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

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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 ± 0.02 s⁻¹ and 3.03 ± 0.32 s⁻¹ for the slow and fast phases of exchange, respectively, compared with 0.47 ± 0.04 s⁻¹ and 19.7 ± 1.3 s⁻¹ for the wild-type. The D1-D170H rates were found to be 0.70 ± 0.16 s⁻¹ and 24.4 ± 4.6 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 (Y₂) 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→S₅. 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)).
Although the results from all of these studies show that the substrate water is indeed bound to the Mn$_4$Ca 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$_4$Ca 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$_4$Ca 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 our 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$_4$Ca 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 helix 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 I 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 I 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,}

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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 CaCl₂ and 5 mM MgCl₂. 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 MgCl₂, 15 mM CaCl₂, 10% glycerol and 1.2 M betaine with the addition of 0.5 mM K₃Fe(CN)_6 acting as an electron acceptor. Samples were individually prepared with a dark adaption/preflash cycle to maximize [S₁] and then the ¹⁸O 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_mix = 174 s⁻¹. The sample was first preset into the S₃ state by two saturating preflashes after which 25 μl of 95% H₂¹⁸O was rapidly injected to give a final ¹⁸O enrichment of 12.0 ± 0.5%. A third saturating flash (the turnover flash) was given to the sample to evolve O₂ 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 O₂ evolved were made at m/e = 34 using an in-line mass spectrometer (Vacuum Generation MM6, Winford, UK).

The O₂ yields were quantified from the peak heights as described earlier (Hillier & Wydrzynski 2004). The exchange rates were based on the assessment of O₂ yield on the third turnover flash (Y₅) as a function of time for ¹⁸O exchange. The Y₅ was corrected by subtracting the background O₂ in the injection (Y_m), while small variations in yield due to membrane permeability and sample activity were corrected by normalizing Y₅ to the sum of the four subsequent flashes (i.e. Y₄₋₇)

\[
Y_N = \frac{[Y_5 - Y_m]}{\sum Y_{m(4-7)}}. \tag{2.1}
\]

For very short exchange times, an additional correction was necessary due to partial mixing of the injected H₂¹⁸O

\[
Y_C = \frac{Y_N}{\epsilon(1 - \exp(-175\epsilon t))} \times \frac{1}{1 + \Delta \text{Chl} \times \exp(-175\epsilon t)}. \tag{2.2}
\]

The first term corrects for the enrichment during the injection and the second the change in chlorophyll concentration (dilution) where k_mix = 174 s⁻¹ and where the chlorophyll is

\[
\Delta \text{Chl} = \frac{[\text{Chl}]_{t=\infty} - [\text{Chl}]_{t=0}}{[\text{Chl}]_{t=0}}.
\]

Finally, each dataset was normalized to give Y_{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 k₂ and slow phase k₁ rate constant.

\[
Y_C = 0.57(1 - \exp(-k_2 t)) + 0.43(1 - \exp(-k_1 t)). \tag{2.3}
\]

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

3. RESULTS

The results from the ¹⁸O exchange measurements of the WT and mutant Synechocystis thylakoid samples are shown in figure 2 as plots of the corrected oxygen yield, Y₅, 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

Figure 2. ¹⁸O exchange measurements in the S₃ 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.

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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.

<table>
<thead>
<tr>
<th>sample</th>
<th>$k_1$ (s$^{-1}$)</th>
<th>$k_2$ (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>0.47±0.04</td>
<td>19.7±1.3</td>
</tr>
<tr>
<td>D1-D61N</td>
<td>0.16±0.02</td>
<td>3.03±0.32</td>
</tr>
<tr>
<td>D1-D170H</td>
<td>0.70±0.16</td>
<td>24.4±4.6</td>
</tr>
<tr>
<td>WT$^{a}$</td>
<td>3.8±1.3</td>
<td>25.9±6.7</td>
</tr>
</tbody>
</table>

$^{a}$ Buffer solution medium: 40 mM MES, pH 6.5, 15 mM MgCl$_2$, 15 mM CaCl$_2$, 10% glycerol and 1.2 M betaine.

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 $k_1 = 0.47±0.04$ s$^{-1}$ and $k_2 = 19.7±1.3$ s$^{-1}$ for the slow and fast phases, respectively. The D1-D61N mutation gave exchange rates of $k_1 = 0.16±0.02$ s$^{-1}$ and $k_2 = 3.03±0.32$ 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-D170N 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 2b (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, $k_1 = 0.70±0.16$ s$^{-1}$ and $k_2 = 24.4±4.6$ 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-D170H 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$_{4\alpha}$ 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$_{4\alpha}$Ca 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 $\sigma$-donation character of the Asp oxygen ligands compared with the stronger $\sigma$-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 in figure 2b 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$_{4\alpha}$Ca cluster and is too distant to function as a direct metal ligand (Ferreira et al. 2004; Loll et al. 2005). The D61N phenotype is photautotrophic 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 2a 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 $\pi$-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 bonding 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 S$_0\rightarrow$S$_1\rightarrow$S$_2\rightarrow$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.

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**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


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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 $40 \text{ kJ mol}^{-1}$ 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.