Evidence That Bicarbonate Is Not the Substrate in Photosynthetic Oxygen Evolution[1]

Juergen Clausen, Katrin Beckmann, Wolfgang Junge*, and Johannes Messinger

Abteilung Biophysik, Universität Osnabrück, 49069 Osnabrueck, Germany (J.C., W.J.); and Max-Planck-Institut für Bioanorganische Chemie, 45470 Muelheim an der Ruhr, Germany (K.B., J.M.)

It is widely accepted that the oxygen produced by photosystem II of cyanobacteria, algae, and plants is derived from water. Earlier proposals that bicarbonate may serve as substrate or catalytic intermediate are almost forgotten, though not rigorously disproved. These latter proposals imply that CO_2 is an intermediate product of oxygen production in addition to O_2. In this work, we investigated this possible role of exchangeable HCO_3^- in oxygen evolution in two independent ways. (1) We studied a possible product inhibition of the electron transfer into the catalytic Mn_4Ca complex during the oxygen-evolving reaction by greatly increasing the pressure of CO_2. This was monitored by absorption transients in the near UV. We found that a 3,000-fold increase of the CO_2 pressure over ambient conditions did not affect the UV transient, whereas the $S_3 \rightarrow S_4 \rightarrow S_0$ transition was half-inhibited by raising the O_2 pressure only 10-fold over ambient, as previously established. (2) The flash-induced O_2 and CO_2 production by photosystem II was followed simultaneously with membrane inlet mass spectrometry under approximately 15% $H_2{}^{18}O$ enrichment. Light flashes that revealed the known oscillatory O_2 release failed to produce any oscillatory CO_2 signal. Both types of results exclude that exchangeable bicarbonate is the substrate for (and CO_2 an intermediate product of) oxygen evolution by photosynthesis. The possibility that a tightly bound carbonate or bicarbonate is a cofactor of photosynthetic water oxidation has remained.

Photosynthetic oxygen production takes place in PSII. The structural arrangement of PSII and of the catalytic Mn_4Ca complex are emerging on the basis of x-ray crystallography and spectroscopy (Zouni et al., 2001; Robblee et al., 2002; Kamiya and Shen, 2003; Biesiadka et al., 2004; Cinco et al., 2004; Ferreira et al., 2004; Messinger, 2004). The Mn_4Ca complex forms together with its ligands the so-called oxygen-evolving complex (OEC), which is a functional unit within PSII. Closely associated with the OEC is one redox-active Tyr, which is known as Y_Z and acts as intermediate electron carrier between the primary donor P680 and the Mn_4Ca complex. Upon excitation of PSII with a series of short laser flashes, the catalytic center (OEC plus Y_Z) can be cycled through a series of redox states. They are coined S_n, where n indicates the number of stored oxidizing equivalents (n = 0–4). Only after reaching S_4, dioxygen is released and the S_0 state is restored (Kok et al., 1970). All studies on the cycling of the catalytic center through its oxidation states (S_n) take advantage of the fact that one state, namely S_1, is the most stable one after long periods of dark adaptation.

Bicarbonate has been clearly shown to bind at the non-heme iron of the acceptor side of PSII (Ferreira et al., 2004) and to be of functional relevance for electron transfer at this position (for a recent review, see van Rensen, 2002). In addition, there is evidence that bicarbonate also affects oxygen evolution directly. PSII of green algae, for example, is inhibited if bicarbonate is removed from the reaction mixture and substituted for by formate (Mende and Wiessner, 1985). Bicarbonate is also reported (1) to stabilize the OEC (for review, see Klimov and Baranov, 2001), (2) to promote the photoassembly of the Mn_4Ca complex (for review, see Ananyev et al., 2001), and (3) to interfere with the damping of the oscillatory pattern of oxygen evolution (Stemler et al., 1974). Finally, in the currently most complete structural model of PSII at 3.5 Å resolution (Ferreira et al., 2004), (bi)carbonate is presented as a ligand of the Mn_4Ca complex.

It seems generally accepted, however, that water serves as the electron donor for the production of reduced organic compounds by cyanobacteria and plants, and that dioxygen, the side product which is pivotal for aerobic life, is derived from water. Alternative concepts like Otto Warburg's (Warburg and Krippahl, 1958; Warburg et al., 1965), who proposed that "activated CO_2" is converted to an aldehyde during oxygen evolution (for a historical survey, see Stemler, 2002), have been discarded mainly because of mass-spectrometric results that show that in PSII preparations the isotopic composition of liberated dioxygen agrees well with the one of the surrounding water even after relatively short mixing times with $H_2{}^{18}O$ (Radmer and Ollinger, 1980; Bader

[1] This work was supported by the Deutsche Forschungsgemeinschaft (grant nos. Ju97/15–3 and Me1623/2–3), the Fonds der Chemischen Industrie (W.J.), the Max-Planck Society (J.M.), and the Land Niedersachsen (W.J.).

* Corresponding author; e-mail junge@uos.de; fax 49–541–969–2262.

The authors responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) are: Wolfgang Junge (junge@uos.de) and Johannes Messinger (messinger@mpi-muelheim.mpg.de).

Article, publication date, and citation information can be found at www.plantphysiol.org/cgi/doi/10.1104/pp.105.068437.

et al., 1987; Messinger et al., 1995; Hillier et al., 1998; Hillier and Wydrzynski, 2000). Strictly speaking, however, these data are still compatible with a catalytic role of bicarbonate if the equilibrium between water, CO_2, and bicarbonate is rapidly installed, as mediated, for example, by a carbonic anhydrase (CA; Silverman and Tu, 1975; Tu and Silverman, 1975). An intrinsic CA activity is indeed linked to the core and/or extrinsic proteins of PSII (Dai et al., 2001; Stemler, 2002; Villarejo et al., 2002; Khristin et al., 2004). The above-cited mass-spectrometric experiments were not designed to specifically address the bicarbonate question, and therefore the CA activity was not assessed in detail. This leaves room for speculations about a possible catalytic role of bicarbonate during oxygen evolution.

Such a catalytic role for bicarbonate was first proposed by Helmut Metzner on thermodynamic grounds (Metzner, 1978). The main idea was that a one-electron oxidation of bicarbonate (HCO_3^-) is energetically possible for $P680^+$, while a one-electron abstraction from water requires far too high oxidizing potentials. Therefore, he assumed that water is delivered to the OEC in a CO_2-bound form, i.e. as bicarbonate. This scheme requires that, in addition to O_2, CO_2 is a product. The formed CO_2 is proposed to be rapidly rehydrated to bicarbonate and therefore to act catalytically. Using today's knowledge about photosynthetic water oxidation, Metzner's idea can be reformulated in a way that appears consistent with presently published data:

$$2HCO_3^- + 2H^+ \xrightarrow{4h\nu} O_2 + 2CO_2 + 4H^+ + 4e^- \quad (1)$$

$$2CO_2 + 2H_2O \rightleftharpoons 2HCO_3^- + 2H^+ \quad (2)$$

$$2H_2O \xrightarrow{4h\nu} O_2 + 4H^+ + 4e^-, \quad (3)$$

wherein $h\nu$ stands for photon-driven processes. In this sequence reaction, (1) describes a series of four light-driven partial reactions involving two bicarbonate molecules (substrate) that are exchangeably bound to the OEC in the S_0 through S_3 states of the OEC. During the $S_4 \rightarrow S_0$ transition, O_2 and 2 CO_2 are formed and initially bound to the Mn_4Ca complex. In the next step they need to be released into the medium to allow another reaction cycle to occur (heterogeneous catalysis).

On the basis of this hypothetical reaction mechanism, two clear predictions can be made. (1) It can be expected that a high partial pressure of either O_2 or CO_2 suppresses oxygen production (this prediction is explained in more detail in the ''Discussion''). (2) Because the reaction equilibrium of reaction 2 ($K = [CO_2]/[H_2CO_3] = 600$; Cotton and Wilkinson 1974) lies to 99.8% on the side of CO_2, a considerable amount of newly formed CO_2 should, at least transiently, escape together with O_2 into solution and become detectable in time-resolved experiments. In contrast, in the generally accepted view of photosynthetic oxygen evolution, with water as sole substrate, no CO_2 evolution and therefore also no inhibition by a high partial pressure of CO_2 are expected.

Given the described remaining chance for a catalytic role of bicarbonate as immediate substrate for photosynthetic oxygen production, we decided to test the modified Metzner model by two complementary approaches.

The stepped progression of the OEC was monitored by UV spectrophotometry under high backpressure of CO_2. In contrast to results from a previous study, where the oxygen-evolving S_4-to-S_0 transition was half-suppressed by only 10-fold elevated oxygen pressure (Clausen and Junge, 2004), the 3,000-fold increase of the CO_2 pressure did not affect the normal turnover of the OEC.

Measurements by membrane inlet mass spectrometry (MIMS), under $H_2{}^{18}O$ enrichment, and at low bicarbonate/CO_2 concentration revealed the normal oscillation of O_2 release, but they gave no evidence, under parallel detection, for any CO_2 release in response to a series of light flashes.

Both results practically exclude that CO_2 is liberated during oxygen evolution and thereby also show that bicarbonate is not an immediate/catalytic substrate as postulated by Metzner. Bicarbonate may, however, be a firmly bound cofactor.

RESULTS

Flash-Spectrometric Experiments

Figure 1 shows UV-absorption transients (wavelength 360 nm) of dark-adapted PSII-core particles from Synechocystis in response to series of short laser flashes given to dark-adapted samples. These transients are composites: They reflect redox reactions of the OEC and of the intrinsic and exogenous electron acceptors (see Lavergne, 1991; Dekker, 1992; Bögershausen and Junge, 1995; Karge et al., 1997; Clausen et al., 2001). With the catalytic center synchronized mainly in its first oxidation state, S_1, before starting the series of flashes, oxygen evolution occurs only after excitation with three flashes of light in a row, i.e. after reaching S_4. From there on it oscillates with period of four as a function of the flash number. Figure 1A shows an overlay of two UV transients. The trace shown in black represents the typical oscillation under atmospheric oxygen pressure (1 bar air, i.e. 0.21 bar O_2, pH 6.7, 20°C). On the first two flashes ($S_1 \Rightarrow S_2$, $S_2 \Rightarrow S_3$) the very rapid (here not resolved) rise of absorption is followed by a slower relaxation of smaller extent, so that each flash gives rise to a positive step. These steps represent oxidations of the OEC (Lavergne, 1991; Dekker, 1992; Bögershausen and Junge, 1995; Karge et al., 1997; Clausen et al., 2001). After the third flash (inducing the transition $S_3 \Rightarrow S_4 \rightarrow X \rightarrow S_0$), these two positive steps are reverted by a negative transient with a typical half-decay time of 1.3 ms. It reflects the reduction of the Mn_4Ca complex that occurs concomitant with the liberation of dioxygen (see Clausen and Junge, 2004; Clausen et al., 2004) and sets the OEC

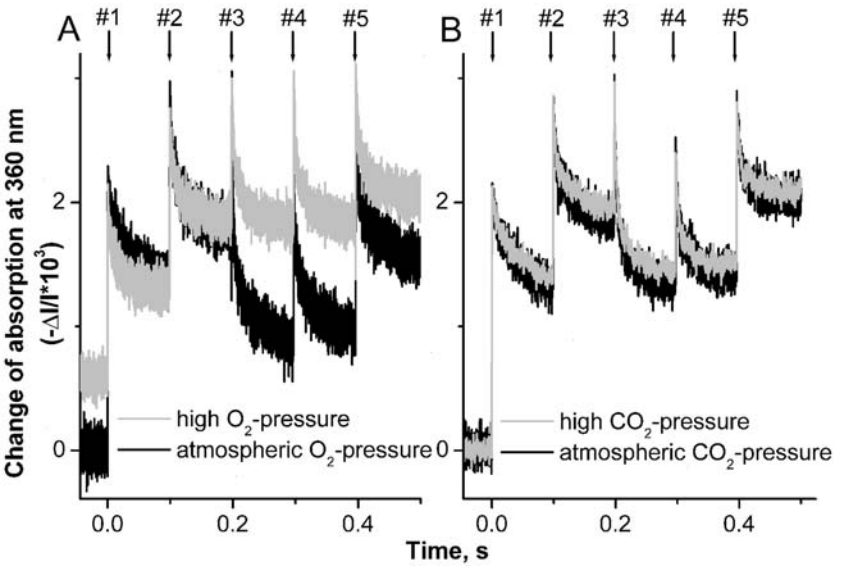

Figure 1. UV absorption transients (360 nm) of dark-adapted PSII-core complexes (normalized to 9 μM Chl, pH 6.6 to 6.7) obtained by the excitation with five short laser flashes (arrows). A, The black trace was obtained after preincubation with 1 bar air [i.e. 0.21 bar p(O_2)], while the gray trace shows the data that were obtained after preincubation with 20 bar pure O_2. The transients were recorded with 50 μs/point and three experiments were averaged. B, The black trace represents again the air control (four transients averaged), while the gray trace gives the data obtained under 1.1 bar carbon dioxide (two transients averaged). These transients were recorded with a time constant of 100 μs/point.

back into the S_0 state. On the fourth flash ($S_0 \Rightarrow S_1$) there is no positive step at 360 nm (Lavergne, 1991), and on the fifth flash a new cycle starts ($S_1 \Rightarrow S_2$) with another positive jump. If the concentration of the product, oxygen, is increased to 20 bar (Fig. 1A, gray trace), then the magnitude of the negative jump decreases because the backpressure of O_2 stabilizes a so far ill-defined intermediate X of the transition $S_3 \Rightarrow S_4 \rightarrow X \rightarrow S_0$ (for details, see Clausen and Junge, 2004).

This type of experiment was repeated under high pressure of CO_2. The ambient carbon-dioxide pressure is about 4×10^{-4} bar (0.04%). Figure 1B compares a typical UV-absorption transient obtained at ambient CO_2 pressure (trace shown in black) with that measured at 3,000-fold increased CO_2 pressure (gray trace, 1.1 bar purified CO_2). Both traces are virtually identical, indicating that greatly elevated CO_2 pressure does not inhibit photosynthetic oxygen production.

The lack of any effect of high CO_2 pressure on oxygen production was corroborated by standard polarographic measurements of the rate of oxygen evolution under continuous illumination. The presence of 50 mM $NaHCO_3$ and 10 mM Na_2CO_3 in the reaction mixture yielded the same rate as observed in control samples without added $NaHCO_3$ and Na_2CO_3. There was neither a stimulatory nor an inhibitory effect of the greatly elevated CO_2 concentration (42.5 mM; Henry constant 38.66 mM bar^{-1} at 20°C) on PSII turnover under steady illumination. This shows that the bicarbonate binding site of the OEC, if present, is already saturated by bicarbonate concentrations formed by ambient amounts of dissolved CO_2.

MIMS

Figure 2A shows mass-spectrometric signals resulting from the excitation of dark-adapted spinach (*Spinacia oleracea*) thylakoids with a series of 12 Xenon (Xe) flashes. They were recorded by MIMS using an isotope-ratio mass spectrometer that was set up to simultaneously measure O_2 and CO_2 at m/z = 32, 34, and 36, and 44, 46, and 48, respectively. For all three mass peaks of each gas, similar results were obtained. For clarity, Figure 2A shows only signals of the singly labeled species (mass 34 and 46, respectively). Before the measurements, the PSII samples (20 μM chlorophyll [Chl]/mL at pH 6.8) were mixed with approximately 15% $H_2{}^{18}O$ (final concentration) and then degassed in the sample chamber under rapid stirring to about 1.4 μM CO_2 (approximately 7% of the initial air-saturated value of 21 μM at 10°C) in order to achieve nearly constant baselines. A preflash and subsequent dark adaptation are used to convert all PSII centers of a given sample into the $S_1Y_D^{ox}$ state. This procedure strongly reduces the S state decay between the required long dark times of 20 s between the flashes because it preoxidizes the endogenous electron donor Y_D that normally causes the fast phase of S_2 and S_3 state decay. The preflashed thylakoids are then illuminated with a train of 12 Xe flashes (approximately 3 μs full width at half maximum [FWHM]). The data in Figure 2A show that O_2 is evolved by a significant number of PSII complexes with high efficiency even at this low dissolved CO_2/HCO_3^- concentration. This is evident from (1) the clear period four oscillation of the O_2 signals, which can be well described with miss and double hit parameters of 14.3% and 3.8%, respectively, and 100% S_1 state dark population, and (2) that from the sum of the absolute amplitudes of the first 12 flashes a number of approximately 700 Chl per active PSII reaction center can be calculated. In contrast, the simultaneously recorded CO_2 trace does not show any flash-induced signals. In this regard it is important to note that the sensitivities of the two Fay cups are identical, and that the

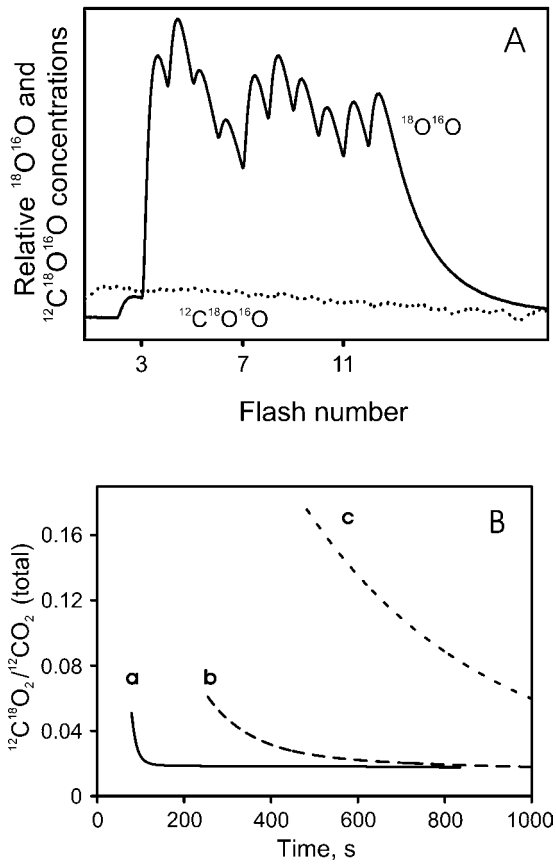

Figure 2. A, Flash-induced $^{18}O^{16}O$ (mass 34; solid line) and $^{12}C^{18}O^{16}O$ (mass 46; dashed line) evolution of spinach thylakoid samples. A series of 12 saturating Xe flashes with intermittent dark times of 20 s was given at 10°C and pH 6.8. The $H_2{}^{18}O$ enrichment was 15% and the Chl concentration 0.02 mg Chl/mL. The signal induced by the third of the flash corresponds to 4.8 pmol O_2. For better comparison, a monoexponentially decaying baseline was subtracted from the CO_2 signal, which had a significantly higher starting value. B, Change in $^{12}C^{18}O_2$ concentration as a function of time after the injection of 28 μL of $H_2{}^{18}O$ (95% initial; 15% final enrichment). The concentration of spinach thylakoids was 0.20 mg Chl/mL (a), 0.02 mg Chl/mL (b), and 0.00 mg Chl/mL (c). Other conditions are as in Figure 2A.

permeability of the silicone membrane for CO_2 is about 6 times greater than for O_2.

Figure 2B displays the decay of the normalized $^{12}C^{18}O^{18}O$ concentration ([48]/([44] + [46] + [48])) as a function of time after injection and rapid mixing (<10 ms) of 28 μL of $H_2{}^{18}O$ into 150 μL of unlabeled, degassed buffer containing different concentrations of spinach thylakoids. The initial rise (data not shown) is due to the ambient ^{18}O-labeled CO_2 content of the $H_2{}^{18}O$-enriched water. Its decay reflects the rather complex isotopic equilibration process between all water and all CO_2, H_2CO_3, HCO_3^-, and CO_2^{2-} molecules in the sample (for details, see e.g. Mills and Urey, 1940). Due to the above-mentioned normalization, the rates are unaffected by the simultaneous consumption of CO_2 by the mass spectrometer. In the absence of thylakoids (Fig. 2B, trace c), the isotopic equilibration is caused by chemical hydration/dehydration reactions of CO_2/H_2CO_3 and the interconversion with HCO_3^-. The observed significant increase in the rate of isotopic equilibration in the presence of thylakoids (Fig. 2B, traces b and a) confirms the previously reported CA activity of thylakoids. First-order rate constants describing the time course of this isotopic equilibration process are given in Table I.

DISCUSSION

The starting point of this study was the possibility of a (so far hidden) role for bicarbonate/CO_2 as substrate/product of photosynthetic oxygen evolution. The nature of a substrate is that it diffuses from the medium to the catalytic site to which it is bound in an exchangeable fashion, i.e. with a certain dissociation constant K_D. Similarly, the formed products are finally released into the surrounding medium in order to allow new substrate to bind. In order to facilitate this process, the products usually have a much smaller affinity to the catalytic site than the substrates. Nevertheless, product inhibition is a well-known phenomenon in catalytic reactions.

Another possible function of bicarbonate is that of a cofactor of photosynthetic water oxidation. In contrast to substrates, cofactors may be relatively tightly associated with the enzyme. Such a possible cofactor role of bicarbonate in photosynthetic water oxidation, which may, for example, involve (1) a function in the proton relay network, (2) a structural role, and/or even (3) provide a binding site for one substrate water molecule, cannot be analyzed by our current approaches.

In the first series of experiments, the reduction of the Mn_4Ca complex during the oxygen-evolving reaction step was followed by monitoring absorption transients in the near UV. For the case that bicarbonate is the immediate substrate of the OEC, it is expected that greatly increased partial pressure of CO_2 should suppress this step. This product inhibition was previously observed for O_2 using moderately increased partial pressures. Nevertheless, for bicarbonate this expectation may not be immediately obvious on the basis of the simple reaction scheme presented in Equations 1 to 3 because increased CO_2 pressure also causes proportionally increased concentrations of HCO_3^- via the reactions shown in Equation 2, which in principle

Table I. *Rates of monoexponential decay of the $^{12}C^{18}O_2$ signal after injection of $H_2{}^{18}O$ into degassed $H_2{}^{16}O$ buffer containing different concentrations of spinach thylakoids at 10°C and pH 6.8*

Chl Concentration	Decay Rate, *k*	Half-Life Time
mg/mL	s^{-1}	*s*
0.00	0.0023	300
0.02	0.0074	95
0.20	0.0764	9

can counterbalance the effect on the product side (Eq. 1). However, because we are concerned with a light-driven heterogeneous catalysis, this cancellation of effects cannot happen. In order to illustrate this important point, we shall concentrate only on the final steps of the postulated reaction sequence that are summarized in more detail in Equation 4:

$$2\cdot\mathrm{BCA} + \mathrm{S_3} \leftrightarrow [\mathrm{S_3}\cdot(\mathrm{BCA})_2] \xrightarrow{h\nu} [\mathrm{S_4}\cdot(\mathrm{BCA})_2] \leftrightarrow \mathrm{X} \leftrightarrow \mathrm{S_0} + \mathrm{O_2} + 2\mathrm{CO_2}. \quad (4)$$

Herein, BCA stands for bicarbonate, S_3 and S_4 for the catalytic center (OEC plus Y_Z) in its third and fourth oxidation states, X for an ill-characterized intermediate, and $h\nu$ for the (usually third) photon that initiates oxygen liberation. Protons are omitted for simplicity. The existence of intermediate X was established elsewhere (Clausen and Junge, 2004), but it can be omitted in the following argument. Equation 4 accounts for three important properties. (1) The right reaction starting from S_4 is heterogeneous in that a solid component cleaves off two soluble components, O_2 and CO_2. Its equilibrium is therefore shifted to the left if the partial pressure of either product is increased. (2) The middle reaction (S_3 to S_4) is photochemical and does not proceed without the extra driving force provided by a quantum of light. Therefore, the equilibrium of the left reaction (binding of bicarbonate to the S_3 state) does not directly bear on the equilibrium of the right because both parts are shielded from each other by the photochemical step. In this particular case, there is additionally good reason to suppose saturation of bicarbonate binding, because (1) the mass-spectrometric experiments show significant O_2 evolution at greatly diminished bicarbonate concentrations and (2) because addition of bicarbonate to PSII suspensions did not increase the rate of oxygen evolution (see above). These arguments show that photosynthetic oxygen evolution should be very sensitive to increased pressure of CO_2, if bicarbonate is the substrate. However, the data of Figure 1B show no inhibition of the $S_3 \rightarrow S_4 \rightarrow S_0$ transition in cyanobacterial core complexes by very large ($\times$3,000) CO_2 overpressure. This result excludes all hypothetical reaction schemes involving exchangeable bicarbonate/CO_2. This conclusion is further corroborated by our MIMS experiments, which give no evidence for any oscillating CO_2 release down to a level of 2% below the one of the oscillating oxygen production.

A weak point of previous time-resolved mass-spectrometric experiments was that they did not determine the CA activity in the samples (see introduction). We show here that the CA activity of spinach thylakoids can be determined from the decay of the mass-spectroscopic signal at a normalized mass of 48 ($^{12}C^{18}O^{18}O$; Fig. 2B). From the data of Table I it can be estimated that under the conditions employed in the previous water exchange experiments (thylakoid concentrations of e.g. 0.25 mg Chl/mL; Messinger et al., 1995) a comparatively long time (>30 s) is required for reaching the isotopic equilibrium between CO_2/HCO_3^- and water. This is in contrast to the rapid isotopic equilibrium in water, which is reached by physical mixing in the microsecond time scale (Messinger et al., 1995; Hillier et al., 1998; Hillier and Wydrzynski, 2000; Hendry and Wydrzynski, 2002). The isotopic equilibration of water is essentially unaffected by the subsequent equilibration with the CO_2 species because of the far smaller concentration of the latter species. Since at 10°C the ^{18}O label quantitatively appears in flash-induced oxygen signals within about 3 s (k_{slow} = 2.2 s^{-1}; Messinger et al., 1995), the combination of these two data sets supports also strongly the view that water, and not bicarbonate, is directly oxidized by PSII. The function of the observed CA activity of PSII and of thylakoid membranes as a whole remains to be elucidated (see Table I and Fig. 2B; for review, see Stemler, 1998, 2002). It has been suggested that the lumenal CA of thylakoids may play a role in the formation of a proton gradient across the thylakoid membrane in the dark in order to support ATP synthesis (van Hunnik and Sultemeyer, 2002). CA may also be involved in regulating the pH of the lumen similar to its known function in blood.

CONCLUSION

The results presented in this article refute any direct role of exchangeable bicarbonate/CO_2 as substrate/intermediate of photosynthetic oxygen evolution. Instead they corroborate the view that water is the direct source of electrons for the production of carbohydrates and, likewise, the source of the oxygen we breathe.

The same results do not rule out the involvement of firmly bound or sequestered bicarbonate in water oxidation. It remains conceivable that bound HCO_3^- may (1) be part of a deprotonation pathway, (2) alter redox properties of the Mn_4Ca complex, (3) stabilize the metal-cluster as a ligand to manganese and/or calcium (Klimov et al., 1995), or (4) provide a binding site for substrate water.

MATERIALS AND METHODS

Flash-spectrometric experiments were carried out with oxygen-evolving PSII-core particles prepared from modified wild-type cells of *Synechocystis* sp. PCC6803 as described elsewhere (Clausen et al., 2001). Aliquots were suspended in standard medium: 1 M Suc, 25 mM $CaCl_2$, 10 mM NaCl, 1 M Gly betaine, 50 mM MES (1 M MES for experiments under 1.1 bar CO_2-pressure), pH 6.6 to 6.7. The temperature was 20°C ± 0.5°C.

The home-built optical cell with fused sapphire windows sustained pressures up to 30 bar (Clausen et al., 2001). The standard reaction medium was pre-equilibrated with purified oxygen or carbon-dioxide gas under the desired partial pressure for at least 40 min. It was then filled into the optical cell (optical pathlength = 1 cm) and re-equilibrated with the pressurized gas phase for another 10 min. A small aliquot (typically 50 μL) of dark-adapted PSII particles from the concentrated stock was then injected into the cell, which was filled with roughly 3 mL of liquid and 20 mL of gas to give final concentrations of 7 to 15 μM Chl and 0.06% (w/v) *N*-dodecyl-β-D-maltoside. The mixture was kept in darkness and equilibrated at the chosen pressure for another 20 min under gentle stirring. After addition of the electron acceptor (200 μM 2,5-dichloro-*p*-benzoquinone), the sample was excited under pressure

with a group of five flashes from a Q-switched Nd-YAG laser (wavelength 532 nm, duration 6 ns FWHM, saturating energy, 100 ms between flashes) and the absorption transients were recorded at a wavelength of 360 nm. This wavelength was chosen to reveal charge transfer bands of the Mn_4Ca complex with minimal contributions from Tyr (Dekker et al., 1984; Schatz and van Gorkom, 1985; Weiss and Renger, 1986; Gerken et al., 1989; Haumann et al., 1999). Data were recorded at an analog bandwidth of 10 kHz and digitized at 50 μs or 100 μs per bin. Control samples were prepared in the same manner, except that they were stirred for 20 min under 1 bar air (0.21 bar oxygen).

MIMS experiments were carried out with spinach (*Spinacia oleracea*) thylakoids, which were suspended in a buffer containing 5 mM $CaCl_2$, 50 mM MES (pH 6.8/10°C), 5 mM $MgCl_2$, 15 mM NaCl, and 400 mM Suc to give a final concentration of 0.02 mg Chl/mL. The experiments were carried out in a home-built cell similar to that described by Messinger et al. (1995), but with 150 μL sample volume and an improved mixing system. The gas inlet system was formed by a silicone membrane (MEM-213) that rests on a porous plastic support, which together separated the aqueous sample from the vacuum (3×10^{-8} bar) of the isotope ratio mass spectrometer (ThermoFinnigan Delta^{plus}XP), which was equipped with seven Fay cups for the simultaneous detection of masses 32, 34, 36, 40, 44, 46, and 48. No artificial electron acceptors were added. Dark-adapted samples were excited with a Xe flash lamp with approximately 3 μs FWHM. The individual flashes were separated by dark intervals of 20 s. The calibration of the CO_2 and O_2 signals was performed by injecting various volumes of air-saturated water samples (21 μM CO_2 and 351 μM O_2 at 10°C) into the sample chamber filled with degassed buffer.

ACKNOWLEDGMENTS

We thank Hella Kenneweg (Osnabrück) and Birgit Nöring (Mülheim/Ruhr) for excellent technical assistance, Holger Heine (Osnabrück) for advise on the construction of the pressure cell, Prof. Rick Debus (Riverside) for the cooperation on Synechocystis, Drs. Ralf Ahlbrink and Armen Mulkidjanian (both Osnabrück) for fruitful discussions and help, and Govindjee (Urbana) and the referees for helpful comments on the manuscript.

Received July 15, 2005; revised September 2, 2005; accepted September 2, 2005; published October 21, 2005.

LITERATURE CITED

Ananyev GM, Zaltsman L, Vasko C, Dismukes GC (2001) The inorganic biochemistry of photosynthetic oxygen evolution/water oxidation. Biochim Biophys Acta **1503:** 52–68

Bader KP, Thibault P, Schmid GH (1987) Study on the properties of the S_3-state by mass spectrometry in the filamentous cyanobacterium *Oscillatoria chalybea.* Biochim Biophys Acta **893:** 564–571

Biesiadka J, Loll B, Kern J, Irrgang KD, Zouni A (2004) Crystal structure of cyanobacterial photosystem II at 3.2 angstrom resolution: a closer look at the Mn-cluster. Phys Chem Chem Phys **6:** 4733–4736

Bögershausen O, Junge W (1995) Rapid proton transfer under flashing light at both functional sides of dark adapted photosystem II core particles. Biochim Biophys Acta **1230:** 177–185

Cinco RM, Robblee JH, Messinger J, Fernandez C, Holman KLM, Sauer K, Yachandra VK (2004) Orientation of calcium in the Mn4Ca cluster of the oxygen-evolving complex determined using polarized strontium EXAFS of photosystem II membranes. Biochemistry **43:** 13271–13282

Clausen J, Debus RJ, Junge W (2004) Time-resolved oxygen production by PSII: chasing chemical intermediates. Biochim Biophys Acta **1655:** 184–194

Clausen J, Junge W (2004) Detection of an intermediate of photosynthetic water oxidation. Nature **430:** 480–483

Clausen J, Winkler S, Hays AMA, Hundelt M, Debus RJ, Junge W (2001) Photosynthetic water oxidation: Mutations of D1-Glu189K, R and Q of *Synechocystis* sp. PCC6803 are without any influence on electron transfer rates at the donor side of photosystem II. Biochim Biophys Acta **1506:** 224–235

Cotton FA, Wilkinson G (1974) Anorganische Chemie. Verlag Chemie, Weinheim, Germany, pp 1–1235

Dai XB, Yu Y, Zhang RX, Yu XJ, He PM, Xu CH (2001) Relationship among photosystem II carbonic anhydrase, extrinsic polypeptides and manganese cluster. Chin Sci Bull **46:** 406–409

Dekker JP (1992) Optical studies on the oxygen-evolving complex of photosystem II. *In* VL Pecoraro, ed, Manganese Redox Enzymes, Ed 1. VCH Publishers, New York, pp 85–104

Dekker JP, van Gorkom HJ, Brok M, Ouwehand L (1984) Optical characterization of photosystem II electron donors. Biochim Biophys Acta **764:** 301–309

Ferreira KN, Iverson TM, Maghlaoui K, Barber J, Iwata S (2004) Architecture of the photosynthetic oxygen-evolving center. Science **303:** 1831–1838

Gerken S, Dekker JP, Schlodder E, Witt HT (1989) Studies on the multiphasic charge recombination between chlorophyll A_{II}^+ (P-680$^+$) and plastoquinone Q_A^- in Photosystem II complexes. Ultraviolet difference spectrum of Chl-a_{II}^+/Chl-a_{II}. Biochim Biophys Acta **977:** 52–61

Haumann M, Mulkidjanian A, Junge W (1999) Tyrosine-Z in oxygen evolving photosystem II: a hydrogen-bonded tyrosinate. Biochemistry **38:** 1258–1267

Hendry G, Wydrzynski T (2002) The two substrate-water molecules are already bound to the oxygen-evolving complex in the S2 state of photosystem II. Biochemistry **41:** 13328–13334

Hillier W, Messinger J, Wydrzynski T (1998) Kinetic determination of the fast exchanging substrate water molecule in the S_3 state of photosystem II. Biochemistry **37:** 16908–16914

Hillier W, Wydrzynski T (2000) The affinities for the two substrate water binding sites in the O(2) evolving complex of photosystem II vary independently during S-state turnover. Biochemistry **39:** 4399–4405

Kamiya N, Shen JR (2003) Crystal structure of oxygen-evolving photosystem II from *Thermosynechococcus vulcanus* at 3.7-angstrom resolution. Proc Natl Acad Sci USA **100:** 98–103

Karge M, Irrgang KD, Renger G (1997) Analysis of the reaction coordinate of photosynthetic water oxidation by kinetic measurements of 355 nm absorption changes at different temperatures in photosystem II preparations suspended in either H_2O or D_2O. Biochemistry **36:** 8904–8913

Khristin MS, Ignatova LK, Rudenko NN, Ivanov BN, Klimov VV (2004) Photosystem II associated carbonic anhydrase activity in higher plants is situated in core complex. FEBS Lett **577:** 305–308

Klimov VV, Allakhverdiev SI, Feyziev YM, Baranov SV (1995) Bicarbonate requirement for the donor side of photosystem II. FEBS Lett **363:** 251–255

Klimov VV, Baranov SV (2001) Bicarbonate requirement for the water-oxidizing complex of photosystem II. Biochim Biophys Acta **1503:** 187–196

Kok B, Forbush B, McGloin M (1970) Cooperation of charges in photosynthetic O_2 evolution. I. A linear four-step mechanism. Photochem Photobiol **11:** 457–475

Lavergne J (1991) Improved UV-visible spectra of the S-transitions in the photosynthetic oxygen-evolving system. Biochim Biophys Acta **1060:** 175–188

Mende D, Wiessner W (1985) Bicarbonate in vivo requirement of photosystem II in the green alga Chlamydobotrys stellata. J Plant Physiol **118:** 259–266

Messinger J (2004) Biophysical studies of Photosystem II and related model systems. Phys Chem Chem Phys **6:** E11–E12

Messinger J, Badger M, Wydrzynski T (1995) Detection of *one* slowly exchanging substrate water molecule in the S_3 state of photosystem II. Proc Natl Acad Sci USA **92:** 3209–3213

Metzner H (1978) Photosynthetic Oxygen Evolution. Academic Press, London

Mills GA, Urey HC (1940) The kinetics of isotopic exchange between carbon dioxide, bicarbonate ion, carbonate ion and water. J Am Chem Soc **62:** 1019–1025

Radmer R, Ollinger O (1980) Isotopic composition of photosynthetic O_2 flash yields in the presence of $H_2{}^{18}O$ and $HC^{18}O_3^-$. FEBS Lett **110:** 57–60

Robblee JH, Messinger J, Cinco RM, McFarlane KL, Fernandez C, Pizarro SA, Sauer K, Yachandra VK (2002) The Mn cluster in the S-0 state of the oxygen-evolving complex of photosystem II studied by EXAFS spectroscopy: Are there three di-mu-oxo-bridged Mn-2 moieties in the tetranuclear Mn complex? J Am Chem Soc **124:** 7459–7471

Schatz GH, van Gorkom HJ (1985) Absorbance difference spectra upon charge transfer to secondary donors and acceptors in photosystem II. Biochim Biophys Acta **810:** 283–294

Silverman DN, Tu CK (1975) Buffer dependence of carbonic-anhydrase catalyzed oxygen-18 exchange at equilibrium. J Am Chem Soc **97:** 2263–2269

Stemler A, Babcock GT, Govindjee (1974) The effect of bicarbonate on photosynthetic oxygen evolution in flashing light in chloroplast fragments. Proc Natl Acad Sci USA **71:** 4679–4683

Stemler AJ (1998) Bicarbonate and photosynthetic oxygen evolution: an unwelcome legacy of Otto Warburg. Indian J Exp Biol **36:** 841–848

Stemler AJ (2002) The bicarbonate effect, oxygen evolution, and the shadow of Otto Warburg. Photosynth Res **73:** 177–183

Tu CK, Silverman DN (1975) Kinetics of exchange of oxygen between carbon-dioxide and carbonate in aqueous-solution. J Phys Chem **79:** 1647–1651

van Hunnik E, Sultemeyer D (2002) A possible role for carbonic anhydrase in the lumen of chloroplast thylakoids in green algae. Funct Plant Biol **29:** 243–249

van Rensen JJS (2002) Role of bicarbonate at the acceptor side of Photosystem II. Photosynth Res **73:** 185–192

Villarejo A, Shutova T, Moskvin O, Forssen M, Klimov VV, Samuelsson G (2002) A photosystem II-associated carbonic anhydrase regulates the efficiency of photosynthetic oxygen evolution. EMBO J **21:** 1930–1938

Warburg O, Krippahl G (1958) Hill-Reaktionen. Z Naturforsch B **13B:** 509–514

Warburg O, Krippahl G, Jetschma C (1965) Widerlegung der Photolyse des Wassers und Beweis der Photolyse der Kohlensäure nach Versuchen mit lebender Chlorella und den Hill-Reagentien Nitrat und $K_3Fe(Cn)_6$. Z Naturforsch B **B20:** 993–996

Weiss W, Renger G (1986) Studies on the nature of the water-oxidizing enzyme. II. On the functional connection between the reaction-center complex and the water-oxidizing system Y in Photosystem II. Biochim Biophys Acta **850:** 173–183

Zouni A, Witt HT, Kern J, Fromme P, Krauss N, Saenger W, Orth P (2001) Crystal structure of photosystem II from *Synechococcus elongatus* at 3.8 Å resolution. Nature **409:** 739–743