

# Bicarbonate Accelerates Assembly of the Inorganic Core of the Water-Oxidizing Complex in Manganese-Depleted Photosystem II: A Proposed Biogeochemical Role for Atmospheric Carbon Dioxide in Oxygenic Photosynthesis<sup>†</sup>

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**ABSTRACT:** The proposed role for bicarbonate ( $\text{HCO}_3^-$ ) as an intrinsic cofactor within the water-oxidizing complex (WOC) of photosystem II (PSII) [Klimov et al. (1997) *Biochemistry* 36, 16277–16281] was tested by investigation of its influence on the kinetics and yield of photoactivation, the light-induced assembly of the functional inorganic core ( $\text{Mn}_4\text{O}_y\text{Ca}_1\text{Cl}_x$ ) starting from the cofactor-depleted apo-WOC–PSII center and free  $\text{Mn}^{2+}$ ,  $\text{Ca}^{2+}$ , and  $\text{Cl}^-$ . Two binding sites for bicarbonate were found that stimulate photoactivation by accelerating the formation and suppressing the decay, respectively, of the first light-induced assembly intermediate,  $\text{IM}_1$  [apo-WOC– $\text{Mn}(\text{OH})_2^+$ ]. A high-affinity bicarbonate site ( $K_D \leq 10 \mu\text{M}$ ) stimulates both the rate of recovery of  $\text{O}_2$  evolving centers and decreases (by a factor of 1.2–3)  $t_{\text{lag}}$ , the time for formation of  $\text{IM}_1$ . This stimulation involves enhanced binding of the initial  $\text{Mn}^{2+}$  and occurs only at concentrations of  $\text{Mn}^{2+}$  at or below the stoichiometric requirements for water oxidation ( $\leq 4 \text{ Mn/PSII}$ ) and disappears above 4  $\text{Mn/PSII}$ . The absence of an effect from added bicarbonate on photoactivation kinetics and yield at saturating concentrations of  $\text{Mn}^{2+}$  and  $\text{Ca}^{2+}$  may be due to the availability of atmospheric bicarbonate dissolved in the buffers ( $\sim 4 \mu\text{M}$  at pH 6.0) sufficient for photoactivation. The second bicarbonate site also stimulates the rate of formation of  $\text{IM}_1$  but has much lower affinity ( $K_D$  approximately millimolar) and becomes observable only at low concentrations of  $\text{Ca}^{2+}$  that are limiting for photoactivation. This stimulation effect appears to occur by complexation of free  $\text{Ca}^{2+}$ , thereby reducing its activity in competing with  $\text{Mn}^{2+}$  in the formation of  $\text{IM}_1$ . Bicarbonate had no effect on the calcium effector site responsible for the rate-limiting dark step of photoactivation ( $\text{Ca}^{2+}$  binding to  $\text{IM}_1$ ). Four interpretations of the high-affinity bicarbonate effect may be advanced as testable hypotheses: bicarbonate may (1) act as an integral cofactor within the WOC (possible ligand to the first Mn), (2) act as a Bronsted base to accelerate proton release during formation of either the dark precursor [apo-WOC– $\text{Mn}(\text{OH})^+$ ] or  $\text{IM}_1$  [apo-WOC– $\text{Mn}(\text{OH})_2^+$ ], (3) directly deliver one or more hydroxide ions during formation of the latter two species (with release of  $\text{CO}_2$ ), or (4) act as a membrane-soluble anion that electrostatically elevates the local concentration of  $\text{Mn}^{2+}$  in PSII. These results support a possible biogeochemical role for bicarbonate in the evolution of the first oxygenic photosynthetic organism. An improvement in the illumination method for photoactivation is presented in which light flashes of increasing duration are used to extend the pre-steady-state lag phase and to suppress photoinhibition, thereby improving the accuracy of  $t_{\text{lag}}$  determination.

Carbon dioxide and its hydrolysis products in water, the carbonate anion and bicarbonate, most likely had important biogeochemical roles in influencing the solubility and speciation of metal ions during the pre-Cambrian epoch, owing to the much higher atmospheric  $\text{CO}_2$  concentration that is predicted to have existed then ( $\times 10^2$ – $10^4$  above the current level at 3–4 billion years ago) (1). This considerably higher concentration would have had profound influences

on the evolution of biological function. Particularly, the influence that bicarbonate had on the speciation of manganese and calcium in the archaean oceans may have altered the course of biological evolution by permitting the chelation of these cofactors in the first successful mutation that produced an  $\text{O}_2$ -evolving photosynthetic organism on earth.

Bicarbonate is known to be required for expressing the maximum activity of photosystem II (PSII)<sup>1</sup> (for recent review see ref 2). However, the variety of effects that

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<sup>1</sup> Abbreviations: Chl, chlorophyll; DCBQ, 2,5-dichloro-*p*-benzoquinone; LED, light-emitting diode; MES, 2-(*N*-morpholino)ethanesulfonic acid; PSII, photosystem II; TPDBA, *N,N,N',N'*-tetrapropionate-1,3-bis(aminomethyl)benzene; WOC, water-oxidizing complex;  $Y_{400}$ , yield of oxygen evolution under pulsed light after 400 flashes;  $t_{\text{lag}}$ , pre-steady-state lag phase of pulsed light photoactivation of oxygen evolution.

bicarbonate exerts on PSII ("bicarbonate effect") appear to be diverse, as well as the identity of the bicarbonate-specific binding sites within PSII. Initially, the site of bicarbonate action on PSII was ascribed to the water-oxidizing complex (WOC) on the donor side of PSII (3, 4) and a model including bicarbonate as a mediator for photosynthetic water oxidation has been suggested (4, 5), which, however, was in contradiction with the results of isotopic experiments (6).

Later, however, strong evidence for the action of bicarbonate on the electron acceptor side of PSII and its requirement for electron transfer between the primary ( $Q_A$ ) and secondary ( $Q_B$ ) plastoquinone molecules was presented (7) and further confirmed by a number of data (for review see ref 2). The non-heme Fe located between  $Q_A$  and  $Q_B$  was shown to be the site for bicarbonate binding on the acceptor side (8, 9).

On the other hand, the bicarbonate effect on the donor side of PSII has been demonstrated comparatively recently (10–15). The most pronounced stimulating effect of bicarbonate is revealed during reconstitution of the Mn cluster after a complete removal of manganese and calcium from PSII preparations (10, 11, 13–15). However, it was unclear what specific step(s) during reconstitution of the  $Mn_4$  cluster were stimulated by bicarbonate ions. The reconstitution process is a natural process that occurs during biogenesis of the inorganic cluster, as well as following the repair of damaged PSII protein subunits. This process is called photoactivation and involves multiple steps that require both light-induced  $Mn^{2+}$  oxidation and dark binding of a  $Ca^{2+}$  ion for reactivation of  $O_2$  evolution (16–23). Recently, progress has been made in examination of the mechanism of photoactivation due to new experimental developments, which have enabled kinetic resolution of the first three intermediates formed during assembly of the core (20–23). Evidence was presented that demonstrated the essential role of  $Ca^{2+}$  in the assembly of the  $Mn_4$  core (22). This approach has been used here to examine the particular step(s) of photoactivation that may use bicarbonate.

## MATERIALS AND METHODS

PSII-enriched membrane fragments were prepared from market spinach by the method of Berthold et al. (24). Samples (at 4 mg of Chl/mL) were stored with 0.4 M sucrose–MES/NaOH buffer (pH 6.0) in liquid  $N_2$  until they were slowly thawed and washed in a medium containing 300 mM sucrose, 35 mM NaCl, and 25 mM MES/NaOH buffer (pH 6.0) (assay medium). Under saturating continuous illumination, the oxygen evolution rate of untreated PSII membrane fragments with 0.8 mM  $K_3Fe(CN)_6$  and 0.5 mM 2,5-dichloro-*p*-benzoquinone (DCBQ) as electron acceptors was 400–450  $\mu\text{mol}$  of  $O_2$ /(mg of Chl·h).

Manganese and calcium were removed from PSII membrane fragments (apo-WOC–PSII) at a concentration of 0.25–1 mg of Chl/mL in the normal assay medium along with 25 mM *N,N,N',N'*-tetrapropionate-1,3-bis(aminomethyl)benzene (TPDBA) and 1 mM ascorbate as described earlier (20). Photoactivation of apo-WOC–PSII membranes by light pulses from an ultrabright LED with a wavelength maximum of 660 nm and  $I_p$  of 800 mW/cm<sup>2</sup> (HLMP-8102, Hewlett-Packard) and amperometric detection of  $O_2$  in the assay medium containing 0.8 mM  $K_3Fe(CN)_6$  were performed simultaneously in a Clark-type microcell of 5  $\mu\text{L}$

volume covered with a silicone-type membrane (21). All the experiments were conducted in the standard assay medium containing 1  $\mu\text{M}$  apo-WOC–PSII membranes (on a per reaction center basis) and 0.8 mM  $K_3Fe(CN)_6$  as an electron acceptor, as well as  $MnCl_2$ ,  $CaCl_2$ , and  $NaHCO_3$  and other salts at concentrations indicated in the text or figure legends.

Oxygen-evolving PSII core particles were prepared from spinach as described in ref 25. When illuminated with saturating light in the presence of 0.5 mM DCBQ, these particles evolved oxygen at typical rates of 800–900  $\mu\text{mol}$  of  $O_2$ /(mg of Chl·h).

A new approach has been used to initiate and observe the photoactivation process. Instead of pulses with constant light duration ( $t_{\text{light}} = 40$  ms) (23), we applied light pulses of gradually increasing duration from 5 to 205 ms (each step of the pulse duration increases by 0.5 ms). The dark time between flashes ( $t_{\text{dark}}$ ) was equal to 3 s, which as shown earlier (22) is optimal for the photoactivation yield. The voltage signal from the preamplified measuring cell passed through a precision low-noise current-to-voltage converter, was further amplified after filtering by adjustable high-and-low-frequency filters, and then was digitized by a 16-bit data acquisition interface under an original program written in the LabView acquisition and analysis software for Windows. The program served also to regulate the duration and the number of pulses.

## RESULTS

(1) *Comparison of the Two Photoactivation Procedures.* The new method of photoactivation by increasing the flash duration diminishes the possibility of photodamage, known to be especially problematic when the manganese cluster is absent such as in the early stages of photoactivation (26–28). Figure 1A shows that the new method with increasing duration pulses (curve 1) lengthens the pre-steady-state lag phase ( $t_{\text{lag}}$ ), which enables more accurate measurement of the time period before  $O_2$  is produced compared to the method with fixed-duration pulses (curve 2). Additionally, the use of shorter light pulses during the initial stage of assembly helps to reduce the flash artifact from the LED pulse, thereby increasing the accuracy of  $O_2$  yield measurement when the yield is low. Another advantage of the new method is the possibility of direct graphical determination of the lag time by extrapolation of the linearized second phase of the photoactivation. [Clear demonstration of  $t_{\text{lag}}$  determination is presented in Figure S1 (Supporting Information), where a semilogarithmic plot (fraction of inactive centers vs time) was used.] The second or rate-limiting phase in which  $O_2$  evolution is restored was previously found to be governed by a dark process that obeys a single-exponential rate law when fixed-duration pulses are used. The convolution of the exponentially increasing population of photoactivated centers (fixed total population), along with the linear increase in illumination time, leads to a linear increase in the total amount of  $O_2$  that is produced on each pulse. The  $O_2$  yield per unit flash duration reflects the normalized  $O_2$  produced per photon and was calculated by division by the duration of each flash as shown in Figure 1B. Comparison of the two illumination methods shows that the use of flashes of increasing duration produces a faster rate of photoactivation (after the lag phase is over) and thus a higher  $O_2$  yield

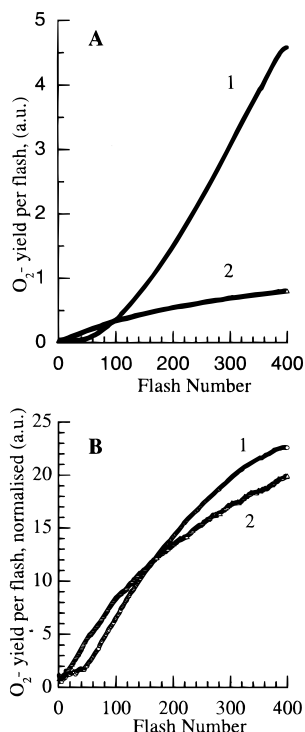


FIGURE 1: Comparison of typical kinetics of the rate of oxygen evolution recovery by photoactivation in apo-WOC-PSII membrane using two methods for illumination by light pulses (A). Illumination method 1, increasing light pulse duration;  $t_{\text{light}} = 5$  ms (initial) to 205 ms (final) with increasing pulse duration of 0.5 ms each pulse,  $t_{\text{dark}} = 3$  s. Illumination method 2, constant pulse length:  $t_{\text{light}} = 40$  ms,  $t_{\text{dark}} = 3$  s. Total number of pulses in both cases is 400,  $I_{\text{peak}} = 800$  mW/cm<sup>2</sup>, and  $\lambda = 660$  nm. (B) Normalization to constant pulse duration by division by pulse duration. Assay conditions: apo-WOC-PSII concentration of 0.25 mg of Chl/mL, which corresponds to 1  $\mu\text{M}$  reaction centers of PSII, 25 mM MES/NaOH buffer (pH 6.0), 35 mM NaCl, 300 mM sucrose, 0.8 mM  $\text{K}_3(\text{FeCN})_6$ , 8  $\mu\text{M}$   $\text{MnCl}_2$ , and 8 mM  $\text{CaCl}_2$ .

per flash reached at the end of the pulse regime (400 pulses). Both methods would eventually produce identical yields of photoactivated PSII if it were not for the somewhat greater loss due to photoinhibition that occurs with the fixed-pulse method. This improvement also enables detection of lower levels of  $\text{O}_2$  reactivation and thus should help in all future studies.

We examined intact  $\text{O}_2$ -evolving PSII core complexes and PSII membrane fragments that had not been extracted to remove the inorganic cofactors. We found no evidence for photoactivation of these samples in the presence of added cofactors (no lag period for oxygen evolution, a constant increasing slope, and no photoactivation) (Supporting Information, Figure S2).

(2) *Effect of Bicarbonate on Photoactivation.* Figure 2 shows that addition of bicarbonate changes the kinetics of photoactivation in a concentration-dependent manner. Figure 2A (curve 2) shows that addition of 25  $\mu\text{M}$   $\text{NaHCO}_3$  causes an acceleration of the rate of the second step of photoactivation (slope) and a decrease in  $t_{\text{lag}}$  when compared to the control (curve 1). [It is clearly seen in Figure S3 (Supporting Information), where a semilogarithmic plot (fraction of inactive centers vs time) was used to determine  $t_{\text{lag}}$ .] By contrast, at 4 mM  $\text{NaHCO}_3$  (curve 3) the rate of the second step of photoactivation is slowed relative to the control while the lag period is not appreciably changed. In Figure 2B these

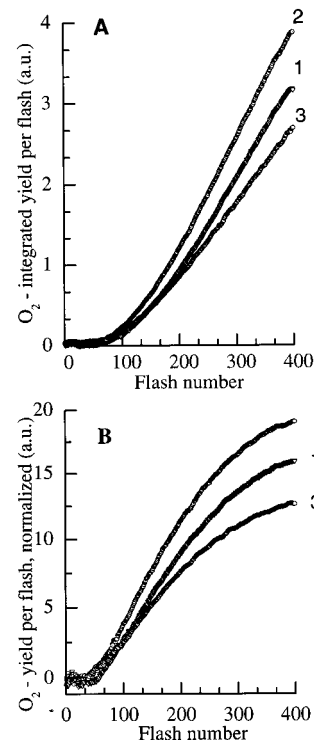


FIGURE 2: Kinetics of photoactivation of apo-WOC-PSII membranes with flashes of increasing flash duration. (A) Curve 1, without added bicarbonate; curve 2, after addition of 25  $\mu\text{M}$   $\text{NaHCO}_3$ ; curve 3, after addition of 4 mM  $\text{NaHCO}_3$ . (B) Normalized  $\text{O}_2$  yield per unit flash length from panel A (normalized by dividing by the light pulse duration of each flash). Assay conditions are same as in Figure 1 except the concentration of  $\text{MnCl}_2$  was 2  $\mu\text{M}$  (2 Mn/PSII).

data are normalized by division by the flash length. From this normalization we observe that the yield of  $\text{O}_2$  per unit flash duration is increased in the case of 25  $\mu\text{M}$   $\text{NaHCO}_3$ , while the 4 mM  $\text{NaHCO}_3$  concentration reduces the yield. There is no major effect on the lag when saturating concentrations of manganese and calcium are used such as those employed in Figure 2. However, at lower Mn concentrations this situation changes.

Figure 3A illustrates the trends in  $t_{\text{lag}}$ , while Figure 3B illustrates the effect on the  $\text{O}_2$  yield observed as a function of bicarbonate and manganese concentrations. In Figure 3A the lag period is seen to decrease as the manganese concentration increases from 1 to 8  $\text{Mn}^{2+}$ /PSII, as previously shown and assigned to the uptake of the first  $\text{Mn}^{2+}$  in the assembly process (20). Bicarbonate addition in the range below 100  $\mu\text{M}$  is found to decrease the lag period, but only in a concentration window between 2 and 4  $\text{Mn}^{2+}$ /PSII, and no effect is observed with either 1 or 8  $\text{Mn}^{2+}$ /PSII. (At  $\text{Mn}^{2+}$  concentrations between 2 and 4  $\text{Mn}^{2+}$ /PSII, a 50% shortening of the lag period is observed upon addition of 16–25  $\mu\text{M}$   $\text{NaHCO}_3$ , which corresponds to the acting concentration of  $\text{HCO}_3^-$  between 5 and 8  $\mu\text{M}$  due to the equilibrium of added bicarbonate at pH 6.0.) Figure 3B shows a similar effect on the  $\text{O}_2$  yield after 400 pulses ( $Y_{400}$ ), increasing to a peak near 100  $\mu\text{M}$  bicarbonate at 2, 3, and 4  $\text{Mn}^{2+}$ /PSII and decreasing to near the original value in the range 0.1 to 0.5 mM bicarbonate. The effect on yield also becomes very low at 8  $\text{Mn}^{2+}$ /PSII, while at 1  $\text{Mn}^{2+}$ /PSII the stimulation of yield requires slightly higher bicarbonate concentration ( $\sim 200$   $\mu\text{M}$ ). At intermediate bicarbonate concentrations (0.5–4

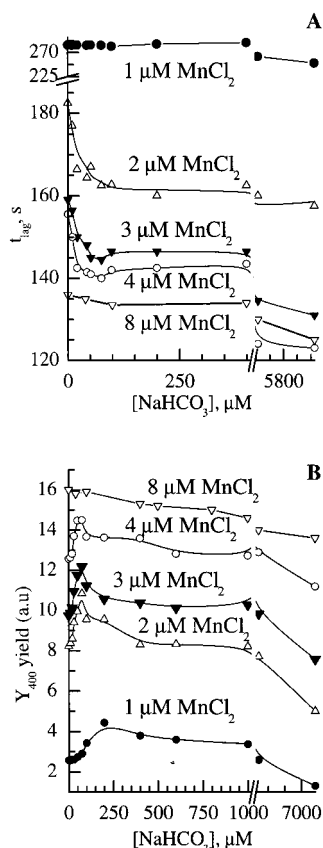


FIGURE 3: Bicarbonate concentration dependence of the photoactivation lag period ( $t_{lag}$ ) (A) and the  $O_2$  yield per unit flash duration measured at flash number 400 ( $Y_{400}$ ) (B). Manganese concentration: (●) 1  $\mu M$ , ( $\Delta$ ) 2  $\mu M$ , ( $\nabla$ ) 3  $\mu M$ , (○) 4  $\mu M$  and ( $\nabla$ ) 8  $\mu M$  MnCl<sub>2</sub>. Photoactivation conditions are the same as in Figure 2 except for concentrations.

mM) only small changes or no change occurs in the lag period, while higher concentrations of bicarbonate (above 4 mM) cause a further decrease.

Figure 4A shows that in the case of saturating amounts of manganese (8 Mn/PSII), addition of bicarbonate accelerates photoactivation by shortening the lag period for recovery of oxygen evolution when subsaturating amounts of CaCl<sub>2</sub> are used. At the optimal concentration of calcium (8 mM) for recovering oxygen evolution yield the effect of bicarbonate is almost absent, only a 10% acceleration of  $t_{lag}$  up to 8 mM bicarbonate. However, at lower calcium concentrations bicarbonate produces a considerably greater acceleration of  $t_{lag}$ ; 1.4-fold acceleration at 0.5 mM CaCl<sub>2</sub> and at 0.25 mM CaCl<sub>2</sub>. The acceleration of  $t_{lag}$  by bicarbonate becomes difficult to observe below 0.1 mM CaCl<sub>2</sub> due to loss of the  $O_2$  signal. The acceleration of  $t_{lag}$  is biphasic in bicarbonate concentration with a higher affinity phase below 100  $\mu M$  bicarbonate. By contrast, bicarbonate produces no effect on the yield of photoactivated centers at any concentration of calcium (Figure 4B). The previously determined calcium concentration that leads to a 50% stimulation of  $t_{lag}$  and photoactivation yield is 1–2 mM (29).

As a further characterization of the bicarbonate effect we examined the influence of formate, which is known to compete with bicarbonate binding to the electron acceptor side of PSII at a low-affinity site. The effect of formate was investigated on the kinetics of electron donation from Mn(II) to apo-WOC–PSII, measured by photoinduced fluores-

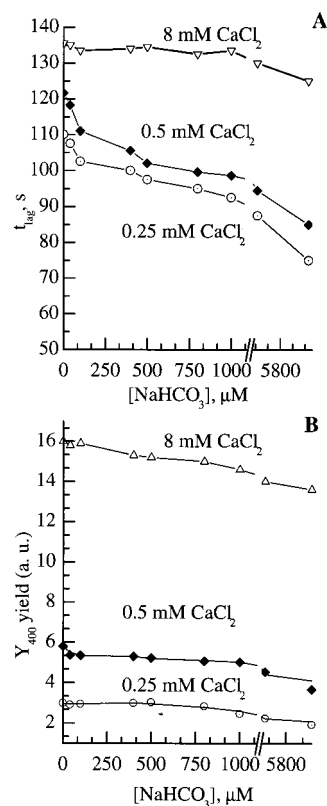
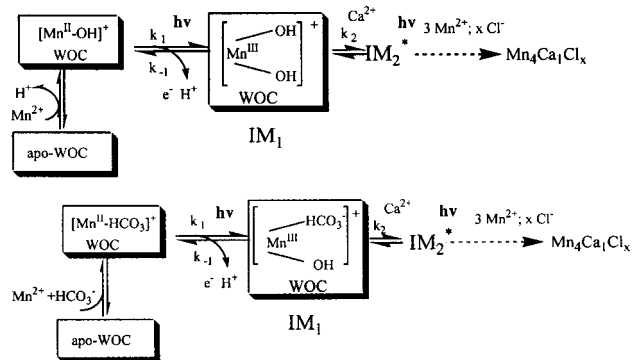


FIGURE 4: Bicarbonate concentration dependence of the photoactivation lag period (A) and the  $O_2$  yield per unit flash duration measured at flash number 400 ( $Y_{400}$ ), (B) at various calcium concentrations. Photoactivation conditions are the same as in Figure 2 except that the concentration of MnCl<sub>2</sub> was 8  $\mu M$ .

#### Scheme 1: Model of Photoactivation in Apo-WOC–PSII (23) and Modification Based on the Data of Bicarbonate Involvement in the First Steps of Reassembly of Active WOC



cence,  $\Delta F$ . At concentrations up to 5 mM, formate did not affect the electron donation rate by Mn(II), indicating that the bicarbonate effect on electron donation to PSII occurs at a distinct site from that where formate interacts at these concentrations.

#### DISCUSSION

The stimulating effect of bicarbonate on the kinetics of the first step of photoactivation seen in Figure 3 indicates that it is involved in accelerating the rate of formation of IM<sub>1</sub> or suppressing deactivation of IM<sub>1</sub> (refer to Scheme 1). The binding affinity for bicarbonate (apparent  $K_D \approx 6.5 \mu M$ ), as obtained from both the effect on  $t_{lag}$  and the increase of



the yield of active centers occurs only in the range of Mn/PSII stoichiometries that are needed for assembly of the WOC (i.e.,  $\leq 4$  Mn/PSII). The dissociation constant agrees well with previous estimates of bicarbonate stimulation of Mn-dependent electron transfer in apo-WOC–PSII samples as measured by Chl fluorescence and photoactivation yield [20–34  $\mu$ M for the major component and  $< 10$   $\mu$ M for the minor component (13)]. The absence of an effect from added bicarbonate on photoactivation kinetics and yield at saturating concentrations of Mn(II) ( $> 4$  Mn/PSII) and Ca(II) may be due to the availability of sufficient ambient bicarbonate dissolved in the buffers ( $\sim 4$   $\mu$ M at pH 6.0) arising from atmospheric CO<sub>2</sub>. This amount would be sufficient for saturation of the requirements for IM<sub>1</sub> formation if bicarbonate is involved in a chemical equilibrium that is coupled to the binding of Mn<sup>2+</sup>. The binding constant for formation of Mn(HCO<sub>3</sub>)<sup>+</sup> in aqueous solution at 25 °C and zero ionic strength is 1.27 [log *K*; *K* is in units of liters per mole (M<sup>−1</sup>)]. Therefore, the bicarbonate effect on IM<sub>1</sub> kinetics could not be due to changes in the chemical form of Mn<sup>2+</sup> in solution, as this occurs only at considerably higher concentrations. At 1 Mn/PSII the rate of IM<sub>1</sub> formation is very slow and the bicarbonate effect on the rate disappears. This may be due to a change in the rate limitation from binding of Mn<sup>2+</sup> to the high-affinity site to its diffusion to the site. Low levels of residual Mn<sup>2+</sup> not removed by the extraction procedure also interfere with kinetic measurements below 1 Mn/PSII.

The high-affinity bicarbonate binding site involved in the kinetics of formation of IM<sub>1</sub> also becomes detectable as the concentration of Ca<sup>2+</sup> is decreased below that which competes for binding to the high-affinity Mn<sup>2+</sup> site [*K*<sub>M</sub> = 1.5 mM (22)] (this occurs even at saturating Mn<sup>2+</sup> concentrations above 4 Mn/PSII). This is a kinetic effect exclusively, as there is no effect observable on the yield of photoactivated centers or on the rate-limiting step (step *k*<sub>2</sub> in Scheme 1, the dark binding of Ca<sup>2+</sup> at its effector site). At 8 mM Ca<sup>2+</sup>, where calcium binding to the high-affinity Mn<sup>2+</sup> site is saturated, the high-affinity effect of bicarbonate disappears. We conclude that the high-affinity bicarbonate site is involved exclusively in promoting the binding of Mn<sup>2+</sup> in one or more of the steps leading to formation of IM<sub>1</sub> and has no detectable influence on the binding affinity of Ca<sup>2+</sup> at its effector site. Bicarbonate binding does produce a further acceleration of the *t*<sub>lag</sub> process in the range between 100 and 7500  $\mu$ M added bicarbonate. This much weaker binding site appears to correspond to the complexation of free Ca<sup>2+</sup> in the buffer to form Ca(HCO<sub>3</sub>)<sup>+</sup> and Ca(CO<sub>3</sub>) [log *K* = 1.26 and 3.15, respectively (30)], thereby decreasing the amount of free Ca<sup>2+</sup> available for competition with Mn<sup>2+</sup> binding.

In Scheme 1 we present two models to account for these effects of bicarbonate on the formation of IM<sub>1</sub>. In the upper panel we suggest that bicarbonate increases the probability of the light reaction that produces IM<sub>1</sub> or increases the stability of IM<sub>1</sub> against subsequent deactivation. This model is identical to the previous model for photoactivation (23), except that it includes bicarbonate acting as a Bronsted base to remove protons released during the hydrolysis of water ligands bound to Mn<sup>2+</sup>. Hydrolysis occurs at two steps, in the formation of the dark precursor to IM<sub>1</sub> [apo-WOC–Mn(OH)<sup>+</sup>] and upon photooxidation to IM<sub>1</sub> [(apo-WOC–Mn(OH)<sub>2</sub>)<sup>+</sup>]. In Scheme 1 (lower panel), bicarbonate is proposed to serve as an integral cofactor essential for the binding of

the initial Mn<sup>2+</sup> (possible ligand). A third possibility is that bicarbonate may deliver one or more hydroxide ions needed during assembly of the Mn<sub>4</sub>O<sub>y</sub> core (instead of water hydrolysis). A fourth possibility is that bicarbonate may partition into the PSII membrane at a higher level than is present in the surrounding aqueous buffer. This increased surface charge would lead to an increase in the local concentration of Mn<sup>2+</sup> and Ca<sup>2+</sup> in the aqueous medium near the interface and thus cause an acceleration of the rates of utilization of these cations observable only at concentrations that remain below saturation of their respective binding sites.

Electrochemical stabilization of IM<sub>1</sub> upon coordination of bicarbonate may be the origin of the kinetic effects observed. The potential for reduction of Mn(III) decreases from 1.2–1.4 V for the aquo ion to 0.92 and 0.63 V for the corresponding mono- and dibicarbonate complexes, forming Mn(HCO<sub>3</sub>)<sup>+</sup> and Mn(HCO<sub>3</sub>)<sub>2</sub>, respectively (31). The latter potentials are sufficiently low that direct oxidation by the photooxidized tyrosine-Z radical would be possible even without further stabilization due to binding to the high-affinity site. This appears not to occur during photoactivation, as was pointed out above, since the bicarbonate effects occur at concentrations much lower than the complexation reaction for Mn<sup>2+</sup> in solution. However, bicarbonate binding could easily stabilize IM<sub>1</sub> as follows. On the basis of the decrease in the reduction potential for Mn(III) upon coordination of bicarbonate to Mn, the predicted difference in dissociation constants for bicarbonate from Mn(III) vs Mn(II) is more than 10 orders of magnitude. Therefore, if binding of both Mn<sup>II</sup> and HCO<sub>3</sub><sup>−</sup> to the apo-WOC–PSII complex were to occur, then subsequent photooxidation to IM<sub>1</sub> forming Mn<sup>III</sup>–(HCO<sub>3</sub>)<sup>2+</sup> or [Mn<sup>III</sup>(HCO<sub>3</sub>)(OH)]<sup>+</sup> (via hydrolysis of bound water) would yield a thermodynamically more stable form of Mn<sup>III</sup>. By increasing the thermodynamic driving force for formation of IM<sub>1</sub>, we can expect an acceleration in the lag period owing to a slowing of the back reaction (*k*<sub>−1</sub>). A similar argument can be made if bicarbonate acts as a Bronsted base to remove a proton, forming Mn<sup>III</sup>(OH)<sup>2+</sup>, which has a reduction potential of 0.5–0.7 V vs 1.2–1.4 V for aquo Mn(III). Put another way, the equilibrium constant for dissociation of hydroxide from Mn<sup>III</sup>(OH)<sup>2+</sup> vs Mn<sup>II</sup>OH<sup>+</sup> is smaller by a factor of 10<sup>10.5</sup>.

In earlier works we showed that bicarbonate enhances the binding affinity of Mn<sup>II</sup> to apo-WOC–PSII; it permits rapid photooxidation of this bound Mn<sup>II</sup> to Mn<sup>III</sup> by tyrosine-Z<sup>+</sup> and suppresses the reduction and release of this Mn<sup>III</sup> (10, 11, 14). These results were interpreted as evidence in favor of direct binding of bicarbonate to Mn<sup>II</sup> and Mn<sup>III</sup> in the presence of apo-WOC–PSII. The present results are consistent with these earlier observations but allow for at least the two distinct interpretations given in Scheme 1.

In conclusion, we have revealed that bicarbonate stimulates the rate of assembly of the inorganic core responsible for water oxidation by two distinct mechanisms. This occurs at the very first step in the binding of the first Mn<sup>2+</sup> via a high-affinity bicarbonate site. Bicarbonate binding at a second site (low-affinity) also accelerates the assembly process at the first step by disrupting the competitive binding of Ca<sup>2+</sup> at the high-affinity Mn<sup>2+</sup> site. There is no effect of bicarbonate on the calcium effector site that controls the rate-limiting uptake of Ca<sup>2+</sup> in the dark. Because bicarbonate exerts a positive influence on the earliest steps of assembly, this

suggests that it may have had a dominant role in the formation of the first O<sub>2</sub>-evolving organisms that evolved in the primitive archaean oceans, where the bicarbonate concentrations would have been greatly elevated owing to the  $\times 10^2$ – $10^4$  fold higher atmospheric CO<sub>2</sub> partial pressure than today (1). This may have been promoted via formation in solution of manganese–bicarbonate clusters of composition Mn<sub>x</sub>(HCO<sub>3</sub>)<sub>y</sub>(OH)<sub>z</sub> as more stable building blocks for photooxidation and binding by the nonoxygenic photosynthetic bacterial precursor which is hypothesized to have given rise to the first O<sub>2</sub>-evolving organisms (32).

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## SUPPORTING INFORMATION AVAILABLE

Three figures showing the fractions of inactive centers versus flash number and dependence of the yield of oxygen evolution per flash on the duration of light pulses. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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