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Interactions of photosystem II with bicarbonate, formate and acetate

Dmitriy Shevela · Vyacheslav Klimov · Johannes Messinger

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Abstract In this study, we probe the effects of bicarbonate (hydrogencarbonate), BC, removal from photosystem II in spinach thylakoids by measuring flash-induced oxygen evolution patterns (FIOPs) with a Joliot-type electrode. For this we compared three commonly employed methods: (1) washing in BC-free medium, (2) formate addition, and (3) acetate addition. Washing of the samples with buffers depleted of BC and CO_2 by bubbling with argon (Method 1) under our conditions leads to an increase in the double hit parameter of the first flash (β_1), while the miss parameter and the overall activity remain unchanged. In contrast, addition of 40–50 mM formate or acetate results in a significant increase in the miss parameter and to an ~50% (formate) and ~10% (acetate) inhibition of the overall oxygen evolution activity, but not to an increased β_1 parameter. All described effects could be reversed by washing with formate/acetate free buffer and/or addition of 2–10 mM bicarbonate. The redox potential of the water-oxidizing complex (WOC) in samples treated by Method 1 is compared to samples containing 2 mM bicarbonate in two ways: (1) The lifetimes of the S_0, S_2, and S_3 states were measured, and no differences were found between the two sample types. (2) The S_1, S_0, S_{-1}, and S_{-2} states were probed by incubation with small concentrations of NH_2OH. These experiments displayed a subtle, yet highly reproducible difference in the apparent S_i/S_{-i} state distribution which is shown to arise from the interaction of BC with PSII in the already reduced states of the WOC. These data are discussed in detail by also taking into account the CO_2 concentrations present in the buffers after argon bubbling and during the measurements. These values were measured by membrane-inlet mass spectrometry (MIMS).

Keywords Flash-induced oxygen evolution pattern (FIOPs) · Membrane-inlet mass spectrometry (MIMS) · S states · Water splitting · Oxygen evolution · Bicarbonate · Hydrogencarbonate · Acetate · Formate

Abbreviations

α	Miss parameter
β	Double hit parameter
β_1	Double hit parameter of the first flash
BC	Bicarbonate = hydrogencarbonate
BC(–)	Bicarbonate depleted
BC(+)	Bicarbonate containing
FIOP	Flash-induced oxygen evolution pattern
MES	2-(N-morpholino) Ethanesulfonic acid
MIMS	Membrane-inlet mass spectrometry
PSII	Photosystem II
S_i states	Oxidation states of the WOC, where i is the number of stored oxidizing equivalents
WOC	Water oxidizing complex
Y_2	Oxygen yield induced by the second flash
Y_D	Redox-active tyrosine 160 of the D2 polypeptide of PSII
Y_Z	Redox-active tyrosine 161 of the D1 polypeptide of PSII

Introduction

Oxygenic photosynthesis in cyanobacteria, algae, and higher plants created the aerobic atmosphere on earth.

D. Shevela · J. Messinger (✉)
Max-Planck-Institut für Bioanorganische Chemie, Stiftstrasse 34-36, 45470 Mülheim an der Ruhr, Germany
e-mail: messinger@mpi-muelheim.mpg.de

V. Klimov
Institute of Basic Biological Problems, Russian Academy of Science, 142290 Pushchino, Moscow Region, Russia

These organisms use electrons from water to reduce CO_2 to carbohydrates, liberating molecular oxygen (O_2) as a side product. Water oxidation is catalyzed by the water-oxidizing complex (WOC) within photosystem II (PSII) and is energetically driven by light-induced charge separations within the reaction center of PSII (Renger and Holzwarth 2005). The heart of the WOC is formed by a metal-oxygen cluster that comprises four manganese ions, one calcium ion, and at least five bridging oxygen's. The structure of this Mn_4O_xCa cluster was recently characterized by polarized EXAFS spectroscopy on PSII single crystals (Yano et al. 2006). In addition, Cl^- and HCO_3^- have been suggested to be of functional relevance for water splitting (recently reviewed in (Wydrzynski and Satoh 2005)).

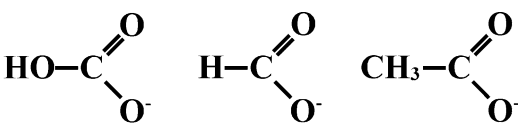
Excitations of dark-adapted PSII complexes by short ('single turn-over') light flashes result in flash-induced oxygen evolution patterns (FIOPs) with a periodicity of four (Joliot et al. 1969). Kok and coworkers concluded that the WOC cycles during water oxidation through five different redox states, named S_i states (i = 0–4), where i is the number of oxidizing equivalents stored within the WOC (Kok et al. 1970). In well dark-adapted samples practically all PSII centers are in the S_1 state. The S_0 state is slowly (tenth of minutes) oxidized to the S_1 state by the oxidized form of tyrosine D, Y_D^{ox}, of polypeptide D2 (Messinger and Renger 1993; Styring and Rutherford 1987; Vermaas et al. 1984). The S_2 and S_3 states are meta-stable and are reduced within seconds to minutes into the S_1 state (Isgandarova et al. 2003; Messinger and Renger 1994; Messinger et al. 1993; Shevela et al. 2006a; Vass et al. 1990a; Vass and Styring 1991) by the reduced tyrosine Y_D (fast phase) and/or by slower donors that include the reduced acceptor side quinone $Q_B^{-/2-}$ (slow phase) (Diner 1977; Nugent et al. 1987; Rutherford et al. 1982; Rutherford and Inoue 1984; Vermaas et al. 1984; Vermaas et al. 1988). The S_4 state is thought to spontaneously decay under the release of O_2 into the S_0 state (for review see (Hillier and Messinger 2005)).

The water oxidation cycle does not work 'perfectly,' and the damping of FIOPs is accounted for by 'miss' (α) and 'double-hit' (β) probabilities that are connected with each flash-induced reaction sequence (Forbush et al. 1971; Kok et al. 1970). For simplicity it is often assumed that these parameters are S_i state and flash number independent. However, the value of α is expected to depend on the redox equilibria of the donor and acceptor sides of PSII (de Wijn and van Gorkom 2002; Renger and Hanssum 1988; Shinkarev and Wraight 1993; Shinkarev 1996). In case of the β probability, it was shown that it depends (i) on the rate of the Q_A^- reoxidation (Messinger et al. 1993) and (ii) on the flash profile (Hillier and Messinger 2005; Jursinic 1981).

The Mn oxidation states of the S_1 state are Mn_4(III,III,IV,IV) (Iuzzolino et al. 1998; Kulik et al. 2005; Messinger et al. 2001b; Yachandra et al. 1993). In agreement with these relatively high-oxidation states of the manganese ions it was shown that the WOC can also be poised in states more reduced than the S_0 state (for review see (Debus 1992; Hillier and Messinger 2005). Such treatments usually involve the incubation of PSII samples with small, hydrophilic reductants such as NH_2OH, NH_2NH_2 or $NO^{\cdot}$ (Bouges 1971; Ioannidis et al. 1998; Messinger et al. 1991; Sarrou et al. 2003). The most reduced S_{-i} state that is stable is the S_{-3} state (Messinger et al. 1997). Indications for the existence of short-lived S_{-4} and the S_{-5} states were presented (Messinger et al. 2001a; Messinger et al. 1997).

There are many experimental results that are consistent with the notion that bicarbonate (BC) is required by PSII for maximal activity; however, interpretation of this effect has a history full of controversies (for recent reviews see (Stemler 2002; van Rensen and Klimov 2005)). The problems are caused (i) by several different protocols for BC-depletion and (ii) by the absence (most of the studies) of monitoring of the residual inorganic carbon levels (CO_2, HCO_3^-(BC) and H_2CO_3). Typically three different methods were employed for BC-depletion: (1) Dilution of samples into buffers that were depleted of BC by bubbling with CO_2-free air, N_2 or argon (Klimov et al. 1995a; Klimov et al. 1995b). This method (Method 1) can be combined with brief boiling of the buffer (Govindjee et al. 1997). (2) Incubation of the samples at pH 5.0 which is well below the pK_a = 6.3 of BC (Good et al. 1966), induces protonation of BC and subsequent dissociation of H_2CO_3 into CO_2 and water, which facilitates BC removal (Method 2). (3) Addition of chemical analogs of BC, such as formate (Stemler and Radmer 1975) or acetate (Stemler et al. 1974) (see Scheme 1) is thought to replace BC from its binding site(s) in PSII (Method 3). In this study we carefully monitor the inorganic carbon levels in the buffers during sample incubation and under measurement conditions by membrane-inlet mass spectrometry (MIMS).

The first report on BC-effects on the electron flow in chloroplasts (Hill reaction) dates back to the year 1958 (Warburg and Krippahl 1958). In the early 1970s the stimulating effects of BC was thought to arise from interactions of BC with the WOC (Stemler et al. 1974; Stemler and Govindjee 1973) and even different models including BC as substrate for photosynthetic water oxidation were



Bicarbonate (BC) **Formate** **Acetate**

Scheme 1

suggested (Metzner 1978; Warburg 1964). The latter suggestions were disproved by mass spectrometric experiments employing ^{18}O-labled HCO_3^- or $H_2^{18}O$ (Clausen et al. 2005; Hillier et al. 2006; Radmer and Ollinger 1980). In 1975 it was shown that BC-depletion affects the electron transfer kinetics on the acceptor side of PSII (Wydrzynski and Govindjee 1975). This interpretation became the dominating view (for review see (van Rensen 2002; van Rensen and Klimov 2005; van Rensen et al. 1999), and it is also supported by two recent PSII crystal structures that display BC as a ligand of the non-heme Fe between the two acceptor side quinones Q_A and Q_B (Ferreira et al. 2004; Loll et al. 2005).

Starting from 1995 the hypothesis for an additional role of BC at the donor side of PSII was revived by experiments showing that BC is required for both maximal activity and stability of the WOC (Allakhverdiev et al. 1997; Klimov et al. 1995a; Klimov et al. 1995b; Klimov et al. 1997). The stimulating effects of BC ions are especially pronounced during photoactivation (Baranov et al. 2000; Baranov et al. 2004). Electrochemical and EPR characterizations of BC complexes with Mn^{II} and Mn^{III} ions show that these ions form electro neutral complexes, and that the dissociation constant (K_d) of the Mn^{III}-BC complex is nearly 10 orders lower than the K_d of the Mn^{II}–BC complex (Kozlov et al. 2004). It has been suggested that these properties of Mn^{II}-BC complexes may facilitate the photo-induced assembly of the inorganic core of the WOC (Dismukes et al. 2001; Kozlov et al. 2004). These results are consistent with several proposals (Klimov and Baranov 2001; van Rensen and Klimov 2005): (1) BC is bound to or is a structural part of the assembled Mn_4O_xCa cluster, (2) BC remains bound in the vicinity of Mn_4O_xCa cluster or (3) BC is required during photoactivation and then leaves the site.

If BC is a direct ligand to the Mn_4O_xCa cluster it can be assumed that its removal will change the redox-potential of the WOC or may affect the accessibility of the WOC to exogenous reductants like NH_2OH and NH_2NH_2. In this study we probe these ideas by a thorough analysis of FIOPs obtained at 2 mM BC and under BC-depleted conditions by studying (i) the life times of the S_0, S_2, and S_3 states and (ii) the reduction rates of the WOC by hydroxylamine.

Materials and methods

Sample preparation

Thylakoid membranes were isolated from market spinach as described previously (Messinger and Renger 1993; Winget et al. 1965). After isolation the thylakoids were frozen in small aliquots in liquid N_2 and then stored at –70°C until used. After prolonged storage (several months) thylakoids are enriched in the reduced form of Y_D (Messinger and Renger 1990; Vass et al. 1990b). Such samples will be referred to as 'S_1Y_D'-thylakoids. Before the measurements, the thylakoids were thawed in the dark on ice and diluted to [Chl] = 1 mg/mL with MCMM buffer (400 mM mannitol, 20 mM $CaCl_2$, 10 mM $MgCl_2$, and 50–100 mM MES/NaOH at pH 6.5). '$S_1Y_D^{ox}$'-thylakoids were obtained from S_1Y_D-thylakoids by excitation with one saturating flash and subsequent 15 min dark-incubation on ice as described earlier (Messinger and Renger 1990).

Bicarbonate/CO_2-depletion

BC depletion from thylakoids was carried out as described in (Klimov et al. 1995a; Klimov et al. 1995b) with some modifications. CO_2/HCO_3^- was removed from the MCMM medium by flushing with argon (BC(–) medium) for 40–60 min. Thylakoids were depleted of CO_2/HCO_3^- by 50–80-fold dilutions with the BC(–) medium and subsequent dark-incubations on ice for 2–10 h under argon atmosphere. Thereafter the thylakoids were collected by centrifugation and washed at least twice in the BC(–) medium ('BC(–) thylakoids').

Treatment of thylakoids with sodium formate

Formate treatment of the $S_1Y_D^{ox}$-thylakoids was done according to the method described in (Stemler and Radmer 1975) with some modifications. Samples were treated in the MCMM buffer containing 50 mM $NaHCO_2$ at 20°C and pH 5.0 for 15 min. Then the treated samples were diluted 50-fold in MCMM BC(–) medium (pH 6.4) containing 50 mM $NaHCO_2$. Subsequently the thylakoids were collected by centrifugation, washed once in the same medium and finally resuspended to 1 mg Chl/ml. In reversibility experiments 10 mM $NaHCO_3$ was added to the samples ~1 min prior to the FIOP measurements, or the samples were washed in formate-free BC(–) buffer.

Treatment of thylakoids with sodium acetate

Acetate treatment of $S_1Y_D^{ox}$-thylakoids was performed according to the method described in (Stemler et al. 1974) in MCMM buffer containing 40 mM CH_3COONa (30 min at 20°C and pH 5.0 or pH 6.5). In cases, where acetate is removed prior to FIOP measurements, the treated samples were washed twice in a 50-fold excess of MCMM BC(–) medium (pH 6.5). Subsequently the samples were resuspended in BC(–) medium to 1 mg Chl/ml. In order to test the reversibility of the treatments 10 mM BC was added to the acetate-treated samples about ~1 min before the start of the FIOP measurements.

Treatment of thylakoids with NH_2OH

NH_2OH-treatment of the samples was done according to the method described in (Messinger et al. 1991, 1997). $S_1Y_D^{ox}$-BC(–) thylakoids were prepared as described above to give a final concentration of 2 mg Chl/ml. The reaction was then started by the addition of NH_2OH solutions in BC(–) MCMM buffer or MCMM buffer containing 2 mM BC (BC(+) medium). The NH_2OH solutions were prepared and adjusted to pH 6.5 shortly before the addition to PSII. The NH_2OH incubation was performed in the dark on ice. After the indicated incubation times 10 µl aliquots were taken in very dim green light and rapidly transferred to the bare platinum cathode of the Joliot-type-electrode. In some cases NH_2OH was removed from the samples prior to taking the FIOPs. This was done by washing the NH_2OH treated sample in a 50-fold excess of MCMM medium (either BC(–) or BC(+)).

FIOP measurements

The FIOPs were measured with a home-built Joliot-type bare platinum electrode (Joliot 1972; Messinger 1993), that keeps the temperature of the electrode constant within ±0.3°C. The measurements were performed at an electrode temperature of 20°C. The samples were kept on the Pt-electrode for about 1 min prior to starting the measurements. The polarization voltage (–0.75 V) was switched on 30 s before excitation with a flash train (2 Hz) of short (~5 µs half-width) saturating Xenon flashes (EG&G, model PS 302, light pack FY-604). The amplified amperometric signals were recorded with a personal computer. No exogenous electron acceptors were added. For BC-depleted samples only freshly prepared BC(–) buffer (MCMM, pH 6.5) was used as flow buffer, while for control measurements BC(+) MCMH medium was used that contained 2 mM $NaHCO_3$. Since, it was impractical to operate the Joliot electrode inside a glove box, we flushed for BC(–) measurements the airspace above the flow buffer in the reservoir constantly with argon. Tubing between the reservoir and the electrode was kept to a minimum. Direct exposure of the sample to air was only possible during the ~40 s transfer time, required for the application of the 10 µl aliquots onto the electrode surface and for electrode assembly. For some experiments this transfer was done under an open nitrogen 'tent' to further reduce this possible source of CO_2 contamination. However, the obtained FIOPs were undistinguishable between the two procedures.

S_i lifetime measurements

The S_2 and S_3 lifetimes were measured by illumination of dark-adapted S_1Y_D-thylakoids with one (S_2 formation) or two (S_3 formation) preflash(es) and subsequent recording of the O_2-yields induced by flash trains (20 flashes at 2 Hz) given at various dark-times (from 0.5 to 90 s) after the respective preflash(es). To observe the kinetics of S_0 oxidation to S_1 by Y_D^{ox}, '$S_1Y_D^{ox}$'-thylakoids were excited with three flashes (S_0 formation) and after various dark-incubation times (from 0.5 to 60 min) FIOPs were recorded. The polarization voltage was switched on 30 s before the flash train.

FIOPs analysis

The first 16 flashes of each FIOP were analyzed using a spreadsheet program that is based on an extended Kok model which was previously described (Isgandarova et al. 2003; Shevela et al. 2006a). In addition to the normal Kok parameters, this program also includes (i) a high-double-hit probability in the 1st flash (β_1), (ii) S_{-1}, S_{-2} and S_{-3} states that can be found after reduction with exogenous electron donors (Messinger et al. 1997), and (iii) S_i state-dependent miss parameters. This is summarized in Eq. 1:

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

where, $\gamma_{i,n} = 1-\alpha_i-\beta_n$ is the S_i state and flash number dependent single-hit probability, n is the flash number, and d an activity parameter that compensates for changes in the number of active PSII centers during the flash train (Messinger et al. 1997). In cases where the possibility of a high-double hit in the first flash was analyzed β_n equals β_1 for the first flash ($n = 1$) and β_n equals β for $n > 1$ (in addition we applied the restriction $\beta_1 \geq \beta$). The theoretical O_2-yield of the nth flash (Y_n^{fit}) and the fit quality (fq) were calculated as described previously (Messinger et al. 1991).

For measuring the fast phases of S_2 and S_3 decay samples with a high content of reduced tyrosine D, Y_D, were used. The high-reduction level of about 80% was reached by storage of the thylakoids at –80°C for several months (Messinger and Renger 1990; Vass et al. 1990b). The S_i state life time data were analyzed by taking into account the fast reduction of S_2 and S_3 by Y_D that can occur during the 500 ms dark-times between flashes of a flash train (Isgandarova et al. 2003). Biphasic decay is assumed for

the least square analysis of the S_2 and S_3 state populations. In contrast, the dark-oxidation of S_0 to S_1 by Y_D^{ox} was modeled by a mono exponential decay (Isgandarova et al. 2003).

Membrane-inlet mass spectrometry (MIMS) measurements

The MIMS measurements were performed with an isotope ratio mass spectrometer (ThermoFinnigan Delta^{Plus} XP) that was connected via a cooling trap (dry ice ethanol) to a home built membrane-inlet cell similar to that described by Messinger and coworkers (Messinger et al. 1995). The volume of the cell is 150 µl and the sample was separated from the vacuum of the mass spectrometer by a silicon membrane (MEM-213) resting on a porous plastic support. For further details on MIMS see (Konermann et al. 2007).

Results

Effect of bicarbonate on the Kok parameters in $S_1Y_D^{ox}$-thylakoids

In order to analyze the effects of bicarbonate (BC) on the parameters of the Kok cycle, FIOPs of dark-adapted $S_1Y_D^{ox}$-thylakoids were measured after depletion of BC from the PSII sample (Fig. 1a). The BC-depletion of PSII was achieved by repeated washing of the thylakoids with pH 6.5 buffer, which had a reduced BC/CO_2 content due to extensive bubbling with argon ('BC(–) buffer'). Figure 1b displays a FIOP of thylakoids that were treated as above, but to which subsequently 2 mM $NaHCO_3$ was added. For both samples typical period four oscillations with maxima of O_2 evolution after the 3rd, 7th, and 11th flashes are observed. This indicates that the overall miss and double-hit parameters are unaffected by our BC depletion, BC(–), procedure. A close inspection of the data shows, however, that the O_2-yield induced by the 2nd flash, Y_2, is rather large in BC(–) samples (Fig. 1a). Y_2 is reversed to normal control levels by addition of 2 mM BC (Fig. 1b). Since both samples were preflashed once prior to the measurement in order to oxidize Y_D, at least two mechanism may be responsible for the high O_2-yield induced by the second flash in BC-depleted samples: (i) the S_2 decay is significantly slower in BC(–) samples or (ii) in BC(–) samples the 1st flash is coupled with a high-double-hit probability, β_1. High β_1 values are known, for example, from ferricyanide treated PSII samples (Jursinic 1981) and high-double hits on every second flash were reported for measurements in the presence of phenyl-para-benzoquinone (PPBQ) (Zimmermann and Rutherford 1986). In both cases the non-heme iron (Fe^{2+}) on the acceptor side of PSII is eventually

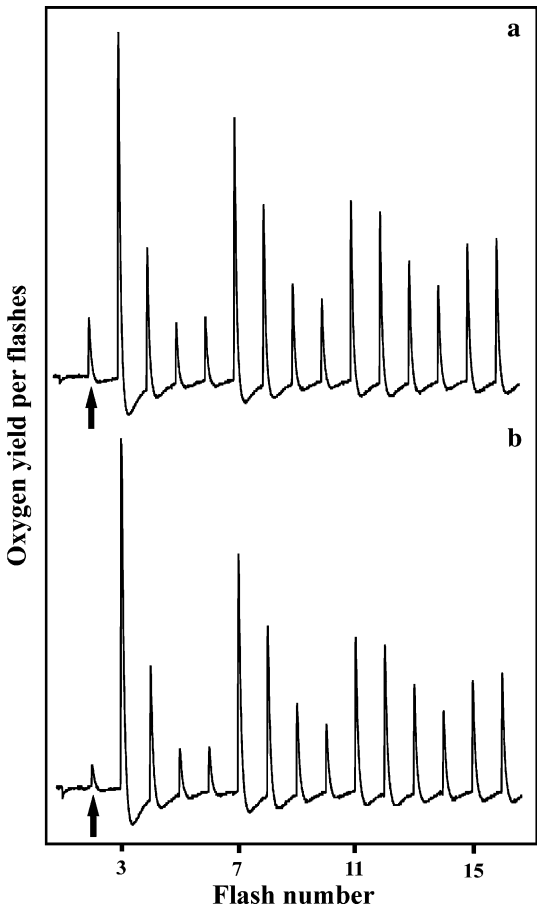


Fig. 1 Original, unnormalized flash-induced oxygen evolution patterns (FIOPs) of dark-adapted spinach thylakoids ($S_1Y_D^{ox}$) that were incubated and washed in CO_2/HCO_3^- depleted buffer (FIOP **a**, BC(–) sample). FIOP **b** (BC(+) sample) was obtained after readdition of 2 mM $NaHCO_3$ to the BC(–) sample. FIOPs were recorded with a flash frequency of 2 Hz at pH 6.5 and 20°C. No exogenous electron acceptors were added

oxidized by the artificial acceptor to Fe^{3+}, which then allows a fast oxidation of Q_A^- within the duration of following xenon flash and thus opens PSII for a second turnover within the same flash. Since, the difference in the 2nd flash O_2 yields persist even after extended dark-times (several hours) between pre-flash and recording of the FIOPs, explanation (i) appears rather unlikely (see also S_2 lifetime measurements below). We, therefore, strongly favor option (ii). It is important to note, however, that our samples do not contain any exogenous electron acceptors.

These qualitative observations are confirmed by a detailed analysis of these FIOPs (see Table 1) employing the extended Kok model described in the experimental section. The fits in Table 1 show that inclusion of the β_1 parameter (fit B) leads to a significant improvement of the fit quality for the FIOP obtained with the BC(–) sample; in contrast, the inclusion of this extra parameter does not lead to an improvement of the fit of the FIOP recorded after

Table 1 Fits of the flash-induced oxygen evolution patterns (FIOPs) of preflashed ($S_1Y_D^{ox}$) spinach thylakoids after bicarbonate depletion and readdition of 2 mM $NaHCO_3$ (displayed in Fig. 1)[a]

Samples	Fit parameters (%)					*fq*
	Fit	α	β	β_1	S_1	
FIOP a	A_1	12.4	4.1	–	(100)	0.000063
	B_1	12.1	2.4	10.5	(100)	0.000046
FIOP b	A_2	12.2	2.6	–	(100)	0.000034
	B_2	12.2	2.3	3.4	(100)	0.000036

[a] The FIOPs were obtained at 20°C and pH 6.5 (Fig. 1a, b). For both fit approaches (A, B) an extended Kok model with S_i state independent miss (α) and double-hit (β) parameters was used. β_1 is the double-hit probability of the first flash. The quality of a fit is represented by the *fq* parameter. Smaller *fq* values indicate a better fit. Dashes denote parameters that were excluded from the fit, while numbers in parentheses give values, which were fixed during optimization. The first 16 flash-induced O_2-yields of each oscillation pattern were analyzed

readdition of bicarbonate (BC(+) sample). We also tested several combinations of S_i state-dependent miss parameters. This did not lead to any new insights, and therefore, only the equal miss approach is shown in Table 1.

Our data differ from previous reports (Jursinic and Stemler 1984; Stemler et al. 1974; Stemler and Lavergne 1997). In these earlier publications effects of BC depletion on the miss parameter, but not on β_1 were observed (unless ferricyanide was added (Jursinic and Stemler 1984)). A detailed comparison of the BC depletion and measuring conditions shows that the most significant difference between the conditions appear to be the presence of either 100 mM formate (Stemler and Lavergne 1997) or 40 mM acetate (Stemler et al. 1974) during depletion at pH 5.0 and also during the FIOP measurements at pH 6.8. We, therefore, added 50 mM formate (Fig. 2) or 40 mM acetate (Fig. 3) to our buffer and pretreated the samples at pH 5.0 as described in the previous publications (Stemler et al. 1974; Stemler and Radmer 1975). Figures 2a and 3a show that under these conditions, indeed high-miss parameters are observed that could be reduced to almost normal values by addition of 10 mM $NaHCO_3$ (Figs. 2b, 3b, and Table 2). Interestingly, in contrast to our BC depletion procedure neither the addition of formate nor that of acetate leads to an increased β_1 parameter (Figs. 2a, 3a, and Table 2). While the increase in the miss parameter is similar for formate and acetate (Table 2), formate leads under our conditions to an inhibition of ~50% of the PSII centers, while acetate leads only to a decrease of ~10% (on the basis of the steady state O_2 yields). Fits employing S_i state-dependent misses gave qualitatively similar results and are, therefore, not presented. These results are in agreement with previous observations (Stemler et al. 1974; Stemler and Lavergne 1997). It should be remarked that we

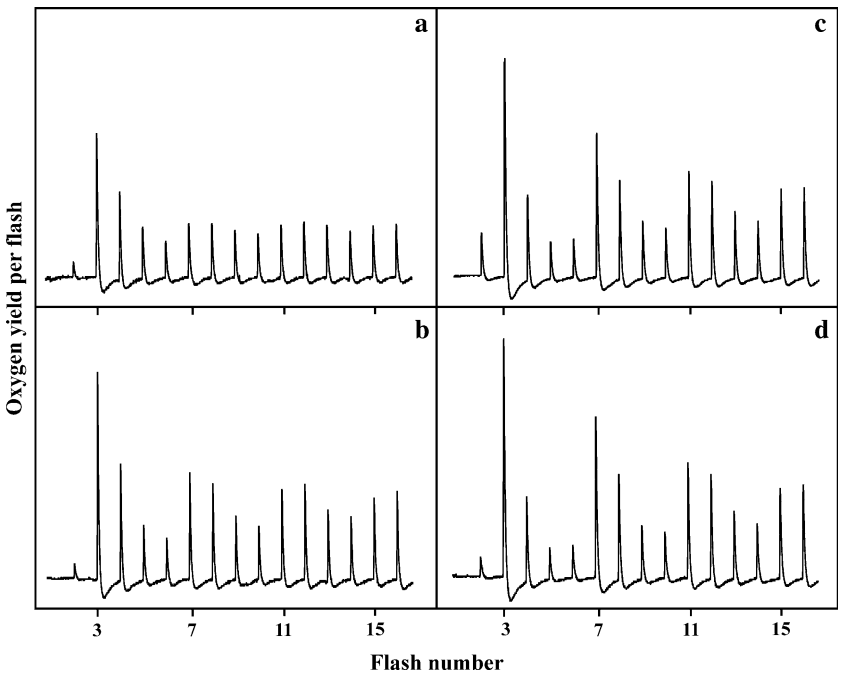


Fig. 2 Original, unnormalized flash-induced oxygen evolution patterns (FIOPs) of dark-adapted spinach thylakoids ($S_1Y_D^{ox}$) that were incubated at pH 5.0 with 50 mM formate (FIOP **a**). FIOP **b** was obtained after addition of 10 mM $NaHCO_3$ to sample **a**. Washing of sample **a** in bicarbonate and formate free buffer yields FIOP **c**. FIOP **d** was recorded after addition of 10 mM bicarbonate to sample **c**. All FIOPs were recorded with a flash-frequency of 2 Hz at pH 6.5 and 20°C. No exogenous electron acceptors were added

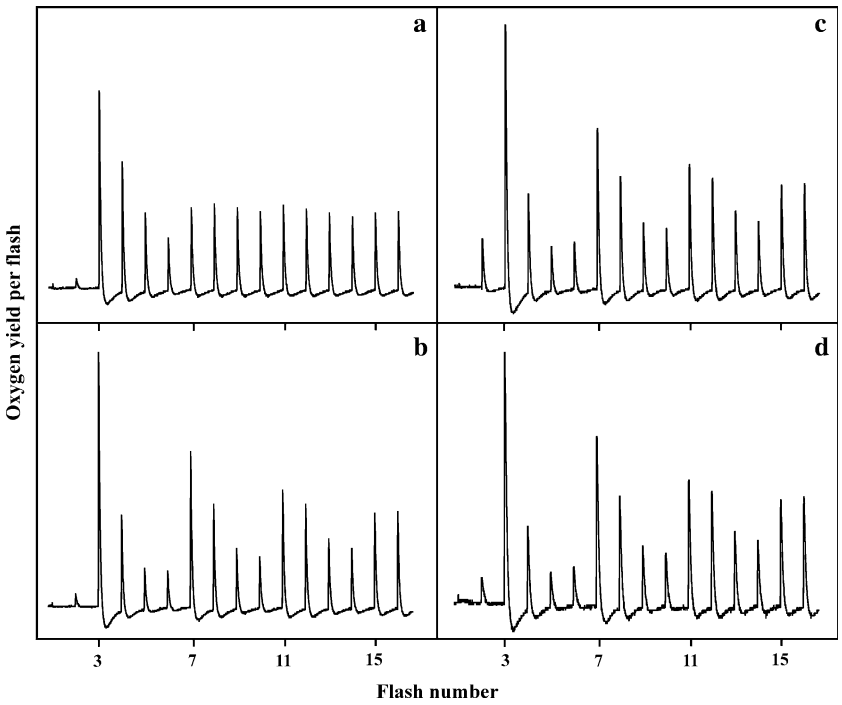


Fig. 3 Original, unnormalized flash-induced oxygen evolution patterns (FIOPs) of dark-adapted spinach thylakoids ($S_1Y_D^{ox}$) that were incubated for 30 min at pH 5.0 with 40 mM acetate (FIOP **a**). FIOP **b** was obtained after addition of 10 mM $NaHCO_3$ to sample **a**. Washing of sample **a** in BC and acetate free buffer yields FIOP **c**. FIOP **d** was recorded after addition of 10 mM bicarbonate to sample **c**. All FIOPs were recorded with a flash-frequency of 2 Hz at pH 6.5 and 20°C. No exogenous electron acceptors were added

Table 2 Fits of the flash-induced oxygen evolution patterns (FIOPs) of $S_1Y_D^{ox}$ thylakoids isolated from spinach after incubation with 50 mM $NaHCO_2$ or 40 mM $NaCH_3CO_2$ at pH 5.0 (see Figs. 2, 3)[a]

	Samples	Fit parameters (%)					*fq*
		Fit	α	β	β_1	S_1	
Formate-treated thylakoids (Fig. 2)	FIOP a	A_1	24.1	6.0	–	(100)	0.000069
		B_1	24.1	4.3	4.3	(100)	0.000087
	FIOP b	A_2	17.6	5.4	–	(100)	0.000054
		B_2	17.2	4.0	4.0	(100)	0.000069
	FIOP c	A_3	12.1	4.0	–	(100)	0.000045
		B_3	11.9	2.8	10.7	(100)	0.000026
	FIOP d	A_4	10.9	2.9	–	(100)	0.000022
		B_4	10.9	2.6	4.3	(100)	0.000023
Acetate-treated thylakoids (Fig. 3)	FIOP a	A_5	26.2	5.6	–	(100)	0.000033
		B_5	26.2	3.3	3.3	(100)	0.000055
	FIOP b	A_6	11.7	3.9	–	(100)	0.000029
		B_6	11.6	3.2	3.2	(100)	0.000034
	FIOP c	A_7	12.4	5.5	–	(100)	0.000057
		B_7	12.0	3.4	9.1	(100)	0.000024
	FIOP d	A_8	11.2	4.1	–	(100)	0.000037
		B_8	10.9	2.9	5.9	(100)	0.000022

[a] The FIOPs were obtained at 20°C in presence of acetate or formate (Patterns a, b), while patterns c, d were obtained after removal of acetate/formate from the samples. Patterns b, d were recorded after addition of 10 mM $NaHCO_3$. The FIOPs were fit using two different approaches (A, B) within an extended Kok model. Parameter: α, S_i state independent miss parameter; β, S_i state independent double-hit parameter and β_1, double-hit probability of the first flash. The quality of a fit is represented by the *fq* parameter. Smaller *fq* values indicate a better fit. Dashes denote parameters that were excluded from the fit, while numbers in brackets give values that were fixed during optimization

obtained an only slightly smaller increase of the miss parameter if the pH 5.0 treatment was omitted and formate or acetate were added directly to the sample at pH 6.5 (data not shown).

The above experiments demonstrate that the information gathered about the function of bicarbonate in PSII is critically dependent on the choice of the BC depletion procedure. To investigate this further, we washed formate or

acetate treated thylakoids with BC(–) buffer (pH 6.5). This procedure removes these additives from the samples, while simultaneously minimizing the rebinding of BC to PSII. The FIOPs obtained under these conditions are shown in Figs. 2c and 3c, respectively. Interestingly, these FIOPs are almost identical to that in Fig. 1a, i.e., they show normal miss parameters and an increased oxygen yield in the second flash. Remarkably, also the formate induced block of PSII centers is fully removed during this washing step. This finding is in agreement with two previous studies (Feyziev et al. 2000; Wiessner et al. 1992). The high β_1 value (Figs. 2c, 3c) can be reversed by addition of $NaHCO_3$ (Figs. 2d, 3d).

S_0, S_2 and S_3 lifetime measurements

For testing the effect of bicarbonate depletion on the redox potential of the Mn_4O_xCa cluster we explored the effect of bicarbonate depletion on the rates of S_2 and S_3 state reduction by endogenous electron donors. The expectation is that removal of BC from a putative binding site at the Mn_4O_xCa cluster should modify the redox potential of the cluster and thereby, alter the stability of the higher S_i states. The S_2 and S_3 lifetimes were determined in the traditional way by giving one (S_2 state) or two (S_3 state) preflashes and varying the dark-time to the FIOP measurements. The obtained FIOPs were then deconvoluted into S_i state populations taking the back reactions of Y_D with S_2 and S_3 into account (for details see (Isgandarova et al. 2003)). The obtained S_2 and S_3 populations are plotted for BC(+) (closed symbols) and BC(–) thylakoids (open symbols) as a function of dark-time in Fig. 4. Lines represent for both sample types biexponential fits. The two phases in the reduction of S_2 and S_3 originate from electron donation by Y_D (fast kinetics) and by the acceptor side of PSII (slow kinetics).

It is clear from Fig. 4 that our BC depletion (method 1) and measuring conditions (see below) do not affect the stability of the S_2 and S_3 states. Within an estimated fit error of about 10% the derived kinetics for the fast and slow decays of these S_i states are found to be independent of the presence or absence of BC (Table 3). Despite the above-discussed significant differences in sample

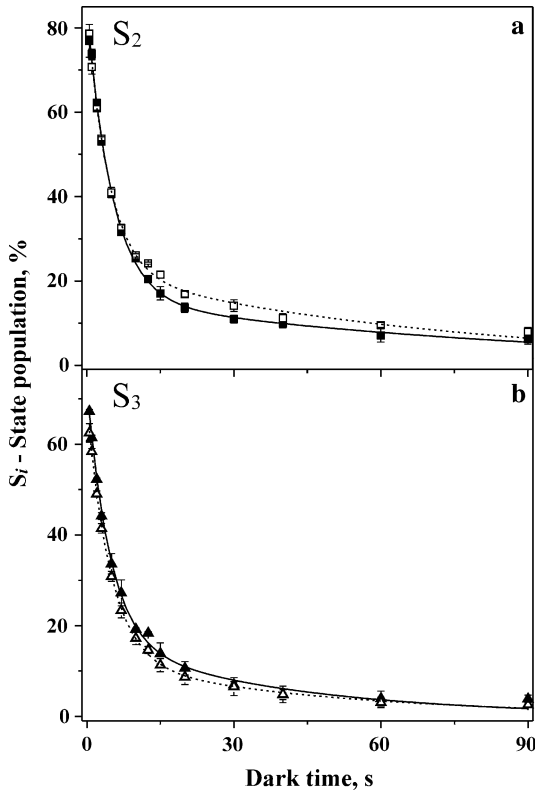


Fig. 4 Relative S_2 (top, **a**) and S_3 (bottom, **b**) state populations of spinach thylakoids (S_1Y_D) as a function of dark-time between one or two preflash(es), respectively, and the main train of saturating single turnover flashes. Closed symbols: thylakoids were incubated over night in a buffer depleted of CO_2/HCO_3^- by bubbling with argon. Open symbols: treated as above but after addition of 2 mM $NaHCO_3$. Symbols and error bars represent the average of 2 or 3 independent measurements. The lines represent biexponential fits (Table 3). The lifetime measurements were performed at 20°C and pH 6.5

Table 3 S_i state decay in BC(+) and BC(–) thylakoids[a]

		CO_2/HCO_3^--depleted medium			In presence of 2 mM $NaHCO_3$		
		k, s^{-1}	$t_{1/2}$, s	A, %	k, s^{-1}	$t_{1/2}$, s	A, %
Slow phase	S_2	0.014	51	26	0.011	59	19
	S_3	0.022	32	18	0.026	27	23
Fast phase	S_2	0.22	3.1	74	0.19	3.6	81
	S_3	0.21	3.3	82	0.21	3.3	77
		k, min^{-1}	$t_{1/2}$, s		k, min^{-1}	$t_{1/2}$, s	
	S_0	0.060	690		0.053	780	

[a] Rate constants (k), half-times ($t_{1/2}$) and amplitudes (A, % of total decay) for S_0 state oxidation by Y_D^{ox} and for the S_2 and S_3 state reduction by Y_D (fast phase) and the acceptor side (slow phase) in spinach thylakoids after over-night dark-incubation (on ice) in CO_2/HCO_3^--depleted medium. The measurements were performed at 20°C and pH 6.5 either in the absence or presence of 2 mM $NaHCO_3$. The fit error for the values is ~10%

treatment, this result is in agreement with the earlier publication of (Stemler et al. 1974) where the slow S_3 state decay was shown to be independent of the BC concentration in a medium containing acetate. It may be remarked, however, that the present data give more direct information, because (i) the complication with acetate is avoided and (ii) the possible mixing of donor and acceptor side effects of BC during the S_2 and S_3 state decay is avoided by studying the interaction of Y_D with the Mn_4O_xCa cluster.

These lifetime measurements were completed by measuring the kinetics of S_0 oxidation to S_1 by Y_D^{ox}. In this case, three preflashes are used to excite $S_1Y_D^{ox}$-thylakoids. Then again the dark-time is varied to the recording of the FIOPs. The results obtained for BC(+) (closed symbols) and BC(–) thylakoids (open symbols) are shown in Fig. 5. Within the fit error of 10% practically no differences exist between the two sample types (Table 3). Therefore, BC does not affect the redox potential of the S_0 state under our conditions.

Reduction of the WOC by NH_2OH

The BC was shown to be a cofactor during photoactivation of PSII and to form complexes with Mn^{2+} (Baranov et al. 2004; Dismukes et al. 2001; Kozlov et al. 2004). It is, therefore, possible that BC binds to the Mn_4O_xCa cluster in the chemically reduced S_{-i} states, because most of the S_{-i} states contain at least one Mn^{2+} ion (except S_{-1}, which may contain only Mn^{3+} ions). Similar to the S_i state lifetime measurements above we assume that binding of BC to the Mn_4O_xCa cluster in the S_{-i} states would modify the redox

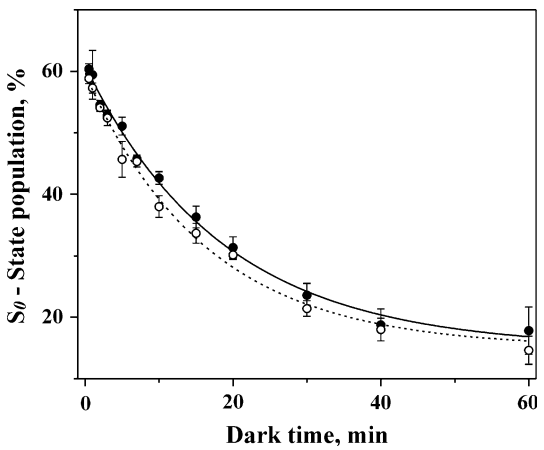


Fig. 5 Relative S_0 state population of spinach thylakoids ($S_1Y_D^{ox}$) as a function of dark-time between three preflashes and a train of saturating single turnover flashes. Closed symbols: thylakoids were incubation over night in BC(–) buffer. Open symbols: treated as above but after addition of 2 mM $NaHCO_3$ shortly before the measurement. Symbols and error bars represent the results of three measurements. The lines show monoexponential fits (see Table 3). The measurements were performed at 20°C and pH 6.5

potential of the reduced states and/or alter their accessibility for NH_2OH, and thereby slow down a possible further reduction.

In a recent report BC was shown to 'retard' the shift to the S_{-i} states during the incubation with small concentrations of the two-electron reductant NH_2NH_2 (Shevela et al. 2006b). In the present study, we employ the one-electron reductant NH_2OH as a probe for the reactivity of the Mn_4O_xCa cluster, because in this way also the S_0 and S_{-2} states can be studied in more detail. In addition, for more precise S_i state deconvolutions, no exogenous acceptors were used ($K_3[Fe(CN)_6]$ was added in the previous study).

Figure 6 presents original (insets) and normalized FIOPs (symbols) that were obtained with spinach thylakoids after 1 min of dark incubation with the indicated concentrations of NH_2OH in BC(–) medium (left) and BC(+) medium (right). The dotted lines show for comparison the respective FIOP of the $S_1Y_D^{ox}$ thylakoids prior to NH_2OH addition. An inspection of the data shows that independent of the presence of BC a progressive shift of the 1st maximum of oxygen evolution toward higher-flash numbers is observed as a function NH_2OH concentration. However, the extent of this shift is at all concentrations smaller in the presence of BC samples (right side) as compared to those obtained with BC(–) thylakoids (left side of Fig. 6). This finding is in agreement with a previous report (Shevela et al. 2006b), where a similar observation was made with NH_2NH_2 as reductant.

Figure 7 displays the calculated normalized S_{-i} state populations as a function of NH_2OH concentration in thylakoids after dark-incubation during 5 min in CO_2/HCO_3^-depleted medium without (a, left side) and after re-addition of bicarbonate (b, right side). Again, an apparent retardation in the reduction of all S_i states is observed in agreement with (Shevela et al. 2006b). It is important to note that the apparent S_i/S_{-i} state distributions after NH_2OH incubation result from a complex sequence of reactions: (i) diffusion of NH_2OH toward the WOC, (ii) reduction of the Mn_4O_xCa cluster, and (iii) the efficiency of light-induced oxidations during the flash train. In addition, back reactions of the formed S_{-i} states with Y_D^{ox} (Messinger and Renger 1993) or other electron acceptors need to be considered.

In order to address the question at which point of the above-described chain of events BC modifies the apparent S_i/S_{-i} state populations, we compare in Fig. 8 FIOPs that were obtained as follows: (1) BC(–)thylakoids were incubated for 10 min with 0.2 mM NH_2OH and then washed and measured in BC(–) medium; (2) BC(+) thylakoids were incubated with NH_2OH and then washed and measured in BC(+) medium, and (3) BC(–) thylakoids were incubated with NH_2OH, but then washed and measured in BC(+) medium. The effect of the washing was 2-fold:

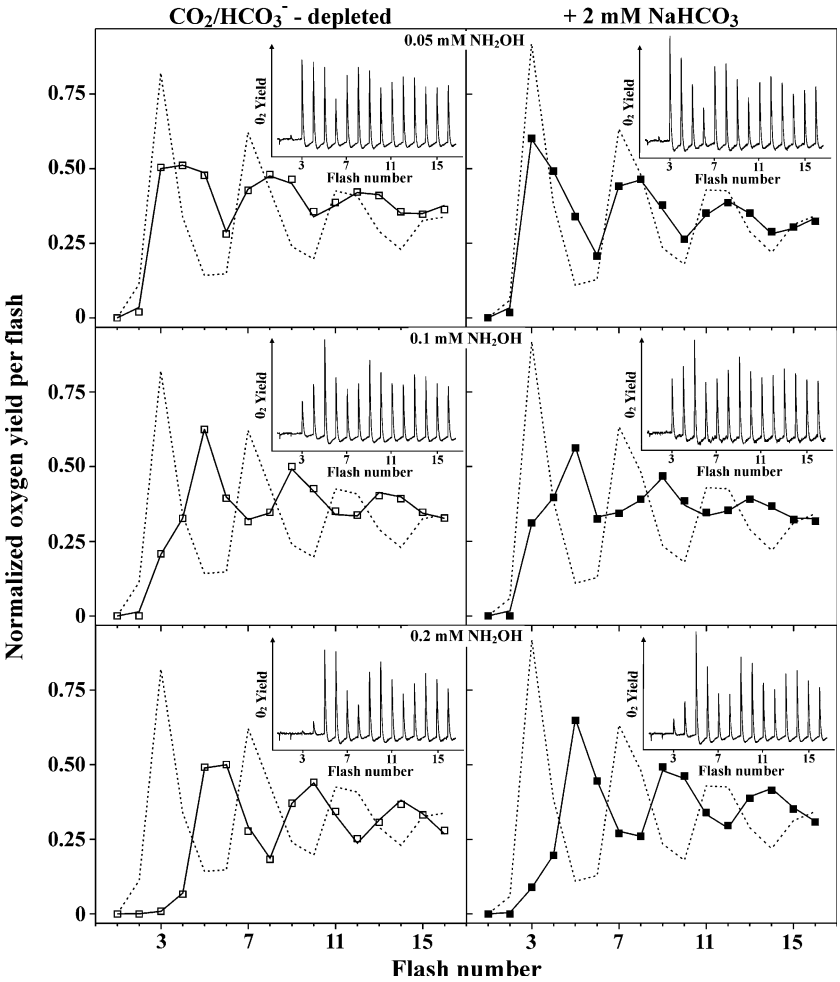


Fig. 6 Normalized flash-induced oxygen yield patterns (FIOPs) of spinach thylakoids ($S_1Y_D^{ox}$) measured after 1 min treatments with 0.05 mM (top), 0.1 mM (middle) and 0.2 mM (bottom) NH_2OH on ice at pH 6.5. BC(–) samples (left side, open symbols) were obtained by washing with CO_2/HCO_3^--depleted buffer medium prior to NH_2OH incubation. For BC(+) samples (right side, closed symbols) 2 mM $NaHCO_3$ was added to BC(–) samples about 1 min before the addition of NH_2OH. FIOP measurements were performed without removal of NH_2OH from the samples. Solid lines show fits with the extended Kok model described in the experimental section. Broken lines represent FIOPs of BC(–) and BC(+) thylakoids, respectively, prior to the addition of NH_2OH. The inserts show the original, unnormalized FIOPs. FIOPs were recorded with a flash-frequency of 2 Hz at pH 6.5 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 average of the O_2-yields induced by flashes 4–7

firstly it allows changing the BC concentration after the NH_2OH incubation and secondly it stops the reduction process by removing the reductant, thus allowing to study the point of action of BC.

The original FIOPs presented in Fig. 8 (left) reveal that a 10-min incubation of $S_1Y_D^{ox}$ thylakoids with 0.2 mM NH_2OH leads to the first maximum of O_2 evolution after the 6th flash, i.e., most centers are shifted into the S_{-2} state. A comparison of patterns 1 and 2 in Fig. 8 and Table 4 reveals that the subtle, yet highly reproducible difference (see below) in the shift between BC(–) and BC(+) samples can be observed again, despite the fact that NH_2OH was removed prior to measuring the FIOPs. FIOP 3 in Fig. 8 was obtained with a sample that was reduced with NH_2OH in BC(–) buffer (like FIOP 1, Fig. 8), but measured after washing with BC(+) medium (like FIOP 2, Fig. 8). It is obvious that pattern 3 more closely resembles FIOP 2 than FIOP 1 in Fig. 8. This indicates that BC does not affect the reaction sequence during NH_2OH reduction, but leads to an apparent shift of the S_i states before or during the measurements. To test, if the differences in apparent S_i/S_{-i} state populations are simply the consequence of the effect of BC on the β_1 parameter, we included this parameter in our fits that are presented in Table 4. Three different scenarios are tested in Table 4: (A) the β_1 parameter is not considered ($\beta_1 = \beta$), (B) β_1 is fixed to 10% as found for BC(–) in Table 1 and (C) β_1 is allowed to vary freely. These fits show that observed differences in S_i/S_{-i} state distribution

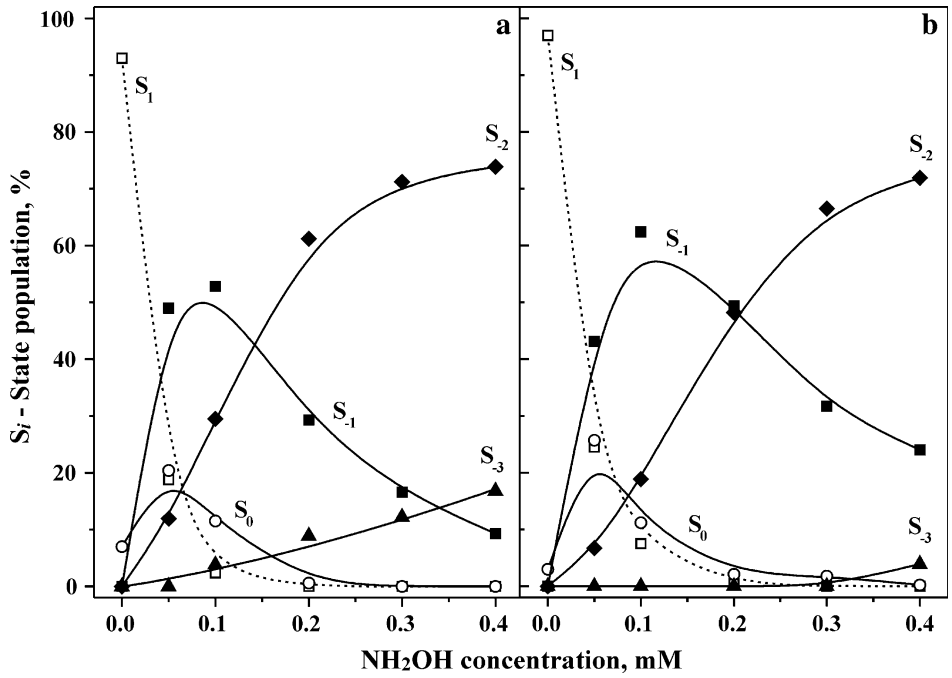


Fig. 7 Normalized S_i state populations of spinach thylakoids ($S_1Y_D^{ox}$) as a function of NH_2OH concentration. The FIOP measurements were carried out with BC(–) (**a**) and BC(+) (**b**) thylakoids. The samples were incubated with NH_2OH for 5 min on ice at pH 6.5. All other conditions are as described in Fig. 6

after NH_2OH incubation of BC(–) and BC(+) thylakoids do not vanish after including the β_1 parameter in the fits. The right side of Fig. 8 displays a summary of three repeats of each experiment. The small error bars show that the experimental error is well below the above-discussed differences.

On the basis of ESEEM measurements it was shown that acetate binds in the vicinity or at Mn_4O_xCa cluster (Clemens et al. 2002). We, therefore, tested if acetate slows down the reduction of the Mn by NH_2OH. Figure 9 shows that no significant slowing of the reduction of the S_1, S_0 and S_{-1} states is observed in presence of 40 mM acetate at pH 6.4. The small differences between the patterns are well explained by the increase in miss parameter caused by acetate addition (compare dashed lines in Fig. 9a, b).

MIMS

In the above experiments BC was depleted from the media by argon bubbling (method 1) and the samples were assayed subsequently on a Joliot-type electrode. These techniques do not allow monitoring the CO_2/BC level during the experiment and, despite extreme care, it cannot be excluded that some CO_2 may have diffused back into the samples during the quick sample transfer and measurements (total time <2 min). Therefore, the question remains what the actual inorganic carbon levels were during the experiments.

The level of BC-depletion from the media can be determined by monitoring the CO_2 level in depleted buffers compared to air-saturated media at given pH. For this 25 µl of these media were injected into the mass spectrometric cell (150 µl volume) that was filled with degassed buffer (pH 6.4). Comparison of trace a (non-depleted buffer) and trace b (argon bubbled buffer) shows that method 1 leads to an about 50-fold reduction of CO_2-levels in the buffer. After a 40 s exposure to air, which simulates the application of the sample onto the Joliot electrode, this level rises to about 5-fold below ambient (Fig. 10).

Discussion

Binding of bicarbonate within the WOC

With the BC-depletion levels reached in this study (~50-times during incubation and ~5-times during measurements) no evidence was found that BC binds to the Mn_4O_xCa cluster. Neither the miss parameter, nor the stabilities of the S_0, (S_1), S_2, and S_3 states during the reaction with endogenous electron donors and acceptors were found to be affected by washing thylakoids in BC(–) buffer obtained by extensive bubbling with argon. In addition, BC did not change the rate of reduction of the WOC by the external reductant NH_2OH. However, a small, yet reproducible shift in the S_i/S_{-i} state distribution occurs that might be related to BC interaction after the reduction occurred or during the light-induced transitions in the flash train. Unfortunately, we are presently unable to provide a conclusive explanation for this latter

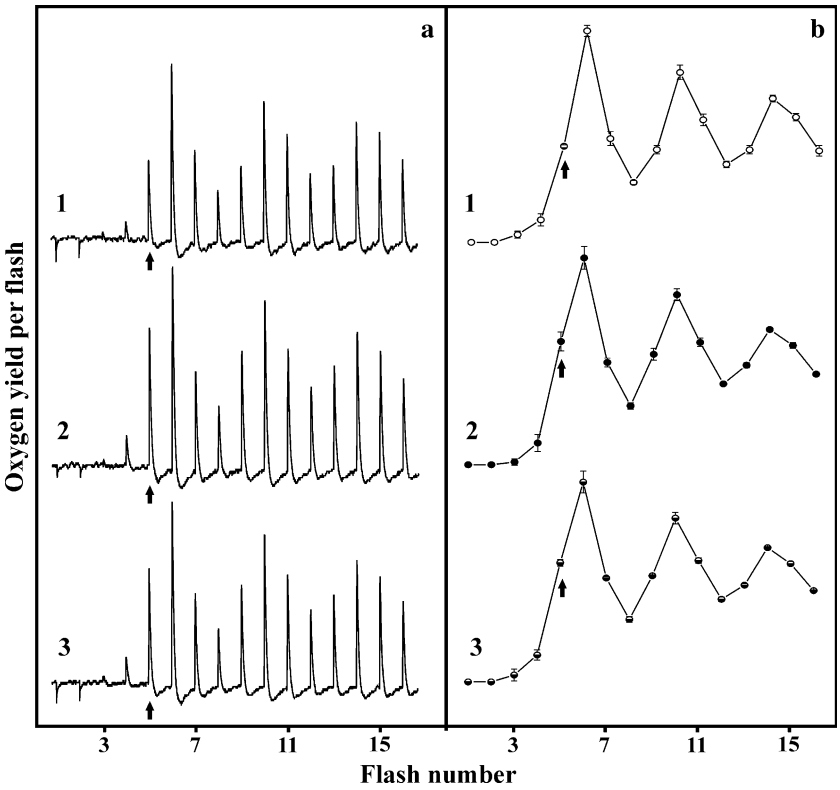


Fig. 8 Original, unnormalized FIOPs (**a**) and the respective normalized O_2 yields per flashes (**b**) of dark-adapted BC(–) spinach thylakoids ($S_1Y_D^{ox}$) obtained after 10-min incubation with 0.2 mM NH_2OH on ice at pH 6.5. In contrast to Fig. 6 all samples were washed after NH_2OH-incubation once in NH_2OH-free medium before the FIOPs were recorded. For FIOP 1 the sample was incubated and measured in the absence of BC, while for FIOP 2 bicarbonate (2 mM) was present throughout the procedure. FIOP 3 was obtained by performing the NH_2OH incubation in absence of BC, while 2 mM BC was added ~1 min before the measurement. Circles and bars (right side, **b**) show the mean values and standard deviations of three measurements. The corresponding fits are given in Table 3. FIOPs were recorded with a flash-frequency of 2 Hz at pH 6.5 and 20°C. No exogenous electron acceptors were added. Normalization of the FIOPs was performed by dividing each flash-induced oxygen yield by the average of the O_2-yields induced by flashes 4–7

Table 4 Fits of the flash-induced oxygen evolution patterns displayed in Fig. 8(a)[a]

Samples	Fit parameters (%)									*fq*
	Fit	S_1	S_0	S_{-1}	S_{-2}	S_{-3}	α	β	β_1	
FIOP 1	A	0.3	3.8	25.1	70.8	0.0	(10.0)	(2.2)	–	0.000004
	B	0.0	2.2	20.0	77.8	0.0	(10.0)	(2.2)	(10.0)	0.000004
	C	0.6	3.4	22.2	70.6	3.2	9.2	2.3	4.1	0.000003
FIOP 2	A_1	0.1	5.4	39.4	55.2	0.0	(10.0)	(2.2)	–	0.000007
	B_1	0.0	2.0	37.7	60.3	0.0	(10.0)	(2.2)	(10.0)	0.000007
	C_1	0.5	4.1	36.0	57.5	1.9	9.1	2.8	2.8	0.000006
FIOP 3	A_2	0.5	5.3	35.2	59.0	0.0	(10.0)	(2.2)	–	0.000005
	B_2	0.2	2.7	32.5	64.6	0.0	(10.0)	(2.2)	(10.0)	0.000005
	C_2	0.7	4.2	31.9	59.6	3.7	9.0	2.7	2.7	0.000003

[a] The extended Kok model described in Materials and methods was used to fit FIOPs of spinach thylakoids. The samples were incubated in the dark for 10-min on ice with 0.2 mM NH_2OH and then washed once in NH_2OH-free medium. For FIOP 1 all steps were performed in CO_2/HCO_3^--depleted medium, while for FIOP 2 bicarbonate (2 mM) was added before NH_2OH incubation and during washing steps. FIOP 3 was obtained from a sample that was treated as described for FIOP 1, but 2 mM BC was added prior to the measurement. Fit parameters: S_1–S_{-3}, normalized S-state populations; α, miss probability; β, double-hit probability and β_1, double-hit probability of the first flash. The quality of the fits is represented by the *fq* parameter. Smaller *fq* values indicate a better fit. Dashes denote parameters that were excluded from the fit, while numbers in parentheses give values, which were fixed during optimization. The first 16 flash induced O_2-yields of each FIOP were analyzed

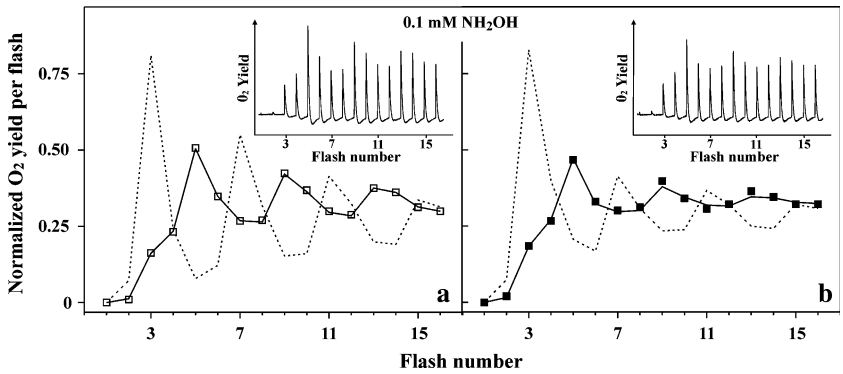


Fig. 9 Normalized flash-induced oxygen yield patterns (FIOPs) after 1-min treatments of spinach thylakoids ($S_1Y_D^{ox}$) with 0.1 mM NH_2OH on ice at pH 6.4. BC(–) samples (**a**, open symbols) were obtained by washing with CO_2/HCO_3^- - depleted buffer medium prior to NH_2OH incubation. Acetate treated samples (**b**, closed symbols) were obtained as described in Fig. 3 by 30-min incubation with 40 mM acetate at pH 6.4. FIOP measurements were performed without removal of NH_2OH from the samples. Broken lines represent FIOPs of BC(–) and acetate thylakoids, respectively, prior to the addition of NH_2OH. The inserts show the original, unnormalized FIOPs at the respective conditions. FIOPs were recorded with a flash-frequency of 2 Hz at pH 6.4 and 20°C. No exogenous electron acceptors were added. Normalization of the FIOPs was performed by dividing each flash-induced oxygen O_2-yield by the average of the O_2-yields induced by flashes 4–7

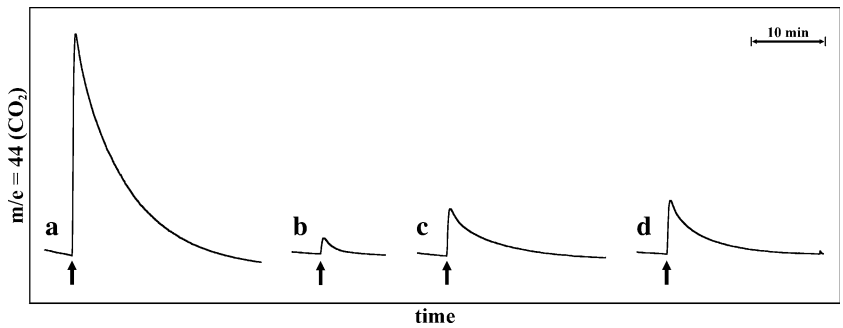


Fig. 10 Membrane-inlet mass spectrometry measurements of the inorganic carbon content (CO_2, HCO_3^-, H_2CO_3) of buffers used in this study. 25 µl aliquots each were injected into 150 µl MMCM medium (pH 6.4) that was thoroughly degassed in the mass spectrometer cell. The time of injection is marked by arrows. Samples: MMCM buffer (pH 6.4) before (**a**) and after (**b**) CO_2-depletion by Ar-bubbling. Curves **c** and **d** show the CO_2 release in CO_2-depleted medium (same as curve b), but after exposure to air on a Joliot type electrode (for 40 s), and in flow buffer tube (for 5 min), respectively

phenomenon. At any case, the effect appears to be too small to allow the conclusion that BC is bound at or near the Mn_4O_xCa cluster.

To further test this question, we examined, whether the BC analog acetate affects the reaction of NH_2OH with the Mn_4O_xCa cluster. The reasoning behind this experiment is that acetate was shown by ESEEM spectroscopy to bind near or possibly even at the Mn_4O_xCa cluster (Clemens et al. 2002). The data of Fig. 9 show that also acetate, at the concentration used in this study (40 mM), does not affect the interaction of NH_2OH with the Mn_4O_xCa cluster, despite the fact that this concentration is high enough to significantly increase the miss parameter. This suggests that the increased miss parameter is a consequence of the interaction of acetate with the acceptor side, and/or that the acetate (BC) and NH_2OH interact at independent sites with the donor side (or WOC).

Although our NH_2OH incubations were performed outside the electrode, i.e., at about ~50-fold decreased BC-levels, they cannot fully exclude very tight BC binding, which might be present if BC were for example a structural component of the WOC.

Bicarbonate binding at the non-heme iron

This study demonstrates for the first time that a clearly increased β_1 parameter is found in BC(–) samples. This effect can be suggested to be coupled to the redox state of the non-heme iron on the acceptor side of PSII. The BC binding to the non-heme iron is suggested by crystallography (Ferreira et al. 2004; Loll et al. 2005) and previous studies (involving mostly formate treatments, see below) have clearly established that the 'BC' effect on the acceptor side involves binding of formate or acetate at the

non-heme iron (for review see (van Rensen 2002; van Rensen and Klimov 2005; van Rensen et al. 1999)). We propose that our BC removal procedure is able to extract the BC molecule bound to the non-heme iron (Fe^{2+}) in a way that it is not replaced by a similar ligand (see below). This appears to alter the redox potential of the non-heme iron so that it can be oxidized to Fe^{3+} by the ambient redox potential. If xenon flashes of a few μs half-width are used for excitation this situation causes a high-double hit only in the first flash, because Fe^{3+} accepts after the first flash very rapidly an electron from Q_A^- and a second turnover can take place in such centers within the same flash. Thereafter, the non-heme iron remains in oxidation state Fe^{2+} for the rest of the flash train and the double hit returns to normal values (Jursinic 1981).

In previous studies, an altered value of β_1 was not found for BC depleted samples (Jursinic and Stemler 1984; Shevela et al. 2006b; Stemler et al. 1974; Stemler and Lavergne 1997). The differences are straight forwardly explained. Shevela and coworkers (Shevela et al. 2006b) used ferricyanide as electron acceptor during the flash experiments that were performed with a highly sensitive-membrane covered (Clark-type) electrode. Therefore, the non-heme iron was oxidized in all samples and no difference was noted between BC(+) and BC(–) thylakoids. In two studies of Stemler BC depletion was achieved by addition of either acetate (Stemler et al. 1974) or formate (Stemler and Lavergne 1997) and in both cases this did not lead to an increase of β_1. These earlier observations are confirmed by the experiments presented in Figs. 2 and 3. We propose that in presence of these carboxylic acids BC is replaced at the non-heme iron by acetate/formate, which prevents its oxidation to Fe^{3+} at ambient redox-potentials. Consistent with this idea it was shown in two previous studies that it is much harder to oxidize the non-heme iron by ferricyanide in presence of formate (Jursinic and Stemler 1984; Radmer and Ollinger 1980). These findings indicate that the redox potential of the non-heme iron dependents on its ligands: it is most easily oxidized to Fe^{3+} in absence of BC, while formate or acetate appear to stabilizes the Fe^{2+} oxidation level as compared to the natural BC ligand.

While the above suggestion gives a coherent explanation for the observed data of this study, we have to remark that we neither directly observed BC binding to PSII (the non-heme iron) nor did we obtain direct information about the redox-state of the non-heme iron. Given the fact that the CO_2/BC levels very quickly rise from ~50-fold depletion to a ~5-fold depletion during sample transfer to the Joliot-electrode, it is also possible that we are unable to observe an increase of the miss parameter similar to that observed with acetate or formate, because of a very rapid (<2 min) re-binding of BC to the non-heme iron during the transfer. This scenario would also be consistent with the above-suggested oxidation of the non-heme iron, because the oxidation of the non-heme iron would in any case occur during the incubation in the sealed vials outside the electrode. We also like to point out that our experiments were performed at a flash frequency of 2 Hz and are therefore unable to detect effects that may be present under rate limiting conditions.

Effects of formate and acetate

Formate and acetate have been added in the past to PSII samples in order to replace BC that may be bound to PSII. This approach is supported by the similar structures of these carboxylic acids as compared to BC (Scheme 1). Consistent with previous studies we find significantly increased miss parameters under these conditions. This raises the question about the mechanistic basis for this effect.

Extensive experimental work by many laboratories has established that there are at least two binding sites for these carboxylic acids within PSII: one at the non-heme iron (Deligiannakis et al. 1994; Diner and Petrouleas 1990; Jajoo et al. 2005; Kühne et al. 1999; Nugent et al. 1992; Wydrzynski and Govindjee 1975; Xiong et al. 1997, 1998) and one between Y_Z and the Mn_4O_xCa cluster (Bock et al. 1988; Clemens et al. 2002; Dorlet et al. 1998, 1999; Feyziev et al. 2000; Force et al. 1997; Govindjee et al. 1997; Jajoo et al. 2005, 2006; Klimov et al. 1995a, b; Kühne et al. 1999; Lakshmi et al. 1999; Lydakis-Simantiris et al. 1998; Maclachlan and Nugent 1993; Mende and Wiessner 1985; Saygin et al. 1986; Szalai and Brudvig 1996a, b; Wincencjusz et al. 1996, 1999). In three further studies also formate binding near Y_D is reported (Hienerwadel et al. 1996, 2005; Kim and Barry 1998). However, it is unclear if formate and acetate indeed replace BC at both binding sites. While on the basis of the cited literature BC replacement appears to be well-established for the binding site at the non-heme iron, binding within the WOC is often reported to occur in competition with Cl^- rather than BC. It was attempted in two previous MIMS studies to quantify the number of bound BC molecules to PSII. While in the first study no evidence was found for CO_2 release after formate injection (Stemler 1989), the subsequent report was able to detect the slow release of one CO_2 per PSII complex after formate injection at pH 6.5 (Govindjee et al. 1991).

Formate (and acetate) binding to the non-heme iron have been reported to slow the Q_A^- to Q_B^- electron transfer, possibly by disrupting the protonation pathway for Q_B^{2-} (Govindjee et al. 1997; van Rensen and Klimov 2005). Binding of acetate (and formate) within the WOC was shown to slow the $Y_Z^\bullet$ reduction kinetics. Especially the $S_2Y_Z^\bullet \rightarrow S_3Y_Z$ transition is slowed significantly at room temperature so that the $S_2Y_Z^\bullet$ state can be traped (Bock et al. 1988; Dorlet et al. 1998, 1999; Feyziev et al. 2000;

Force et al. 1997; Kühne et al. 1999; Lakshmi et al. 1999; Lydakis-Simantiris et al. 1998; Maclachlan and Nugent 1993; Szalai and Brudvig 1996a, b; Wincencjusz et al. 1996, 1999). Both phenomena can give rise to higher-miss parameters and the current study does not allow deciding, which effect is dominating under our conditions. It also remains to be established, whether the high-miss parameters are caused by the absence of BC or the presence of acetate or formate. Our current data appear to favor the latter, but due to the relatively low-depletion levels during the FIOP measurements they are not fully conclusive in this regard.

MIMS measurements

Our MIMS data show that argon bubbling of buffers leads to a significant reduction of the inorganic carbon levels, in our hands ~50-fold. They also show that CO_2 is diffusing back into the depleted solutions very quickly if small aliquots are handled. This may be one reason for the discrepancies in the literature, and monitoring of the CO_2/BC level should be part of any future study.

Conclusion

We show that reducing the BC concentration in the samples 5-fold relative to air saturated buffers does not affect the redox potential of the WOC in PSII as shown by unchanged S_0, S_2, and S_3 life-times. Even at ~50-fold reduced BC level the rate of reduction of the WOC by NH_2OH was unchanged. Therefore, it appears likely that BC, after its probable involvement in the assembly of the Mn_4O_xCa cluster, leaves the WOC. Alternatively BC could remain so tightly bound to the WOC that we were unable to remove it by washing with BC/CO_2-depleted buffer. This question will be addressed in future MIMS studies. The cause for the high miss parameters in presence of acetate of formate remains to be established.

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