Investigation of substrate water interactions at the high-affinity Mn site in the photosystem II oxygen-evolving complex

Sonita Singh¹, Richard J. Debus², Tom Wydrzynski¹ and Warwick Hillier^{1,*}

¹Photobioenergetics Group, Research School of Biological Sciences, The Australian National University, Canberra, Australian Capital Territory 0200, Australia

²Department of Biochemistry, University of California, Riverside, CA 92521-0129, USA

¹⁸O isotope exchange measurements of photosystem II (PSII) in thylakoids from wild-type and mutant *Synechocystis* have been performed to investigate binding of substrate water to the high-affinity Mn₄ site in the oxygen-evolving complex (OEC). The mutants investigated were D1-D170H, a mutation of a direct ligand to the Mn₄ ion, and D1-D61N, a mutation in the second coordination sphere. The substrate water ¹⁸O exchange rates for D61N were found to be $0.16 \pm 0.02 \text{ s}^{-1}$ and $3.03 \pm 0.32 \text{ s}^{-1}$ for the slow and fast phases of exchange, respectively, compared with $0.47 \pm 0.04 \text{ s}^{-1}$ and $19.7 \pm 1.3 \text{ s}^{-1}$ for the wild-type. The D1-D170H rates were found to be $0.70 \pm 0.16 \text{ s}^{-1}$ and $24.4 \pm 4.6 \text{ s}^{-1}$ and thus are almost within the error limits for the wild-type rates. The results from the D1-D170H mutant indicate that the high-affinity Mn₄ site does not directly bind to the substrate water molecule in slow exchange, but the binding of non-substrate water to this Mn ion cannot be excluded. The results from the D61N mutation show an interaction with both substrate water molecules, which could be an indication that D61 is involved in a hydrogen bonding network with the substrate water. Our results provide limitations as to where the two substrate water molecules bind in the OEC of PSII.

Keywords: photosynthesis; photosystem II; water oxidation; ¹⁸O isotope exchange; substrate water-binding affinity; mass spectrometry

1. INTRODUCTION

The generation of molecular oxygen (O₂) from the photosynthetic oxidation of water has left a striking signature on the Earth. By changing the atmosphere to an oxidative O₂-rich environment, it has enabled the biochemical cycles of aerobic respiration to develop and with that, the evolution of complex multicellular life forms (Raymond & Segre 2006; Acquisti *et al.* 2007). The underlying redox chemistry for the photosynthetic oxidation of water to O₂ is performed by photosystem II (PSII) and involves the capture of photons and sequential storage of oxidizing equivalents at a redox-active inorganic Mn₄Ca cluster. The Mn₄Ca cluster is bound in a sub-domain of PSII called the oxygen-evolving complex (OEC). The OEC couples the one-electron reactions of charge separation with the four-electron process of water oxidation via a redox-active tyrosine (Y_Z) residue and effectively lowers the thermodynamic energy for catalysis to produce O₂ (Hillier & Messinger 2005; McEvoy & Brudvig 2006). The exact details of the photosynthetic oxidation of water have remained elusive; however, recent X-ray crystallography (Ferreira *et al.* 2004; Loll *et al.* 2005; Yano *et al.* 2006) and a range of spectroscopic approaches (Clausen & Junge 2004; Debus *et al.* 2005; Haumann

et al. 2005; Kulik *et al.* 2005; Strickler *et al.* 2005) have recently provided unprecedented information towards resolving the structure and operation of the OEC.

The mechanistic framework of photosynthetic water oxidation is based on a model introduced in Kok *et al.* (1970) which describes a cycle of five oxidative states, called the S states and labelled S₀→S₄. The S-state nomenclature refers to intermediates of the OEC as the four oxidizing equivalents that are stored and with O₂ being produced on the transition from S₄ to S₀. This model was developed to explain the period four oscillations in the O₂ yield upon excitation of photosynthetic samples with sequential saturating flashes of light (Joliot *et al.* 1969). The structure of each S-state intermediate and the chemistry of each transition, however, have not yet been explicitly determined. Further information is required to narrow the possibilities (Hillier & Messinger 2005; McEvoy & Brudvig 2006). In seeking to rationalize substrate water interactions in PSII, a range of electron paramagnetic resonance (EPR) and Fourier transform infrared (FTIR) measurements have been undertaken. Both approaches have shed some insights into the substrate-binding properties but are limited when it comes to separating the interactions from non-substrate water. In another approach, ¹⁸O isotope exchange measured by mass spectrometry can be used to report only on the substrate water. But in this approach, there is greater uncertainty in assigning the chemical nature of the substrate at its binding site (for review, see Hillier & Messinger (2005)).

* Author for correspondence (warwick.hillier@anu.edu.au).

One contribution of 20 to a Discussion Meeting Issue 'Revealing how nature uses sunlight to split water'.

Figure 1. Structural models of the OEC from the crystal structures published as (a) 1S5L.pdb (Ferreira *et al.* 2004) and (b) 2AXT.pdb (Loll *et al.* 2005). The structure is presented with Mn ions as purple, Ca green and the psbA protein (D1) is shown as a pale green with side chain residues labelled according to single letter codes. The 'dangling' high-affinity Mn_4 ion is in close proximity to aspartate D170 ligand (1.99 Å 1S5L or 2.39 Å 2AXT) and is proposed to act as a direct ligand to Mn_4 . The residue aspartate D1-D61 is further away (4.53 Å 1S5L or 4.22 Å 2AXT) and was proposed to be a potential H-bonding partner for a water coordinating Mn_4 (Ferreira *et al.* 2004).

Although the results from all of these studies show that the substrate water is indeed bound to the Mn_4Ca cluster, despite many proposals, it is still not known which of the metal ions in the cluster bind the substrate water nor how the O–O bond is formed.

To gain further information on the substrate water-binding sites in the OEC, we have performed ^{18}O exchange measurements in the S_3 state on mutants of selected amino acid residues. These amino acids are predicted to interact with the catalytic Mn_4Ca cluster and form ligands to the bound substrate water based on mutagenesis studies (Debus 2001, 2005) and X-ray crystallographic analysis (Ferreira *et al.* 2004; Loll *et al.* 2005). The most recent crystal structure models of the OEC, 1S5L.pdb (Ferreira *et al.* 2004) and 2AXT.pdb (Loll *et al.* 2005), are shown in figure 1. Both models show a Mn_4Ca cluster with various surrounding amino acid ligands. We have investigated two of the amino acid residues for possible substrate water interactions, D1-D61 and D1-D170. Based on the 1S5L.pdb data, it was proposed that D1-D61 interacts with a substrate water bound to the high-affinity Mn_4 ion through a H-bonding water chain network from the site of water oxidation in the OEC to the outside of the PSII complex (Barber *et al.* 2004; Ferreira *et al.* 2004). The interaction of D1-D61 with substrate water is also supported by the 2AXT.pdb data (Loll *et al.* 2005). The D1-D61N phenotypes in *Synechocystis* PCC6803 exhibit slower electron transfer rates and slower O_2 -release kinetics (Hundelt *et al.* 1998). The other amino acid residue D1-D170 on which we report is believed to be a direct ligand to the high-affinity Mn_4 ion, since it is required for the photoassembly of the Mn_4Ca cluster (Nixon & Diner 1992; Campbell *et al.* 2000; Chu *et al.* 2001; Debus *et al.* 2003, 2005). The high-affinity Mn_4 ion has also been suggested to be a binding site for substrate water, since it is situated at the beginning of a hydrophilic chain of residues that also involves

D1-E65, MSP-K317 and MSP-E31 and has been proposed to function in the transport of protons out of the OEC or water into the catalytic site (Barber *et al.* 2004).

2. MATERIAL AND METHODS

(a) Construction of site-directed mutants

The constructions of the D1-D61N, the D1-D170H mutant and the wild-type* (WT) control strains of *Synechocystis* sp. PCC6803 were described previously (Chu *et al.* 1994, 1995), except that the mutation-bearing and control constructs were transformed into a host strain of *Synechocystis* having a His-tag fused to the C-terminus of CP47 (Debus 2001). The designation 'wild-type*' differentiates this strain from the native wild-type strain that contains all three *psbA* genes, lacks a His-tag on the C-terminus of CP47 and is sensitive to antibiotics. Cells were propagated as described previously (Chu *et al.* 2001; Debus *et al.* 2003), but in 7 l pyrex carboys. To verify the integrity of the D1-D61N and D1-D170H cultures that were harvested for the purification of PSII particles, an aliquot of each culture was set aside and the complete sequence of the *psbA-2* gene was obtained after PCR amplification of genomic DNA. No traces of the wild-type D1-Asp61 or D1-Asp170 codons nor of any spontaneous mutation within *psbA-2* (e.g. second-site revertants) was detected.

(b) Isolation of *Synechocystis* thylakoid membranes

Thylakoid membranes were purified as described previously (Tang & Diner 1994) and modified as described by Strickler *et al.* (2005). Briefly, the cells (in two or three 7 l carboys) were propagated in liquid BG-11 media containing 5 mM glucose until an OD_{730} of 0.9–1.2 was obtained, whereupon they were concentrated and suspended in a buffer containing 50 mM 2-(*N*-morpholino)-ethanesulfonic acid (MES)–NaOH (pH 6.0), 10% (v/v) glycerol, 1.2 M betaine, 5 mM $CaCl_2$, 5 mM $MgCl_2$, 1 mM benzamidine, 1 mM ϵ -amino-*n*-caproic acid, 1 mM phenylmethylsulphonyl fluoride and 0.05 mg ml $^{-1}$ BSA. The cells were broken by nine cycles of (5 s on/15 min off) in a glass bead homogenizer (Bead-Beater, BioSpec Products,

Figure 2. ^{18}O exchange measurements in the S_3 state of PSII thylakoid membranes from WT and mutants of *Synechocystis* sp. PCC6803: (a) D1-D61N and (b) D1-D170H. The WT data are shown in both (a,b) as black circles with the kinetic fit shown by a black line. In (a), the D1-D61N data are shown as red circles with the kinetic fit shown by a red line on the left. In (b), the D1-D170H data are shown by blue circles with the kinetic fit shown by a blue line. Insets are a shorter expanded time to show the fast phase.

Bartlesville, OK). After separation of unbroken cells and debris by centrifugation (10 min at 5000g in a Beckman JA-14 rotor), the resulting thylakoid membranes were concentrated by centrifugation (20 min at 40 000g in a Beckman Ti45 rotor) and suspended to concentrations of 1–1.5 mg of Chl ml^{-1} in a buffer containing 50 mM MES–NaOH (pH 6.0), 10% (v/v) glycerol, 1.2 M betaine, 20 mM CaCl_2 and 5 mM MgCl_2 . The concentrated thylakoid membranes were flash-frozen as 1 ml aliquots in liquid nitrogen and stored at -80°C until use.

(c) Conditions for mass spectrometric assays

Frozen thylakoid samples were thawed and diluted to 0.25 mg ml^{-1} Chl in the assay buffer containing 40 mM MES (pH 6.5), 15 mM MgCl_2 , 15 mM CaCl_2 , 10% glycerol and 1.2 M betaine with the addition of 0.5 mM $\text{K}_3\text{Fe}(\text{CN})_6$ acting as an electron acceptor. Samples were individually prepared with a dark adaption/preflash cycle to maximize $[\text{S}_1]$ and then the ^{18}O exchange experiments were performed. Light flashes were provided by a xenon flash lamp with FWHM = 5.2 μs .

A rapid mixing sample chamber originally described by Messinger *et al.* (1995) having a reaction volume of 160 μl was maintained at 10°C . The mixing rate, as determined by fluorescence (Hillier & Wydrzynski 2004), was $k_{\text{inj}} = 174 \text{ s}^{-1}$. The sample was first preset into the S_3 state by two saturating preflashes after which 25 μl of 95% H_2^{18}O was rapidly injected to give a final ^{18}O enrichment of $12.0 \pm 0.5\%$. A third saturating flash (the turnover flash) was given to the sample to evolve O_2 as a function of the exchange time (Δt) between the injection and the turnover flash. Four saturating flashes were then given 20 s apart for normalizing the signals. The timing sequence for flashes and injection was controlled by a computer. Measurements of the O_2 evolved were made at $m/e = 34$ using an in-line mass spectrometer (Vacuum Generation MM6, Winford, UK).

The O_2 yields were quantified from the peak heights as described earlier (Hillier & Wydrzynski 2004). The exchange rates were based on the assessment of O_2 yield on the third turnover flash (Y_3) as a function of time for ^{18}O exchange. The Y_3 was corrected by subtracting the background O_2 in the

injection (Y_{inj}), while small variations in yield due to membrane permeability and sample activity were corrected by normalizing Y_3 to the sum of the four subsequent flashes (i.e. Y_{4-7})

$$Y_{\text{N}} = \frac{[Y_3 - Y_{\text{inj}}]}{\sum Y_{n(4 \rightarrow 7)}}. \quad (2.1)$$

For very short exchange times, an additional correction was necessary due to partial mixing of the injected H_2^{18}O

$$Y_{\text{C}} = Y_{\text{N}} \frac{\varepsilon}{\varepsilon(1 - \exp(-175t))} \times \frac{1}{1 + \Delta\text{Chl} \times \exp(-175t)}. \quad (2.2)$$

The first term corrects for the enrichment during the injection and the second the change in chlorophyll concentration (dilution) where $k_{\text{inj}} = 175 \text{ s}^{-1}$ and where the chlorophyll is

$$\Delta\text{Chl} = \frac{[\text{Chl}]_{(t=0)} - [\text{Chl}]_{t=\infty}}{[\text{Chl}]_{t=\infty}},$$

Finally, each dataset was normalized to give $Y_{\text{C}(t=\infty)} = 1$.

The biphasic plots of Y_{C} versus Δt at $m/e = 34$ were analysed as the sum of two exponential functions using the following equation (Hillier & Wydrzynski 2004) revealing a fast phase k_2 and slow phase k_1 rate constant.

$$Y_{\text{C}} = 0.57(1 - \exp(-k_2t)) + 0.43(1 - \exp(k_1t)). \quad (2.3)$$

No data were recorded at $m/e = 36$ due to the ^{18}O enrichment and small oxygen signals in the mutant samples.

3. RESULTS

The results from the ^{18}O exchange measurements of the WT and mutant *Synechocystis* thylakoid samples are shown in figure 2 as plots of the corrected oxygen yield, Y_3 , versus the exchange time, Δt . All data plots display biphasic kinetics reflecting the two exchange sites as reported previously for other PSII samples (Messinger *et al.* 1995; Hillier *et al.* 1998, 2001). The WT (black circles) and D61N data (red circles) are presented in figure 2a with the fitted exchange kinetics according to equation (2.3) shown by a solid line. The rates are given

Table 1. Rate constants for ^{18}O exchange in the S_3 state of PSII thylakoid membranes from WT and mutants of *Synechocystis* sp. PCC6803.

sample	k_1 (s^{-1})	k_2 (s^{-1})
WT ^a	0.47 ± 0.04	19.7 ± 1.3
D1-D61N	0.16 ± 0.02	3.03 ± 0.32
D1-D170H	0.70 ± 0.16	24.4 ± 4.6
WT ^b	3.8 ± 1.3	25.9 ± 6.7

^a Buffer solution medium: 40 mM MES, pH 6.5, 15 mM MgCl_2 , 15 mM CaCl_2 , 10% glycerol and 1.2 M betaine.

^b Buffer solution medium: 50 HEPES, pH 7.0, 1 mM sucrose, 250 mM NaCl, 10 mM MgCl_2 and 5 mM CaCl_2 . Result from a previous study (Hillier *et al.* 2001).

in table 1. The inset to figure 2 shows a shorter time scale to better compare the fast phase. The exchange rates for WT thylakoids were found to be $k_1 = 0.47 \pm 0.04 \text{ s}^{-1}$ and $k_2 = 19.7 \pm 1.3 \text{ s}^{-1}$ for the slow and fast phases, respectively. The D1-D61N mutation gave exchange rates of $k_1 = 0.16 \pm 0.02 \text{ s}^{-1}$ and $k_2 = 3.03 \pm 0.32 \text{ s}^{-1}$ and thus affects both water-binding sites. More specifically, D1-D61N mutation results in an approximately threefold slowing in the slow phase k_1 and approximately 6.5-fold slowing in the fast phase k_2 . In both cases, the substrate binding is stronger in the D1-D61N mutation, but the overall effect is not localized to a single substrate water site. Table 1 also lists the calculated rates for samples from previous measurements (Hillier *et al.* 2001). The earlier WT *Synechocystis* thylakoids are somewhat different from the current work, probably due to the different solution buffer compositions.

The effects of the D1-D170H mutation are shown in figure 2*b* (blue circles) as compared with the WT data (black circles) with solid line fits giving the rates in table 1. The exchange rates for D1-D170H were, $k_1 = 0.70 \pm 0.16 \text{ s}^{-1}$ and $k_2 = 24.4 \pm 4.6 \text{ s}^{-1}$ and show less than a twofold change in rate compared with WT, just outside the error margins. Thus, it appears that the D1-D170H mutation does not change the binding properties of substrate water compared with the WT sample.

4. DISCUSSION

Understanding the molecular mechanism by which water is oxidized to O_2 in PSII requires the knowledge of where the two substrate water molecules bind within the OEC. Thus far, the X-ray structural resolution of the PSII complex is limited to 3 Å, which is insufficient to locate individual water molecules. Until the structural resolution can be further refined, other approaches must be applied to determine the coordination geometry of the catalytic Mn ions and the location of the substrate water. Our approach to this problem has been to use water ligand ^{18}O exchange measurements to infer the nature of the substrate-binding sites (Hillier & Wydrzynski 2004; Hillier & Messinger 2005). Since the rate of ^{18}O exchange depends upon the chemical properties of the binding site, some insight into the substrate water interactions with the catalytic site can be made by comparison with inorganic complexes (Hillier & Wydrzynski 2001, 2008) or by conducting computational density

functional theory calculations (Lundberg *et al.* 2003). However, in PSII, it is difficult to abstract from ligand exchange data alone the precise nature of the bound substrate water. As a new strategy, we have begun to characterize the nature of the substrate water-binding sites by measuring the exchange kinetics in various mutations of the OEC. A range of mutants have been generated over the years in *Synechocystis* (Debus 2001, 2005) with varying phenotypes. In this work we have examined the amino acid residues associated with the high-affinity Mn_4 site and have focused on the D1-D170H and D1-D61N mutations.

The D1-D170 residue in PSII has been suggested to be a ligand to Mn based on EPR data (Campbell *et al.* 2000; Debus *et al.* 2003) and has been assigned in the structural models 1S5L and 2AXT to a unidentate ligand to Mn_4 , the high-affinity Mn site (Ferreira *et al.* 2004; Loll *et al.* 2005). The D1-D170H *Synechocystis* mutant exhibits 50% of the WT O_2 evolution activity, which is attributed to a 50% reduction in the assembly of the Mn_4Ca clusters. The remaining active catalytic sites appear to function normally and show the normal mid-frequency S_2/S_1 difference spectrum (Nixon & Diner 1992; Chu *et al.* 1994, 2001). The EPR signals for the S_1 and S_2 states are also relatively unperturbed (Campbell *et al.* 2000; Debus *et al.* 2003). The general phenotype of D1-D170H is not strong and has been discussed at length (Debus *et al.* 2003). In terms of water binding and interactions with the Mn_4 metal ion, the non-isosteric mutational change of aspartic acid to histidine is expected to induce some level of electronic perturbation in the coordinated Mn ion. Specifically, a perturbation is predicted to arise from the weaker σ -donation character of the Asp oxygen ligands compared with the stronger σ -donation from the nitrogen ligand from His (Richens 2005; Hillier & Wydrzynski 2008). Such changes would thus decrease the electron density of the Mn_4 ion and modulate the ligand exchange reactions. The increased electron donation from histidine into the Mn_4 ion would be expected to increase the ^{18}O ligand exchange rates as seen in some model systems (Richens 2005; Hillier & Wydrzynski 2008). However, the results in table 1 and figure 2*b* show there to be minimal effects on the two substrate water exchange rates when the D1-D170H mutant is compared with WT, with relative differences just outside of the fitting errors.

The D1-D61N mutant results in charge neutralization arising from the substitution of aspartic acid with the neutral asparagine residue. The Asp61 residue is located in the second coordination sphere of the Mn_4Ca cluster and is too distant to function as a direct metal ligand (Ferreira *et al.* 2004; Loll *et al.* 2005). The D61N phenotype is photoautotrophic but exhibits slower S -state transitions and O_2 release kinetics (Hundelt *et al.* 1998). The functional change arising from the D1-D61N mutation, when assessed with the structural work, is a perturbation of the H-bonding interactions between the predicted water and the D61 ligand. Such a functional change should be detectable by the ^{18}O exchange measurements (Hillier *et al.* 2001; Hillier & Wydrzynski 2004). The results in table 1 and in figure 2*a* show a decrease into both the fast and slow rates of ^{18}O

exchange, the largest effect being the approximately 6.5-fold reduction in the fast phase of exchange.

The implications from these results are as follows. The Asp170 residue when mutated does not affect either phase of ^{18}O exchange. Thus, the first possible interpretation of these data is that there is no substrate water bound to the high-affinity Mn_4 site. As discussed above, the σ -bonding character from the Asp $\rightarrow$ His mutation is different and would likely generate changes in the effective charge of the Mn ion that ordinarily would perturb ligand exchange rates. As no changes are observed, the absence of substrate water binding to this site is a strong possibility. A second scenario is a reorientation of the substrate water molecule such that binding is equivalent in the mutant and WT and there is no change in ^{18}O exchange. However, given that the ^{18}O exchange rates report S-state changes most likely arising from Mn oxidation steps, the expectation is that the substrate-binding site involves a redox-active site (Hillier & Wydrzynski 2000, 2004). However, the Asp170 to His mutation shows no change during the $\text{S}_0 \rightarrow \text{S}_1 \rightarrow \text{S}_2 \rightarrow \text{S}_3$ transitions in the FTIR spectra (Debus et al. 2005) and would therefore be inconsistent with the S-state-dependent changes in ^{18}O exchange. A final further possibility from these results is that a water molecule is indeed bound to the high-affinity Mn_4 site, but it is not a substrate water. Such an interaction could provide a potential fifth ligand to the Mn ion as needed in the X-ray models (Barber et al. 2004; Ferreira et al. 2004) but would not be an oxygen atom involved in O_2 generation.

Based on the effects of the D1-D61N mutations, there is another possible interpretation of the ^{18}O exchange data where the fast ^{18}O exchange phase in PSII is limited by the diffusion/transport of substrate water through a putative substrate water chain (Barber et al. 2004; Ferreira et al. 2004). In this case, the ^{18}O exchange rates for PSII would reflect: (i) the entry of the substrate into the OEC (the fast phase) and (ii) the exchange at a metal site for one substrate water molecule (the slow site). Using this rationale, the fast phase of exchange is limited by substrate diffusion, and consequently the exchange behaviour of this second substrate site is masked. Potentially then, this site could be the Mn_4 ion coordinated by D1-D170. However, the slow ^{18}O site and the characteristic S-state changes in rate must be derived from a site other than Mn_4 . If D1-D61 is the beginning of this chain (Barber et al. 2004; Ferreira et al. 2004), then the D1-D61N mutation would slow the entry of the substrate sixfold compared with the WT. One caveat is that a decrease in exchange rate with the D1-D61N mutation, although indicative of stronger substrate water binding, has also been observed when the extrinsic polypeptides were removed (Hillier et al. 2001). The origin of such a small change may be small non-specific perturbations to the transition state. Future work with other residues on the putative water chain will be performed to evaluate this possibility.

This study was supported by the Australian Research Council DP0770149 and DP0450421. The authors wish to thank Elmars Krausz, Ron Pace and Rob Stranger at ANU for their valuable discussions.

REFERENCES

- Acquisti, C., Kleffe, J. & Collins, S. 2007 Oxygen content of transmembrane proteins over macroevolutionary time scales. *Nature* **445**, 47–52. (doi:10.1038/nature05450)
- Barber, J., Ferreira, K., Maghlaoui, K. & Iwata, S. 2004 Structural model of the oxygen-evolving centre of photosystem II with mechanistic implications. *Phys. Chem. Chem. Phys.* **6**, 4737–4742. (doi:10.1039/b407981g)
- Campbell, K. A., Force, D. A., Nixon, P. J., Dole, F., Diner, B. A. & Britt, R. D. 2000 Dual-mode EPR detects the initial intermediate in photoassembly of the photosystem II Mn cluster: the influence of amino acid residue 170 of the D1 polypeptide on Mn coordination. *J. Am. Chem. Soc.* **122**, 3754–3761. (doi:10.1021/ja000142t)
- Chu, H.-A., Nguyen, A. P. & Debus, R. J. 1994 Site-directed photosystem II mutants with perturbed oxygen-evolving properties. 1. Instability or inefficient assembly of the manganese cluster *in vivo*. *Biochemistry* **33**, 6137–6149. (doi:10.1021/bi00186a013)
- Chu, H.-A., Nguyen, A. P. & Debus, R. J. 1995 Amino acid residues that influence the binding of manganese or calcium to photosystem II. 1. The lumenal interhelical domains of the D1 polypeptide. *Biochemistry* **34**, 5839–5858. (doi:10.1021/bi00017a016)
- Chu, H.-A., Debus, R. J. & Babcock, G. T. 2001 D1-Asp170 is structurally coupled to the oxygen evolving complex in photosystem II as revealed by light-induced Fourier transform infrared difference spectroscopy. *Biochemistry* **40**, 2312–2316. (doi:10.1021/bi0022994)
- Clausen, J. & Junge, W. 2004 Detection of an intermediate of photosynthetic water oxidation. *Nature* **430**, 480–483. (doi:10.1038/nature02676)
- Debus, R. J. 2001 Amino acid residues that modulate the properties of tyrosine Y_Z and the manganese cluster in the water oxidizing complex of photosystem II. *Biochim. Biophys. Acta* **1503**, 164–186. (doi:10.1016/S0005-2728(00)00221-8)
- Debus, R. J. 2005 The catalytic manganese cluster: protein ligation. In *Photosystem II: the water : plastoquinone oxidoreductase in photosynthesis*, vol. 25 (eds T. Wydrzynski & K. Satoh), pp. 261–284. Dordrecht, The Netherlands: Springer.
- Debus, R. J., Aznar, C., Campbell, K. A., Gregor, W., Diner, B. A. & Britt, R. D. 2003 Does aspartate 170 of the D1 polypeptide ligate the manganese cluster in photosystem II? An EPR and ESEEM study. *Biochemistry* **42**, 10 600–10 608. (doi:10.1021/bi034859f)
- Debus, R. J., Strickler, M. A., Walker, L. M. & Hillier, W. 2005 No evidence from FTIR difference spectroscopy that aspartate-170 of the D1 polypeptide ligates a manganese ion that undergoes oxidation during the S_0 to S_1 , S_1 to S_2 , or S_2 to S_3 transitions in photosystem II. *Biochemistry* **44**, 1367–1374. (doi:10.1021/bi047558u)
- Ferreira, K. N., Iverson, T., Maghlaoui, K., Barber, J. & Iwata, S. 2004 Architecture of the photosynthetic oxygen-evolving center. *Science* **303**, 1831–1838. (doi:10.1126/science.1093087)
- Haumann, M., Liebisch, P., Muller, C., Barra, M., Grabolle, M. & Dau, H. 2005 Photosynthetic O_2 formation tracked by time-resolved X-ray experiments. *Science* **310**, 1019–1021. (doi:10.1126/science.1117551)
- Hillier, W. & Messinger, J. 2005 Mechanism of photosynthetic oxygen production. In *Photosystem II: the water : plastoquinone oxidoreductase in photosynthesis*, vol. 25 (eds T. Wydrzynski & K. Satoh), pp. 567–608. Dordrecht, The Netherlands: Springer.
- Hillier, W. & Wydrzynski, T. 2000 The affinities for the two substrate water binding sites in the O_2 evolving complex of photosystem II vary independently during S-state turnover. *Biochemistry* **39**, 4399–4405. (doi:10.1021/bi992318d)

- Hillier, W. & Wydrzynski, T. 2001 Oxygen ligand exchange at metal sites: implications for the O₂ evolving mechanism of photosystem II. *Biochim. Biophys. Acta Bioenerget.* **1503**, 197–209. (doi:10.1016/S0005-2728(00)00225-5)
- Hillier, W. & Wydrzynski, T. 2004 Substrate water interactions within the photosystem II oxygen evolving complex. *Phys. Chem. Chem. Phys.* **6**, 4882–4889. (doi:10.1039/b407269c)
- Hillier, W. & Wydrzynski, T. 2008 ¹⁸O–water exchange in photosystem II: substrate binding and intermediates of the water splitting cycle. *Coord. Chem. Rev.* **252**, 306–317. (doi:10.1016/j.ccr.2007.09.004)
- Hillier, W., Messinger, J. & Wydrzynski, T. 1998 Kinetic determination of the fast exchanging substrate water molecule in the S₃ state of photosystem II. *Biochemistry* **37**, 16 908–16 914. (doi:10.1021/bi980756z)
- Hillier, W., Hendry, G., Burnap, R. L. & Wydrzynski, T. 2001 Substrate water exchange in photosystem II depends on the peripheral proteins. *J. Biol. Chem.* **276**, 46 917–46 924. (doi:10.1074/jbc.M102954200)
- Hundelt, M., Hays, A.-M. A., Debus, R. J. & Junge, W. 1998 Oxygenic photosystem II: the mutation D1-D61N in *Synechocystis* sp. PCC 6803 retards S-state transitions without affecting electron transfer from Y_Z to P680⁺. *Biochemistry* **37**, 14 450–14 456. (doi:10.1021/bi981164j)
- Joliot, P., Barbieri, G. & Chabaud, R. 1969 Un nouveau modele des centres photochimiques du systeme II. *Photochem. Photobiol.* **10**, 309–329.
- Kok, B., Forbush, B. & McGloin, M. 1970 Cooperation of charges in photosynthetic oxygen evolution. I. A linear four step mechanism. *Photochem. Photobiol.* **11**, 457–475.
- Kulik, L. V., Epel, B., Lubitz, W. & Messinger, J. 2005 ⁵⁵Mn pulse ENDOR at 34 GHz of the S₀ and S₂ states of the oxygen-evolving complex in photosystem II. *J. Am. Chem. Soc.* **127**, 2392–2393. (doi:10.1021/ja043012j)
- Loll, B., Kern, J., Saenger, W., Zouni, A. & Biesiadka, J. 2005 Towards complete cofactor arrangement in the 3.0 angstrom resolution structure of photosystem II. *Nature* **438**, 1040–1044. (doi:10.1038/nature04224)
- Lundberg, M., Blomberg, M. R. A. & Siegbahn, P. E. M. 2003 Modeling water exchange on monomeric and dimeric Mn centers. *Theor. Chem. Acc.* **110**, 130–143.
- McEvoy, J. P. & Brudvig, G. W. 2006 Water-splitting chemistry of photosystem II. *Chem. Rev.* **106**, 4455–4483. (doi:10.1021/cr0204294)
- Messinger, J., Badger, M. & Wydrzynski, T. 1995 Detection of one slowly exchanging substrate water molecule in the S₃ state of photosystem II. *Proc. Natl Acad. Sci. USA* **92**, 3209–3213. (doi:10.1073/pnas.92.8.3209)
- Nixon, P. J. & Diner, B. A. 1992 Aspartate-170 of the photosystem-II reaction center polypeptide D1 is involved in the assembly of the oxygen-evolving manganese cluster. *Biochemistry* **31**, 942–948. (doi:10.1021/bi00118a041)
- Raymond, J. & Segre, D. 2006 The effect of oxygen on biochemical networks and the evolution of complex life. *Science* **311**, 1764–1767. (doi:10.1126/science.1118439)
- Richens, D. T. 2005 Ligand substitution reactions at inorganic centers. *Chem. Rev.* **105**, 1961–2002. (doi:10.1021/cr030705u)
- Strickler, M. A., Walker, L. M., Hillier, W. & Debus, R. J. 2005 Evidence from biosynthetically incorporated strontium and FTIR difference spectroscopy that the C-terminus of the D1 polypeptide of photosystem II does not ligate calcium. *Biochemistry* **44**, 8571–8577. (doi:10.1021/bi050653y)
- Tang, X.-S. & Diner, B. A. 1994 Biochemical and spectroscopic characterization of a new oxygen-evolving photosystem II core complex from the Cyanobacterium *Synechocystis* PCC 6803. *Biochemistry* **33**, 4594–4603. (doi:10.1021/bi00181a021)
- Yano, J. *et al.* 2006 Where water is oxidized to dioxygen: structure of the photosynthetic Mn₄Ca cluster. *Science* **314**, 821–825. (doi:10.1126/science.1128186)

Discussion

G. Brudvig (*Yale University, USA*). How would you rationalize the changes in the exchange rates with S-state changes? In particular, the slowly exchanging water slows down in S₁ → S₂ and speeds up in S₁ → S₂. W. Hillier. The changes with S-state are possibly quite revealing as to the nature of the substrate-binding sites. Certainly the oxidation of metal ions will in general make ligand exchange rates less labile: as the metal ion contracts from oxidation, the larger charge is effectively distributed over a smaller ionic radius. So the decrease in exchange rates of several orders of magnitude is a feature consistent with metal centred oxidation. However, the increase in exchange S₁ → S₂ is not consistent with this view and must be another process. Quite possibly this is a classic ligand *trans* effect associated with the adjacent (opposite) d orbital. Alternatively, this change in rate may be due to a structural change in the coordination of this metal site. However, neither of these interpretations have any particularly strong scientific backing at this point.

V. Batista (*Yale University, USA*). Our quantum mechanics/molecular mechanics modelling from the crystal structures suggests that there are two water channels. One channel seems to provide a H-bonding network linking the Ca site. A second channel provides a H-bonding network to a Mn₄ site. What is interesting is that the water molecules in these channels appear only to have two or three H-bonding partners and are in essence placed in single file organization. This picture suggests that upon O₂ formation, the next water molecule in each channel is advanced forward through the channels. Do you share or support this view?

W. Hillier. Yes. The emerging view is that there are specific substrate channels and these may play some role in the mechanism. The ¹⁸O ligand exchange rates for our fast exchange site are possibly derived from a transport property into the catalytic site. This is one interpretation for this exchange phase. Thus far, it is not known in any model system what the rates of exchange for oxygen are in a one-dimensional array of water molecules. My sense is, this could be quite fast but there is not really any system where this has been tested. However, substrate and proton channels have also appeared in published work of Jim (Barber). He did not specifically allude to them earlier today, but they appear to be mapped in the structural data and it will be interesting to see the interaction of the substrate water in these channels.

F. Ho (*Uppsala University, Sweden*). We also have found water channels within PSII using solvent accessibility calculations. There appears to be a gap between two channels formed by Ca, His190, Glu189, Y_Z, Phe186 and Gln165 which may act as a control gate to regulate water access into the Mn cluster. The gap is very close to the possible diameter and could be possible with the right small shifts in residue positions to bridge.

W. Hillier. Regulated water access is something Tom (Wydrzynski) considered a number of years ago for controlling the reaction coordinate and minimizing deleterious side reactions. The dynamics of this channel or the energy landscape will then control the conduction of water along this pathway in the case of a gap. What then this them may correlates with ^{18}O

exchange measurements is the activation barrier for the exchange, namely a 40 kJ mol^{-1} thermodynamic barrier. Improved structures are needed to see the real connectivity of these channels. Another consideration more critical is the path of protons if they choose to use your proposed pathway. Small gaps in conductivity will disrupt the proton transfer pathway.