Effects of methanol on the S_i -state transitions in photosynthetic water-splitting

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Abstract From a chemical point of view methanol is one of the closest analogues of water. Consistent with this idea EPR spectroscopy studies have shown that methanol binds at—or at least very close to—the Mn_4O_xCa cluster of photosystem II (PSII). In contrast, Clark-type oxygen rate measurements demonstrate that the O_2 evolving activity of PSII is surprisingly unaffected by methanol concentrations of up to 10%. Here we study for the first time in detail the effect of methanol on photosynthetic water-splitting by employing a Joliot-type bare platinum electrode. We demonstrate a linear dependence of the miss parameter for S_i state advancement on the methanol concentrations in the range of 0–10% (v/v). This finding is consistent with the idea that methanol binds in PSII with similar affinity as water to one or both substrate binding sites at the Mn_4O_xCa cluster. The possibility is discussed that the two substrate water molecules bind at different stages of the cycle, one during the $S_4 \rightarrow S_0$ and the other during the $S_2 \rightarrow S_3$ transition.

Keywords Photosystem II · Manganese cluster · Water-splitting · Oxygen evolution · Methanol

Introduction

In all oxygen-evolving photosynthetic organisms (cyanobacteria, green algae, and higher plants) water-splitting takes place at the μ -oxo bridged tetra-manganese calcium cluster (Mn_4O_xCa , with $4 \leq x \leq 8$) of photosystem II (PSII). This process is energetically driven by strongly oxidizing equivalents that are generated one at a time following light absorption in the chlorophyll-containing reaction center of PSII (Renger and Holzwarth 2005). In this way the Mn_4O_xCa cluster is oxidized stepwise and cycles through five oxidation states that are referred to as S_i states (Kok et al. 1970). The index ($i = 0, \dots, 4$) signifies the number of stored oxidizing equivalents. After the accumulation of four oxidizing equivalents the highly reactive S_4 state is reached, which decays under the release of molecular oxygen and rebinding of new substrate water into the S_0 state. After dark-adaptation the predominant state is the S_1 state, since S_0 , S_2 , and S_3 are meta-stable and react to S_1 via different pathways (for details see, Hillier and Messinger 2005; Messinger and Renger 2008). This kinetic scheme (Kok cycle) explains the periodicity of four that is observed when dark-adapted thylakoids are illuminated with a train of saturating single turn-over flashes (Joliot et al. 1969).

The S_i state turnovers are coupled to miss (α) and double hit (β) probabilities. Therefore the period-four oscillation and damps out within a couple of cycles. The double hit parameter (probability for $S_i \rightarrow S_{i+2}$ transitions) mostly originates from double turnovers on the acceptor side and its magnitude is dependent on the half-width of the employed flashes. The miss parameter (probability that no S_i state advancement occurs) maybe affected by all redox-equilibria and kinetics within PSII and is therefore a very sensitive probe for changes within PSII. The magnitude of

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α strongly depends on pH and temperature (Messinger and Renger 1994; Messinger et al. 1993), and is also affected by H/D isotope exchange (Christen et al. 1999).

Due to recent progress important details are known about the structure of the $\text{Mn}_4\text{O}_x\text{Ca}$ cluster and its ligands in the dark-stable S_1 state (Ferreira et al. 2004; Loll et al. 2005; Yano et al. 2006) and some information is also available on the number of substrate water molecules and their binding modes in the various S_i states (Chu et al. 2000; Evans et al. 2004; Hansson et al. 1986; Hillier and Wydrzynski 2000; Kimura et al. 2005a, b; Messinger et al. 1995; Noguchi et al. 1995; Noguchi and Sugiura 2000, 2002; Radmer and Ollinger 1986; Su et al. 2008; Turconi et al. 1997). However, due to the limited resolution and to radiation damage during the collection of the X-ray diffraction pattern (Yano et al. 2005), the substrate binding sites and the S_i state dependent protonation states of the substrate ‘water’ molecules are still a matter of speculation—a fact that currently diminishes the chances of deriving the correct mechanism of photosynthetic water-splitting seriously.

Water is not only the substrate for oxygen evolution, but it is also likely involved in functionally important H-bonding networks that are required for the structural and functional integrity of PSII (Kaminskaya et al. 2003; Noguchi and Sugiura 2002). Therefore, it is not possible to gain direct information on substrate binding by varying the water concentration. Accordingly, isotope labeling, partly in connection with time-resolved methods, has been employed to gather information on water-binding sites and modes at the $\text{Mn}_4\text{O}_x\text{Ca}$ cluster (for summary see, Hillier and Messinger 2005). An alternative approach is to utilize substrate analogs. Prominent examples for molecules structurally related to water are hydrogen sulfide, methanol, ammonia, hydrogen peroxide, hydroxylamine, and hydrazine (Scheme 1).

Water and methanol differ structurally by the replacement of one H-atom by a CH_3 -group (Scheme 1). While this exchange affects (i) molecular properties like polarity and H-bonding capacity, and (ii) bulk properties, such as the boiling point, it leaves one end of the molecule structurally identical and increases the overall size of the molecule only to a relatively small extent. These comparatively conservative differences between methanol and water are reflected by the fact that methanol and water are completely mixable at all molar ratios. Because of these properties methanol can be considered to be one of the closest analogs of water.

In previous EPR-based studies it was noticed that most EPR signals of the $\text{Mn}_4\text{O}_x\text{Ca}$ cluster are affected by the addition of 1–5% methanol. For example, the S_3 state parallel mode EPR signal (Matsukawa et al. 1999) and the $g = 4.1$ perpendicular mode S_2 state EPR signal (for

Scheme 1 Molecules structurally related to water. Top left to bottom right: hydrogensulfate (H_2S), methanol (CH_3OH), ammonia (NH_3), water (H_2O), hydrogenperoxide (H_2O_2), hydroxylamine (NH_2OH), and hydrazine (NH_2NH_2)

review see, Haddy 2007) cannot be observed in presence of methanol or ethanol, while in PSII membrane fragments from higher plants the S_0 multiline signal can only be resolved after methanol addition (Messinger et al. 1997a). An ESEEM study employing ^2H -labeled methanol clearly showed that methanol (and ethanol) binds at—or at least very close to—the $\text{Mn}_4\text{O}_x\text{Ca}$ cluster (Force et al. 1998). Consistent with this result it was recently demonstrated by molecular mechanics simulations that an access channel exists that is big enough in diameter to allow methanol (and of course the smaller water) to diffuse from the lumen to the catalytic $\text{Mn}_4\text{O}_x\text{Ca}$ cluster (Ho and Styring 2008; see, also Murray and Barber 2007).

In case of ammonia the oxygen of water is exchanged against the isoelectronic NH -group (Scheme 1). Several EPR and FTIR studies suggest that ammonia has one or two binding sites at the $\text{Mn}_4\text{O}_x\text{Ca}$ cluster (for review see, Debus 1992; Hillier and Messinger 2005). At high enough concentrations ammonia inhibits O_2 evolution and alters the S_2 state EPR multiline signal (Haddy 2007). The possible competition between ammonia and methanol for binding sites at the water-splitting $\text{Mn}_4\text{O}_x\text{Ca}$ cluster of photosystem II has been studied by two groups. Based on FTIR measurements Chu and co-workers concluded that methanol is unable to replace ammonia from its binding site at the $\text{Mn}_4\text{O}_x\text{Ca}$ cluster (Fang et al. 2005), whereas for the reverse

exchange experiment Evans et al. found employing electron-spin echo envelope modulation (ESEEM)-spectroscopy for detection that ammonia can displace methanol from its binding site (Evans et al. 2005). These data indicate that ammonia binds more strongly to the $\text{Mn}_4\text{O}_x\text{Ca}$ cluster than methanol, and that these two water analogs have at least one common binding site.

The question remains whether one or more of these sites are substrate water binding sites. If that were the case one would expect that increasing methanol concentrations should affect the efficiency of oxygen-evolution and possibly also oxidation products of these water-analogs maybe created due to side reactions. The effect of methanol on the oxygen-evolving activity of PSII has so far only been probed by measurements with Clark-type oxygen electrodes. In such experiments suspensions of PSII samples are illuminated for about 1 min with strong white light in the presence of artificial electron acceptors. These kinds of measurements have been performed by several groups and consistently did not reveal any inhibitory effects of methanol at concentrations of up to 5–10% (v/v) (Bernat et al. 2002; Deak et al. 1999; Force et al. 1998). This finding raises the question as to whether or not methanol really acts as substrate analog in PSII. However, one other study (Frasch et al. 1988) reports that methanol can be oxidized by the $\text{Mn}_4\text{O}_x\text{Ca}$ cluster in the presence of H_2O_2 —a result consistent with methanol binding at a substrate water-binding site.

A third group of molecules structurally related to water is formed by hydrogen peroxide, hydroxylamine, and hydrazine (Scheme 1, lower row). These molecules are, however, not isoelectronic to water. Instead, they are redox-active and known to reduce stepwise the $\text{Mn}_4\text{O}_x\text{Ca}$ cluster (see, for example Frascch and Mei 1987; Mano et al. 1987; Messinger et al. 1991). While it is still possible that these molecules react via binding to a substrate water-binding site at the $\text{Mn}_4\text{O}_x\text{Ca}$ cluster, hydrogen peroxide, hydrazine, and hydroxylamine do not appear to bind for long periods of time at the $\text{Mn}_4\text{O}_x\text{Ca}$ cluster. Hydrogen sulfide has been reported to react in a similar fashion with PSII as hydroxylamine (Sivaraja et al. 1988).

In this study we employ flash-induced oxygen-evolution measurements to probe in detail the effects of small (0–10%, v/v) concentrations of methanol on the efficiency of photosynthetic water-splitting.

Materials and methods

Sample preparation

Spinach thylakoids were isolated from leaves of market spinach as described previously (Winget et al. 1965) with

slight modifications outlined in Messinger and Renger (1993). After the final isolation step, thylakoids were frozen as small aliquots in liquid nitrogen and then stored at -80°C until used. These samples were manipulated as described below to populate the reduced or/and oxidized form of tyrosine D (Y_D and $\text{Y}_\text{D}^{\text{ox}}$, respectively). Before the measurements of flash-induced oxygen-evolution patterns (FIOPs) were performed, the thylakoids were thawed in the dark on ice and diluted to $[\text{Chl}] = 1 \text{ mg/ml}$ with medium A (0.4 M sucrose, 15 mM NaCl, 5 mM MgCl_2 , 5 mM CaCl_2 , and 50 mM MES/NaOH at pH 6.0). Methanol was added to give final concentrations of 0, 3, 5, 7, and 10% (v/v) about 5 min before the samples were applied onto the Joliot-type electrode (see below).

PSII membrane fragments of ‘BBY’ type were prepared from fresh thylakoid membranes according to the method of Berthold et al. (1981) with minor modifications (Ono and Inoue 1983).

Preparation of ‘ $\text{S}_1\text{Y}_\text{D}$ ’- and ‘ $\text{S}_1\text{Y}_\text{D}^{\text{ox}}$ ’-thylakoids

- (i) $\text{S}_1\text{Y}_\text{D}$ thylakoids contain a high percentage ($\geq 80\%$) of the reduced form of tyrosine D (Y_D). These preparations were obtained by long-term (several months) dark-storage at -80°C (Messinger and Renger 1990, 1993; Vass et al. 1990b).
- (ii) $\text{S}_1\text{Y}_\text{D}^{\text{ox}}$ thylakoids with Y_D oxidized in about 90% of the centers were obtained by excitation of $\text{S}_1\text{Y}_\text{D}$ samples (pH 6.0; 20°C) with one saturating flash and subsequent 5 min dark-incubation (Messinger and Renger 1990).

Measurements of flash-induced O_2 -evolution patterns (FIOPs)

FIOPs were obtained in the absence of exogenous electron acceptors with an unmodulated home-built Joliot-type (bare platinum) electrode (Joliot 1972; Messinger 1993) at 20°C ($\pm 0.3^\circ\text{C}$). Sample aliquots of 10 μl were transferred to the electrode in very dim green light. Prior to starting the measurements thylakoids were kept for 3 min on the electrode at given temperature to ensure complete sedimentation and temperature equilibration. The pH of the flow buffer, which also contained the indicated methanol concentrations, was adjusted to the value of 6.0. The polarization voltage of -750 mV was switched on 40 s before flash excitation. Saturating flashes at a frequency of 2 Hz were provided through a polished plexi-glass rod by a xenon flash lamp (Perkin Elmer, LS-1130-4). The amplified amperometric signals were recorded with a personal computer at a sampling rate of 3 ms/point.

S₂/S₃ lifetime measurements

The S₂ and S₃ state lifetimes were measured with the Joliot-type electrode. Dark-adapted S₁Y_D-thylakoids were excited with one (S₂ state formation) or two (S₃ state formation) pre-flash(es). After various dark times, t_d (0.5 to 120 s) the O₂-yields induced by flash train were recorded and deconvoluted into S_{*i*} state populations (Isgandarova et al. 2003; Shevela et al. 2006, 2007).

Analysis of FIOPs

The first 16 flashes of each FIOP were analyzed within the framework of different extended Kok models using Excel spreadsheet programs (Kebekus et al. 1995; Messinger et al. 1991, 1997b). All fit programs are based on the formulas:

$$S_n = KS_{n-1}d \quad (1)$$

and

$$Y_n^{\text{fit}} = (1 - \alpha)[S_3]_{n-1} + \beta[S_2]_{n-1} \quad (2)$$

where, S_{*n-1*} and S_{*n*} are vectors of the S_{*i*} state populations before and after *n*th flash of the train, K is the matrix containing the Kok parameters α (miss probability) and β (double hit probability), *d* is an activity parameter that compensates for changes in the number of active PSII centers during the flash train, Y_{*n*}^{fit} is the oxygen yield due to the *n*th flash, and [S₃]_{*n-1*} and [S₂]_{*n-1*} are the normalized S₃ and S₂ state populations immediately before the *n*th flash, respectively. In the most commonly used fit procedure, misses and double hits are assumed to be S_{*i*} state independent and the S vectors contain only redox states from S₀ to S₃, i.e.

$$[S]_n = \begin{bmatrix} [S_0]_n \\ [S_1]_n \\ [S_2]_n \\ [S_3]_n \end{bmatrix} \text{ and } K = \begin{bmatrix} \alpha & 0 & \beta & \gamma \\ \gamma & \alpha & 0 & \beta \\ \beta & \gamma & \alpha & 0 \\ 0 & \beta & \gamma & \alpha \end{bmatrix} \quad (3)$$

where, γ is the single hit probability ($\gamma = 1 - \alpha - \beta$). The computer program minimizes the expression

$$dy_n^2 = \sum_{n=1}^F \left[Y_n^{\text{exp}} - Y_n^{\text{fit}} \left(\frac{\sum_{n=1}^F Y_n^{\text{exp}}}{\sum_{n=1}^F Y_n^{\text{fit}}} \right) \right]^2 \quad (4)$$

where, Y_{*n*}^{exp} is the relative O₂ yield of the *n*th flash and *F* equals the number of analyzed flashes. The normalization is given by

$$\sum_{i=0}^3 [S_i] = 1 \quad (5)$$

The fit quality is calculated according to Eq. 6:

$$fq = \frac{dy_n^2}{F - P} \quad (6)$$

where, *P* is the number of free parameters used in the fit. In a more advanced approach, the fit program also includes (i) the reduced S₋₁ state and (ii) S_{*i*} state dependent misses (Isgandarova et al. 2003; Messinger et al. 1997b; Shevela et al. 2006). This extended Kok model is summarized in Eq. 7:

$$\begin{bmatrix} [S_{-1}]_n \\ [S_0]_n \\ [S_1]_n \\ [S_2]_n \\ [S_3]_n \end{bmatrix} = \begin{bmatrix} \alpha_{-1} & 0 & 0 & 0 & 0 \\ \gamma_{-1,n} & \alpha_0 & 0 & \beta_n & \gamma_{3,n} \\ \beta_n & \gamma_{0,n} & \alpha_1 & 0 & \beta_n \\ 0 & \beta_n & \gamma_{1,n} & \alpha_2 & 0 \\ 0 & 0 & \beta_n & \gamma_{2,n} & \alpha_3 \end{bmatrix} \times \begin{bmatrix} [S_{-1}]_{n-1} \\ [S_0]_{n-1} \\ [S_1]_{n-1} \\ [S_2]_{n-1} \\ [S_3]_{n-1} \end{bmatrix} \times d \quad (7)$$

where, for example, $\gamma_{1,n} = 1 - \alpha_1 - \beta_n$ is the S_{*i*} state and flash number (*n*) dependent single-hit probability. In case of S_{*i*} state dependent misses, for the sake of simplicity, the α -values for the transitions S₀ → S₁ and S₁ → S₂ were fixed to 0 ($\alpha_1 = \alpha_2 = 0$) since they are assumed to be insignificantly small. The α -values of S₂ → S₃ and S₃ → S₀ were set as free running parameters, but fixed to be equal, i.e., $\alpha_2 = \alpha_3$ (Isgandarova et al. 2003; Shevela et al. 2006). For β_n it is assumed that the double hit of the first flash (β_1) is greater or equal to the value of β in the remaining flashes, which are assumed to then cause identical double hits; i.e., $\beta_1 \geq \beta_2 = \beta_3 \dots$. Accordingly, the value of Y_{*n*}^{fit} was calculated using Eq. 8:

$$Y_n^{\text{fit}} = (1 - \alpha_3)[S_3]_{n-1} + \beta_2[S_2]_{n-1} \quad (8)$$

The S_{*i*} state lifetime data were analyzed by further extending the Kok model by also taking into account the fast reduction of S₂ and S₃ by Y_D that can occur during the 500 ms dark-times between flashes of a flash train (Messinger and Renger 1993; Vass et al. 1990a). The required first order rate constants (k_2^f , k_3^f) and the percentage of Y_D in the samples were determined from lifetime measurements in an iterative process that is described in detail in (Isgandarova et al. 2003).

Rate measurements of photosynthetic O₂ evolution

The oxygen-evolving activity of PSII membrane fragments was measured with a Clark-type oxygen electrode (Hansatech) at 25°C under continuous actinic illumination (250 W halogen lamp) at the saturating intensity through a Schott GG 455 (2 mm) and Schott KG 3 (2 mm) filters, 8-cm water filter, and 2-cm CuSO₄ solution (5%) as a heat

filter. Measurements were done at 20 μg Chl/ml in 1-ml chamber filled with buffer A containing the designated MeOH concentrations, and 200 μM PPBQ and 500 μM $\text{K}_3[\text{Fe}(\text{CN})_6]$ as artificial electron acceptors. The electrode was calibrated using air-saturated water.

Results

Previous reports showed that methanol concentrations up to 10% (v/v; 2.5 M) do not affect the oxygen-evolution rates (Bernat et al. 2002; Deak et al. 1999; Force et al. 1998), while at 20% (v/v; 5 M) a small, reversible decline was reported (Force et al. 1998). The data in Fig. 1 confirms this finding for PSII membrane (BBY) preparations under our experimental conditions.

The top row of Fig. 2 displays original FIOPs obtained with dark-adapted $\text{S}_1\text{Y}_\text{D}^{\text{ox}}$ thylakoids (spinach) that contain none (A), 3% (B), or 10% (C) methanol. The control sample without methanol (A) exhibits a pronounced period-four oscillation with a maximum at the 3rd, 7th, 11th, and 15th flash. Inspection of this data readily reveals that with increasing methanol concentration this oscillation becomes less pronounced. These raw data also show that the overall oxygen yields (steady state yields) of the thylakoids decrease only slightly at higher methanol concentrations.

To quantify the increased damping caused by the presence of methanol the FIOPs were analyzed by various extended Kok models as described in “Materials and methods.” The obtained fit parameters are listed in Table 1. The fits assuming equal miss probabilities (A1, A2, and A3

in Table 1) are visualized for each condition as solid line in the lower part of Fig. 2 (for comparison the dashed line shows pattern A). Although this fit approach (A1–A3, Table 1) comprises several simplifying assumptions (see “Materials and methods”) it still allows a quite good description of the data (compare solid lines with symbols). While the double hit parameter is hardly affected by methanol (see also Fig. 3b), the miss parameter increases significantly from 9.7% in the control to 19.4% at a methanol concentration of 10%. Figure 3a (closed symbols) includes the results of several intermediate concentrations and shows that the increase in the miss parameter is practically linearly related to the methanol concentration. Importantly, the open symbols in Fig. 3a show that the increased miss probability can be practically fully reversed to normal values by washing the samples in methanol free buffer A. The enhanced miss probability is therefore not due to for example, an irreversible extraction of pigments or denaturation.

A significant improvement of the fit quality can be achieved if the initial S_1 state population is not fixed to 100% and the states S_0 and S_{-1} are included (fits B1–B3). While in this case the miss parameter is lower, the overall trend remains the same. A similar good fit quality can be reached if the miss parameter is assumed to be zero for the $\text{S}_0 \rightarrow \text{S}_1$ and $\text{S}_1 \rightarrow \text{S}_2$ state transitions, and the $\text{S}_2 \rightarrow \text{S}_3$ and $\text{S}_3 \rightarrow (\text{S}_4) \rightarrow \text{S}_0$ transitions are connected with larger miss parameters (fits C1–C3). For simplicity it is assumed that $\alpha_{23} = \alpha_{30}$. Since it is unlikely that the preflashed ($\text{S}_1\text{Y}_\text{D}^{\text{ox}}$) samples contain centers in the S_0 and S_{-1} states, we favor fit approach C over B. For fits C1–C3, the values α_{23} and α_{30} are higher than the average miss α in the other two approaches. This is expected since the overall miss factor of the cycle is distributed over only two instead of four S_i state transitions (if divided by two a very similar value is obtained as for A and B). Also with fits C1–C3 a linear increase of the miss parameter with the methanol concentration is observed (data not shown in Fig. 3).

The above data were obtained with pre-flashed samples, in which most centers are in the state $\text{S}_1\text{Y}_\text{D}^{\text{ox}}$. Therefore, it is unlikely that fast S_2 or S_3 state decay can explain the observed increase in the miss parameter. However, it cannot be fully excluded that methanol affects the dark-stability of the $\text{Y}_\text{D}^{\text{ox}}$ radical, which normally is stable for several hours at the employed conditions. In such a scenario both the amplitude and the rate of fast S_2 or S_3 decay, which are ascribed to the reactions $\text{S}_2\text{Y}_\text{D} \rightarrow \text{S}_1\text{Y}_\text{D}^{\text{ox}}$ and $\text{S}_3\text{Y}_\text{D} \rightarrow \text{S}_2\text{Y}_\text{D}^{\text{ox}}$, might be functions of the methanol concentration. To test these possibilities S_2 and S_3 state lifetime measurements were performed with samples enriched in either Y_D or $\text{Y}_\text{D}^{\text{ox}}$. The results of the $\text{S}_{2/3}\text{Y}_\text{D}$ measurements show that only small changes in the rates of fast S_2/S_3 decay can be discerned (Table 2). The

Fig. 1 Dependence of oxygen-evolving activity on concentrations of methanol in BBY samples as measured by Clark-type electrode after 2-min incubation of the samples in medium A with different concentrations of methanol at pH 6.0 and 25°C under continuous actinic illumination. The assays were performed in the presence of 200 μM PPBQ and 500 μM $\text{K}_3[\text{Fe}(\text{CN})_6]$ as electron acceptors. The rate of oxygen evolution of control samples (0% of methanol) was $440 \pm 30 \mu\text{mol O}_2 (\text{mg Chl})^{-1} \text{h}^{-1}$

Fig. 2 Normalized FIOPs of spinach thylakoids ($S_1Y_D^{ox}$) measured before (a), after an addition of 3% (b), and 10% (c) of methanol on ice at pH 6.0. Methanol was added to the samples about 3–5 min before FIOP measurements. The measurements were performed without removal of MeOH from the samples. Solid lines correspond to fits A of Table 1, while symbols show experimental values of FIOPs obtained. Broken gray lines with open symbols in (b) and (c)

represent FIOPs prior to the addition of MeOH (the same as in a). The inserts show the original, unnormalized FIOPs that were recorded with a flash-frequency of 2 Hz at pH 6.0 and 20°C. No exogenous electron acceptors were added. Normalization of the FIOPs was performed by dividing each flash-induced O_2 -yield by the O_2 -yield induced by 3rd flash in ‘control’ sample (a)

Table 1 Fits of the FIOPs of preflashed $S_1Y_D^{ox}$ spinach thylakoids in the presence of 0% (FIOP A), 3% (FIOP B), and 10% (FIOP C) of methanol (% v/v) (revealed in Fig. 1)^a

Sample	Fit parameters (%)								$f_q \times 10^6$
	Fit	α	α_{23}	α_{30}	β	S_1	S_0	S_{-1}	
0% MeOH (FIOP A)	A ₁	9.7	–	–	2.4	100*	–	–	22.6
	B ₁	7.9	–	–	1.9	86.6	8.0	5.4	6.5
	C ₁	–	16.4	16.4	2.0	100*	–	–	8.0
3% MeOH (FIOP B)	A ₂	11.3	–	–	2.3	100*	–	–	20.4
	B ₂	9.4	–	–	1.8	85.6	8.8	5.3	5.6
	C ₂	–	19.0	19.0	1.8	100*	–	–	5.2
10% MeOH (FIOP C)	A ₃	19.4	–	–	2.5	100*	–	–	25.7
	B ₃	16.2	–	–	1.5	81.0	9.4	9.6	9.4
	C ₃	–	30.6	30.6	1.4	100*	–	–	7.5

^a The FIOPs were obtained at 20°C and pH 6.0 (Fig. 1) and fit using different approaches. For fits A and B an extended Kok model (for details, see, “Materials and methods”) with S_i state-independent miss and double-hit parameters was used. In fits C₁–C₃ the miss parameters of the $S_2 \rightarrow S_3$ and $S_3 \rightarrow S_0$ transitions (α_{23} and α_{30} , respectively) were used as free parameters (forced to be equal), while those for the $S_0 \rightarrow S_1$ and $S_1 \rightarrow S_2$ transitions (α_{01} and α_{12} , respectively) were fixed to 0. Dashes indicate that parameters were excluded from the fit, while stars mark values that were fixed during the optimization. The quality of a fit is represented by the f_q parameter. Smaller f_q values indicate a better fit. The first 16 flash-induced oxygen yields of each FIOP were analyzed

experiments performed with the pre-flashed (Y_D^{ox}) samples confirmed that the rates of fast and slow S_2 decay are virtually unchanged in presence of 3% methanol (data not shown in Table 2). Interestingly, they also revealed a small increase of the amplitude of the fast phase from 20% (control) to 36% (3% methanol). Calculations with our fit program show (data not presented) that inclusion of the fast decay into the fitting of the FIOPs only insignificantly reduces the above described differences in the miss parameters of control and methanol containing samples.

This is also confirmed basically by the full reversibility of the described methanol effect by washing the samples with methanol-free buffer (see Fig. 3)—a treatment that is unable to re-oxidize Y_D .

Discussion

In this report we describe a surprisingly strong linear relationship between the methanol concentration in the

Fig. 3 Dependence of the miss (top, **a**) and double hit (bottom, **b**) probabilities on the presence of different methanol concentrations (% v/v) in $S_1Y_D^{ox}$ (spinach) thylakoids (closed symbols). Parameters obtained after removal of methanol (by washing the samples twice in methanol free buffer A) from the samples are represented by open symbols. The measurements were recorded at 20°C and pH 6.0. The S_i state independent misses (α) and double hits (β) were calculated from FIOPs using the extended Kok model described in “Material and methods.” Symbols and error bars represent the average of 2 or 3 independent measurements. All other conditions are as described in Fig. 1

Table 2 S_2 and S_3 state decay in S_1Y_D -thylakoids in the absence and the presence of 3% of methanol^a

		No MeOH			3% MeOH		
		k (s ⁻¹)	$t_{1/2}$ (s)	A (%)	k (s ⁻¹)	$t_{1/2}$ (s)	A (%)
Fast phase	S_2	0.05	14.1	96	0.08	8.6	89
	S_3	0.07	10.0	94	0.09	7.4	89
Slow phase	S_2	0.007	107	4	0.007	105	11
	S_3	0.006	120	6	0.008	92	11

^a Rate constants (k), half-times ($t_{1/2}$), and amplitudes (A, % of total decay) for the S_2 and S_3 state reduction by Y_D (fast phase) and the acceptor side (slow phase) in spinach thylakoids in medium A. The FIOPs measurements were performed at 20°C and pH 6.0 either in the absence or presence of 3% methanol. The fit error for the values is ~10%

sample and the miss parameter of photosynthetic water-splitting. This methanol induced increase of α is fully reversible at concentrations of up to at least 10% methanol.

The magnitude of the α -value depends on equilibria of both the donor and the acceptor sides of PSII (Renger and Hanssum 1988; Shinkarev and Wraight 1993; Shinkarev 1996, 2005). The almost complete invariance of the double hit parameter β to methanol addition (see Fig. 3b) reveals that acceptor-side effects are negligible. Therefore, the increase of α is ascribed to donor-side effects. It seems most plausible to assume that methanol acts as an effective competitor of water for its binding site that suppresses S_i -state advancement when bound. The results also show that methanol exchanges among different PSII complexes during the flash sequence. We have previously used the FIOPs technique to study the exchange rates of herbicides at the Q_B -site (Vermaas et al. 1984). The present study reveals an exchange rate of methanol that is fast when compared to the dark-time between the flashes (0.5 s), but slow when compared to the lifetime of Y_Z^{ox} (order of 100 μ s). At 10% (v/v) methanol the molar ratio of methanol to water is about 5% and gives rise to an increase of α from 9.7% in the control to 19.4% (see Fig. 3a). One could expect an increase of α of about 5% provided that functional water and methanol exhibit the same binding affinity to the Mn_4O_xCa cluster and if all clusters containing one bound methanol molecule are temporarily blocked in the S_i -state advancement. In fact, our data show an increase of about 10% thus suggesting that methanol binds somewhat stronger than water (factor of about 2). The proposal of direct binding of methanol to Mn is consistent with two recent ESEEM studies employing isotopically labeled (CD_3-OH) methanol (Åhring et al. 2006; Force et al. 1998).

While the above interpretation is very tempting, further studies are required to probe these ideas. At this stage of the investigation other specific or unspecific interactions of methanol with PSII cannot be fully ruled out.

An increase of α by about 10% is expected to give rise to a decrease of the average oxygen yield per single turnover flash of a similar extent (Messinger and Renger 1994; Christen et al. 1999). Indeed a small decrease of oxygen yield is observed in the FIOPs displayed in Fig. 2. A similar effect cannot be seen up to 10% (v/v; or 2.5 mM) when measuring the oxygen rate under excitation with saturation continuing with white light (Fig. 1, Force et al. 1998), as in this case the activity of PSII is limited by the turnover at the acceptor side. Even if present, an effect of 10% would be within the error of Clark-type measurements. Therefore, the results of Figs. 1 and 2 are not in contradiction with theoretical expectations.

Recently the first ^{17}O -HYSCORE detection of bound (substrate) water was reported (Su et al. 2008). The results obtained resolved only one type of bound water and the possibility was discussed that the presence of 3% methanol may prevent the observation of the second substrate

binding site. On the basis of the data from the present study this scenario can be ruled out, since only about 6% of the centers would have been undetectable due to methanol binding to a substrate site. Therefore, the more likely option is that there exists only one type of ^{17}O -exchangeable substrate water-binding site in the S_1 and S_2 states that can be detected under the conditions of the ^{17}O -HYSCORE technique. On the other hand, results of time-resolved mass spectrometric $\text{H}_2^{16}\text{O}/\text{H}_2^{18}\text{O}$ -substrate water exchange experiments suggest that both substrate water molecules are already bound in the S_2 state (Hendry and Wydrzynski 2002). However, the reported rate for the fast exchange in S_2 is close to the limit of the time resolution of the experiment in which structurally perturbed samples (two extrinsic proteins were removed) have been employed. Therefore, at present the possibility cannot be fully excluded that the two substrate water molecules bind in the Kok cycle at different redox levels S_i of the water oxidizing complex (WOC): the first one during the $S_4 \rightarrow S_0$ transition and the second one may bind during the $S_2 \rightarrow S_3$ transition that is known to be coupled with significant structural changes (Boussac and Rutherford 1988; Boussac et al. 1990; Dau et al. 2001; Guiles et al. 1990; Haumann et al. 2005; Liang et al. 2000; Messinger et al. 1991; Noguchi and Sugiura 2002; Pushkar et al. 2008; Renger and Hanssum 1992; Siegbahn 2008). To decide between both possibilities further advanced EPR, FTIR/Raman, and time-resolved membrane-inlet mass spectrometry measurements will be required.

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