_2^{18}O$ incubation time dependence of the $m/z$ 34 and $m/z$ 36 signals\textsuperscript{26,42}. Assessing whether the two substrate waters can still exchange with the bulk water just before O–O bond formation, that is, in the transient $S_1Y_Z$ and $S_2Y_Z$ states, is expected to provide additional information on the nature and the binding sites of the two substrate waters, and therefore on the chemistry of water oxidation.

Thus far, only very few time-resolved studies were able to probe these two transient states\textsuperscript{22,24,25,43,44} and no information about substrate–water binding in the $S_2Y_Z$ and $S_3Y_Z$ states is available as yet. This is mainly because the half-times of these states in native PSII samples are too short ($\leq 1–2$ ms) with respect to the mixing dead time in the water-exchange experiments ($t_{1/2} = 3$ ms; see Supplementary Fig. 1). Recent studies set conditions though whereby the lifetimes of these transient states can be extended while preserving the overall function of the enzyme\textsuperscript{45,46}. It was found that Thermosynechococcus elongatus cells growing on Sr\textsuperscript{2+} containing media devoid of Ca\textsuperscript{2+} incorporate Sr\textsuperscript{2+} in place of Ca\textsuperscript{2+} into the OEC and that Cl$^-$ can be exchanged biochemically against Br$^-$ or I$^-$ (Fig. 1a); importantly, these substitutions have only minor structural effects\textsuperscript{47–50}, but extend the half-lifetime of the $S_3YZ$ state to 7 ms (Sr/Br-PSII) or even 45 ms (Sr/I-PSII)\textsuperscript{45,46}. These samples thereby provide the opportunity to probe the rates of substrate–water exchange in this last transient before O$_2$ formation (Fig. 1c).

Here we show that the exchange of both substrate waters is strongly retarded in the transient $S_3Y_Z$ state as compared with the semi-stable $S_1Y_Z$ state. Four possible mechanisms for this simultaneous retardation of the exchange of both substrate waters induced by $Y_Z$ oxidation and the subsequent deprotonation of the catalytic site are presented and evaluated. On the basis of this evaluation and present literature data we conclude that W2 is most likely to be the fast exchanging substrate water ($W_f$), while O5 (or W3) can be assigned to be the slowly exchanging substrate water ($W_s$).

**Results**

**Substrate–water exchange in the $S_1Y_Z$ state.** Substrate–water exchange measurements were performed for the $S_1Y_Z$ state to determine the influence of co-factor substitution on the substrate–water binding affinity in PSII core samples of $T$. elongatus. For this, the dark-adapted PSII samples were excited with two saturating flashes to advance the PSII complexes from the dark-stable $S_1Y_Z$ state into the semi-stable $S_1Y_Z$ state. $H_2^{18}O$ was then injected at defined times before inducing dioxygen formation by giving one additional flash to the enzyme\textsuperscript{26,28,37}.

Figures 3a,b compare the substrate–water exchange kinetics in the $S_1Y_Z$ state of native Ca/Cl samples of $T$. elongatus with samples in which these cofactors were replaced by either Sr/Br or Sr/I. The native Ca/Cl-PSII shows the typical biphasic kinetics for the rise of the mixed labelled $^{16,18}O_2$ (Fig. 3a), and the corresponding monophasic rise for the doubly labelled $^{18,18}O_2$ species (Fig. 3b). As described previously, the fast $^{16,18}O_2$ rise reflects the exchange of the fast exchanging substrate water ($W_f$), while the subsequent slow increase of the $^{16,18}O_2$ signal and the

\begin{figure}[h]
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\caption{**Figure 2 | Conceivable O–O bond formation mechanisms in the ‘S4’ state of PSII.** (a) Nucleophilic attack by a bulk water onto a Mn\textsuperscript{IV} = O or Mn\textsuperscript{IV}OXY radical\textsuperscript{29}, (b) nucleophilic attack by a Ca bound water onto a Mn\textsuperscript{IV} = O or Mn\textsuperscript{IV}OXY radical\textsuperscript{26,32–35}, (c) coupling of a Ca-hydroxyl radical with a Mn-bound radical substrate\textsuperscript{14}, (d) direct coupling between a terminal Mn-oxyl radical with an oxo bridge between Ca and Mn\textsuperscript{6,28,30,31,36}.}
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\begin{figure}[h]
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\caption{**Figure 3 | Substrate–water exchange in $T$. elongatus PSII core particles containing different ionic cofactors.** (a,b) The substrate water exchange in the $S_1Y_Z$ state at $m/z$ 34 ($^{16,18}O_2$: a) and $m/z$ 36 ($^{18,18}O_2$: b), while c displays the exchange in the $S_3Y_Z$ state at $m/z$ = 34. Symbols mark the data points (green triangles, Ca/Cl-PSII; black squares, Sr/Br-PSII; blue circles, Sr/I-PSII), while full lines are fits representing the fast and slow substrate–water exchange (a,b; for rate constants see Table 1) or simulations of the expected experimental outcome assuming the exchange rates are identical in the $S_1Y_Z$ and $S_3Y_Z$ states (c). The blue dashed and dotted lines in c represent simulations where either the slow (dashed) or fast (dotted) rate of exchange was set to be 1,000 times slower than that measured in the $S_1Y_Z$ state (Table 1). All data points (n = 1) are normalized to values reached after complete isotopic equilibration. Each time course was measured once, but consists of many separately measured data points that were in part obtained on different days.}
\end{figure}
respectively, if the exchange rates were the same as in the S3 state. The exchange rate needs to be additionally slowed by a factor of 10 for Wf (if Wf is slowed by a factor of 1,000) and of 10,000 for Ws (if Ws is slowed by a factor of 1,000), to make them consistent with the data.

This specific effect on the slow substrate–water exchange is useful, as it allows the detection of the exchange of both substrate waters in the S3YZ state (see below). Finally, the substitution of Br− for I− has only a modest effect on the water exchange in the S3YZ state as evidenced by the similar exchange characteristics obtained with the Sr/Br- and Sr/I-PSII samples (Fig. 3a,b).

**Substrate–water exchange in the S3YZ state.** On the basis of the exchange rates determined for the semi-stable S2YZ state, substrate water-exchange experiments in the transient S3YZ state (Fig. 3c) were performed for all three sample types (Ca/Cl, Sr/Br, Sr/I). In these experiments, the dark-adapted PSI samples were advanced from the S1YZ state by three saturating flashes into the transient S2YZ state to initiate the O2-forming reaction sequence (Fig. 1c). After this third flash, H218O was injected at various delay times, and the 18O incorporation into the product O2 was monitored. The symbols in Fig. 3c show that no incorporation of the 18O-label occurred for any of the three sample types, not even in the relatively long-lived S3YZ state as evidenced by the similar exchange characteristics obtained with the Sr/Br- and Sr/I-PSII samples (Fig. 3a,b).

The extent to which the substrate-water exchange is slowed in the S3YZ state versus S3 state is illustrated by the computed transitions (Fig. 2c)28,32–35 or coupling of a Ca-bound oxyl radical with a high valent Mn-oxo group (Fig. 2b)36–38 or coupling of a Ca-bound oxy radical with a high valent Mn-oxo group (Fig. 2c)14 may take place, if conditions are present in PSI that slow down the exchange of the Ca-bound substrate water seven to eight orders of magnitude over the exchange rates reported for water ligated to Ca2+ in aqueous solutions37,39,40,58. Alternatively, both substrate waters may be Mn-ligated and form the O−O bond via radical coupling (Fig. 2d)6,30,31,36.

The lack of 16,18O2 and 18,18O2 production (Fig. 3c) demonstrates that the exchange of both substrate waters is significantly slower than the decay of the S3YZ state into S2YZ + O2, even when the O2 production is severely slowed by cofactor exchange. The fact that the exchange of both substrate waters is slowed down so significantly despite a constant redox state of the Mn4CaO5 cluster is important, and we discuss below the four possible mechanisms for ‘arresting’ both substrate waters under these conditions.

As a consequence, the exchange rate of Wf in the S3YZ state is <0.83 s−1, and is thus at least tenfold slower than the exchange rate of Ws in the S2YZ state of the Sr/I samples, and at least as slow as the exchange of Ws in the Ca/Cl-PSII. It is emphasized that neither the overall oxygen production yield nor the period four oscillations were markedly affected by the cofactor substitutions45,46 (see also Supplementary Fig. 3), showing that this observation is made with enzyme that despite the slowed final O2-producing transition functions normally.

### Discussion

In catalysis, either in biology or in chemistry, isotope labelling studies are instrumental for elucidating reaction mechanisms52. In such experiments the substrate is labelled with a (stable) isotope and the propagation of this label into intermediates and/or the product(s) is followed. When studying the mechanism of water oxidation, H218O is typically added to a reactive species that has been pre-formed in unlabelled water. As an example, this method provided the demonstration that in a synthetic Mn-oxo complex, H218O is produced by nucleophilic attack of hydroxide on MnV18O (ref. 53). The characteristic signature for this mechanism is the evolution of 16,18O2 at a ratio equal to the 18O-enrichment of bulk water. In contrast, we did not observe here any incorporation of 18O into the dioxygen product above the natural abundance level, when injecting H218O into PSI suspensions poised in the S3YZ state, even when the lifetime of the S3YZ state was significantly lengthened by the exchange of Ca2+ by Sr2+ and Cl− by I− (Fig. 3c). It is noted that the S3YZ state of PSI is comparable to MnV18O in the above model complex in the sense that the O−O bond is formed without the acquisition of any additional oxidizing equivalents. In absence of an unprecedented diffusion barrier54,55, which would need to arise during the S3YZ → S2YZ → S3YZ + H+ transitions, the observed lack of 16,18O2 formation directly excludes that in PSI the O−O bond is formed in the same way as in the MnV18O model system56. In other terms, the nucleophilic attack of free water onto an electrophilic oxygen species (Fig. 2a) does not occur in PSI. This conclusion is consistent with previous data that showed that both substrate waters are bound to the OEC already in the S2 and S3 states56,57. However, nucleophilic attack of a Ca-bound water/hydroxo onto a MnV18O/MnV18O → O/MnV18O → O group (Fig. 2b)36,37,39,40,58 or coupling of a Ca-bound oxyl radical with a high valent Mn-oxo group (Fig. 2c)14 may take place, if conditions are present in PSI that slow down the exchange of the Ca-bound substrate water seven to eight orders of magnitude over the exchange rates reported for water ligated to Ca2+ in aqueous solutions37,39,40,58. Alternatively, both substrate waters may be Mn-ligated and form the O−O bond via radical coupling (Fig. 2d)6,30,31,36.

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The simplest possibility (mechanism 1) for slowing down significantly the exchange of both substrate waters would be the existence of the O−O bond (peroxide) already in the S3YZ state39,60. At this point, the 18O-label from the injected water could not be incorporated into the product and thus no 16,18O2 would be observed. Although internally consistent, this hypothesis is at odds with a recent time resolved X-ray spectroscopy experiment, which excluded Mn reduction.

| Table 1 | Rate constants of proton and electron transfer and water exchange during O2 formation in PSI. |
|-------------------|------------------|------------------|------------------|
|                     | Ca/Cl-PSII       | Sr/Br-PSII       | Sr/I-PSII        |
| S2YZ → S3YZ         | S2YZ → S0YZ      | S2YZ → S3YZ      | S2YZ → S3YZ      |
| k1, s−1             | 6030 ± 60        | 3010 ± 30        | > 700            |
| k2, s−1             | 630 ± 6          | 96 ± 9           | 15 ± 3           |
| Water exchange, S3YZ |                   |                  |                  |
| k1, s−1             | 40 ± 4           | 29 ± 3           | 25 ± 3           |
| k2, s−1             | 0.69 ± 0.06      | 6.6 ± 0.5        | 6.3 ± 0.3        |

The rate constants of proton and electron transfer and water exchange during O2 formation in PSI are shown in Table 1. The rates of both substrate waters are significantly retarded during the S3YZ state. The exchange rates were determined for the semi-stable S2YZ state, substrate water-exchange experiments in the transient S3YZ state (Fig. 3c) were performed for all three sample types (Ca/Cl, Sr/Br, Sr/I). In these experiments, the dark-adapted PSI samples were advanced from the S1YZ state by three saturating flashes into the transient S2YZ state to initiate the O2-forming reaction sequence (Fig. 1c). After this third flash, H218O was injected at various delay times, and the 18O incorporation into the product O2 was monitored. The symbols in Fig. 3c show that no incorporation of the 18O-label occurred for any of the three sample types, not even in the relatively long-lived S3YZ state as evidenced by the similar exchange characteristics obtained with the Sr/Br- and Sr/I-PSII samples (Fig. 3a,b).

The extent to which the substrate-water exchange is slowed in the S3YZ state versus S3 state is illustrated by the computed exchange curves in Fig. 3c. The longer lifetimes of both the S1YZ and S2YZ states should have resulted in relative 16,18O2 signals of 51(±6)% and 18(±2)% in the Sr/I-PSII and Sr/Br-PSII samples, respectively, if the exchange rates were the same as in the S3YZ state. Such yields are well above the detection limit, which allows the detection of 16,18O2 formation even at natural abundance (Fig. 3c and Supplementary Fig. 2). Interestingly, the dashed and dotted blue curves show that a 1,000-fold slowing of only one of the exchange rates, while keeping the other one unchanged, cannot explain the data. Thus, our results imply that the exchange rates of both substrate waters are significantly retarded during the S3YZ → S2YZ → S0YZ + H+ transitions. These simulations demonstrate that the corresponding other substrate water exchange rate needs to be additionally slowed by a factor >30 for Wf (if Wf is slowed by a factor of 1,000) and >10 for Ws (if Ws is slowed by a factor of 1,000), to make them consistent with the data.
(formation of the formal $S_1^2(O_2)^{-2} - Y_2^5$ intermediate) during the transient phase (Fig. 1c). This implies that the O–O bond is formed at a later stage of the reaction cycle\textsuperscript{33,34}, and rules out peroxide formation in the $S_1 Y_2^5$ state as an explanation for the arrested water change.

An alternative interpretation (mechanism 2) is that the oxidation state of $Y_2$ strongly influences the substrate–water exchange via the H-bonding network around the Mn$_4$CaO$_5$ cluster, which includes the two water ligands of Ca (W3 and W4; Fig. 1a). The oxidation of $Y_2$ (that is, $Y_2^5$ formation) is coupled to the transfer of its phenolic proton to the nearby D1-His190 (Fig. 1a)\textsuperscript{61,62}. This proton movement undoubtedly changes the H-bonding network\textsuperscript{43,63,64} around the $Y_2$/Ca site and, as a consequence, may affect the exchange of the two substrate waters. This line of thought is partially supported by earlier data obtained with the alkaline-induced $S_2^0 Y_2$ state (the dash denotes a likely difference in protonation state and/or water ligation with respect to the $S_2^0 Y_2$ state), which was generated by addition of base to the preformed $S_2^0 Y_2$ state\textsuperscript{62}. These studies demonstrated that the substrate–water exchange is 5- to 20-fold slower in the $S_2^0 Y_2$ state than in the $S_3^0 Y_2$ state\textsuperscript{32,65}. The magnitude of this change is, however, too small to account for the 1000-fold decrease needed for at least one of the two substrates to explain the lack of $^{18}$O-labelling of the dioxygen observed here (Fig. 3c). Nevertheless, this option cannot be completely ruled out, and, if true, would be a remarkable demonstration of the interconnectivity of all components of the OEC.

A third option (mechanism 3) correlates the arrest of the substrate–water exchange with the deprotonation event on the Mn$_4$CaO$_5$H$_2$O$_4$ cluster that was previously reported to occur during the $S_3^0 Y_2$ state\textsuperscript{22} and is demonstrated here to also occur in the Sr/Br-PSII samples (Supplementary Figs 4–10, Supplementary Note 1 and Supplementary Methods). This proton release could explain a 1,000-fold decrease of one of the substrate–water exchange rates, if $W_f$ or $W_s$ is deprotonated. Notably, this could also account for a simultaneous slowing of the other substrate water (that is, at least 10- to 30-fold; see above), if the exchanges of both substrate waters are coupled (see ref. 28), or if the exchange of the other substrate is simultaneously slowed by mechanism 2.

Finally (mechanism 4), a recent theoretical study concluded that substrate–water exchange can only occur in PSII, if at least one Mn ion within the Mn$_4$CaO$_5$ cluster is in the Mn$_{III}$ redox state\textsuperscript{40}. Thus, to exchange a water ligand in the $S_3^0 Y_2$ state, the Mn$_4$CaO$_5$ cluster must first be transiently reduced by $Y_2^5$ to generate the exchange-competent $S_2^0 Y_2$ state (which may differ from the $S_2^0 Y_2$ and $S_3^0 Y_2$ states discussed above in its protonation state and/or water binding). This requires the $S_3^0 Y_2$ state and $S_3^0 Y_2$ state to be almost isoenenergetic with low transition barrier, allowing for a fast redox equilibrium with an appreciable probability to form the $S_2^0 Y_2$ state. If this is the mechanism for substrate exchange in the $S_3^0 Y_2$ state, then the substrate–water exchange in the $S_3^0 Y_2$ state would indeed be expected to be impeded, simply because in this state the Mn$_4$CaO$_5$ cluster cannot be transiently re-reduced by $Y_2^5$. As such, our data provide the first experimental support to this theoretical prediction. We note, however, that mechanism 4 critically depends on the condition that both substrate waters are ligated to Mn and that all Mn ions in the $S_1 Y_2^5$ and $S_2^0 Y_2$ states are in the oxidation state Mn$^{IV}$, while the alternative explanations (mechanisms 2 and 3) do not.

Experiments and theoretical calculations conducted by several groups have suggested that W2, W3, O5 (Fig. 1a) or WX (a water that is proposed to bind to the Mn$_4$CaO$_5$ cluster during the $S_1 Y_2^5$ state) are likely candidates for the two substrate waters\textsuperscript{4,6,28,39,40,66,67}. In a very recent work, WX was ruled out as the immediate substrate by clearly demonstrating that both substrates are already bound in the $S_2^0 Y_2$ state\textsuperscript{32}. Our present substrate–water exchange experiments in the $S_1 Y_2^5$ state (Fig. 3a,b and Table 1) show that the exchange of $W_f$ is only marginally affected by biological Ca/Sr substitution, while the exchange of $W_s$ occurs ten times faster in the Sr-PSII sample. The fact that the difference between $W_f$ and $W_s$ is even stronger after biological substitution than after chemical substitution\textsuperscript{31} considerably strengthens the previous suggestion\textsuperscript{30,31} that $W_f$ is not a ligand of Ca$^{2+}$. This point is further supported by the data presented here on water exchange in the $S_3^0 Y_2$ state, which show that exchange rate of $W_f$ is at least commensurate with the exchange rate of $W_s$ in the $S_3^0 Y_2$ state. Thus, from the short list above, $W_2$ appears to be the most probable candidate for $W_f$\textsuperscript{39,57}. In contrast, the tenfold dependence of the binding affinity of $W_f$ on biosynthetic Ca/Sr substitution reported here provides additional strong support for a direct bond between Ca/Sr and $W_s$. This makes W3 (ref. 39) and O5 the most likely candidates for $W_s$ with O5 being favoured, owing to the $S_2^0 Y_2$ state dependence of the $W_s$ exchange rate\textsuperscript{28,36,58,67}, even though a definitive assignment will require additional experimental support.

For the first time, the exchangeability of the substrate–water molecules has been probed in the transient state before the O–O bond formation. This provides important additional constraints for the ongoing identification of the substrate–water binding sites at the Mn$_4$CaO$_5$ cluster and for the elucidation of the mechanism of water oxidation in PSII. The discovery that both substrate waters are non-exchangeable in the transient state before O$_2$ formation suggests that arresting the exchange of both substrate water molecules, rather than just one, is a mechanistic requirement. We propose, in line with the finding that the slowing down of oxygen evolution on Ca/Sr substitution stems from a change in entropy\textsuperscript{68}, that this lack of exchange with the bulk water reflects a highly ordered arrangement of the OEC that is essential for low-energy O–O bond formation.

### Methods

**Preparation of the PSII samples.** The *T. elongatus* strain used was the ΔphsA1ΔphsA2 deletion mutant\textsuperscript{69} constructed from the *T. elongatus* 43-H strain that had a His6-tag on the carboxy terminus of CP43 (ref. 70). The biological Ca/Sr and the biochemical Ca/Br exchanges were achieved as previously described\textsuperscript{61,62,63,64,65}. Ca/Cl-PSII, Sr/Cl-PSII and Sr/Br-PSII were purified with the protocol already described\textsuperscript{68}. For the Cl$^-$/I$^-$ exchange, Sr/CJ-PSII’s binding to the Ni column was washed overnight with ~8–10 column volumes of a buffer containing 10% glycerol, 1 M betaine, 15 mM CaCl$_2$, 15 mM MgCl$_2$, 40 mM MES, 1 M l-histidine, 0.03% β-dodecyl maltoide, pH 6.5 (pH adjusted with NaOH).

Next, the PSI core complexes bound to the resin were washed overnight with a buffer containing, 1 M betaine, 15 mM Ca(OH)$_2$, 15 mM Mg(OH)$_2$, 1 M l-histidine, 0.03% β-dodecyl maltoide, pH 6.5 (adjusted by addition of MES powder). The eluted PSI samples were then washed by using Amonin-ultra-15 100 K concentrators in a buffer containing 1 M betaine, 15 mM Ca(OH)$_2$, 15 mM Mg(OH)$_2$, 1 M l-histidine, 0.03% β-dodecyl maltoide, MES 40 mM, pH 6.5 (adjusted by addition of NaOH). The PSIIs were then eluted with a buffer containing, 1 M betaine, 15 mM Ca(OH)$_2$, 15 mM Mg(OH)$_2$, 1 M l-histidine, 0.03% β-dodecyl maltoide, MES 40 mM, pH 6.5 (adjusted with NaOH). PSII samples were frozen at 77 K in liquid nitrogen until use.

**Substrate–water exchange measurements.** An isotope ratio mass spectrometer (ThermoFinnigan Delta plus XP) connected to a membrane-inlet cuvette (165 μl) via a cooling trap (liquid N$_2$) was used to measure substrate–water exchange at 20$^\circ$C\textsuperscript{26,28,41}. The substrate–water exchange in the $S_1 Y_2$ state was studied by illuminating PSII, highly enriched in the $S_1 Y_2$ state by a preflash and subsequent dark-adaptation, with two saturating Xe-flashes (2 Hz), followed by H$_2^{18}$O injection at various times before the third flash, which was given at a fixed time after the second flash (6 s for Ca/Cl-PSII, 3 s for Sr/Br-PSII, 2 s for Sr/Cl-PSII). Four flashes (2 Hz) were given 5 min after the third turnover flash for normalization purpose. For the $S_1 Y_2$ state measurements the $S_1 Y_2$-enriched PSII samples were illuminated with three flashes (2 Hz) followed by H$_2^{18}$O injection at various times after the third flash, and the normalizing flash sequence. The $S_1 Y_2$ data were treated and fit within an Excel spread sheet employing $J_{Y_2} = 0.66 \times (1 - e^{-b Y_2(t)}) + 0.34 \times (1 - e^{-b Y_2(t)})$ and $J_{Y_2} = 1 - e^{-b Y_2(t)}$.

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where $^{32}$O and $^{34}$O signify the incineration time dependent $^{16}$O$_2$ and $^{18}$O$_2$ yields, respectively$^{26,39}$. The expected $^{16}$O$_2$ yields for the S2-Y$_5$ state experiments were calculated in 1 ms intervals and then summed up over the whole decay (3,000 ms) within Excel by folding the monoexponential S3-Y$_5$ decay with the increasing $^{18}$O$_2$ enrichment in the two binding sites. By varying the delay time between S3-Y$_5$ formation (third flash) and start of H$_2^{18}$O enrichment (injection), the expected $^{18}$O$_2$ yield was calculated for delays up to 210 ms. Injection artefacts, Gd dilution and H$_2^{18}$O mixing were accounted for as in the S3-Y$_5$ experiments.

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5. Chl dilution and H$_2^{18}$O mixing were accounted for as in the S3 expectation $^{16,18}$O$_2$ yield was calculated for delays up to 210 ms. Injection artefacts, Gd dilution and H$_2^{18}$O mixing were accounted for as in the S3-Y$_5$ experiments.

From the references mentioned, it can be inferred that the study focuses on understanding the water oxidation mechanism in photosystem II, particularly the role of manganese complexes and the energetics involved. The work involves detailed kinetic analysis using isotope exchange experiments and computational modeling to elucidate the redox transitions and the role of substrate water molecules in the oxygen-evolving cycle. The references cited provide a rich context for the experimental techniques and theoretical approaches used in the study. The references cover a range of topics from the crystal structures of the oxygen-evolving complex to the specific oxidation states of manganese and calcium required for water oxidation.

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Author contributions
The experiment was conceived by F.R., A.B. and J.M. The samples were prepared by A.B. and the water-exchange experiments were performed and analysed by H.N. under the supervision of J.M. Proton release measurements were performed by F.R. and A.B. The manuscript was written by F.R., A.B. and J.M. with contributions of H.N.

Additional information
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