Rapid Reports

Evidence against Bicarbonate Bound in the O_2-Evolving Complex of Photosystem II[†]

Gözde Ulas,[‡] Gerard Olack,[§] and Gary W. Brudvig*[,‡]

Department of Chemistry and Department of Geology and Geophysics, Yale University, New Haven, Connecticut 06520-8107

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ABSTRACT: The oxidation of water to molecular oxygen by photosystem II (PSII) is inhibited in bicarbonate-depleted media. One contribution to the inhibition is the binding of bicarbonate to the non-heme iron, which is required for efficient electron transfer on the electron-acceptor side of PSII. There are also proposals that bicarbonate is required for formation of O_2 by the manganese-containing O_2-evolving complex (OEC). Previous work indicates that a bicarbonate ion does not bind reversibly close to the OEC, but it remains possible that bicarbonate is bound sufficiently tightly to the OEC that it cannot readily exchange with bicarbonate in solution. In this study, we have used NH_2OH to destroy the OEC, which would release any tightly bound bicarbonate ions from the active site, and mass spectrometry to detect any released bicarbonate as CO_2. The amount of CO_2 per PSII released by the NH_2OH treatment is observed to be comparable to the background level, although N_2O, a product of the reaction of NH_2OH with the OEC, is detected in good yield. These results strongly argue against tightly bound bicarbonate ions in the OEC.

Photosystem II (PSII) is a pigment–protein complex that spans the thylakoid membranes of photosynthetic organisms (*1, 2*). Upon photoactivation, the Mn_4Ca-containing active site of PSII catalyzes the splitting of water (eq 1) to release O_2 as the first step of oxygenic photosynthesis, which is hence called the "oxygen-evolving complex" (OEC). Research to broaden our understanding of the natural water-oxidizing mechanism identified catalytically important metal ions and their protein-ligated environment in the active site, along with redox-active cofactors in the electron-transfer pathway, among many other important developments (*1*). However, no general consensus about the mechanism at the molecular level has yet been reached.

$$2H_2O + 4h\nu \rightarrow O_2 + 4H^+ + 4e^- \quad (1)$$

PSII is reversibly inactivated in media completely depleted of bicarbonate ion, a phenomenon known as the "bicarbonate effect" (*3, 4*). The activity is restored following addition of bicarbonate to the medium. There is a known binding site for a bicarbonate ion on the non-heme iron, which is necessary for turnover of the electron-acceptor side of PSII (Figure 1a) (*5, 6*). However, another bicarbonate ion has been proposed to function on the electron-donor side of PSII as an electron donor (*7*), a base to transport protons (*8*), a stabilizing cofactor (*9*), a ligand to Mn (*10, 11*), and also a substrate in the water-splitting mechanism (*3, 12*).

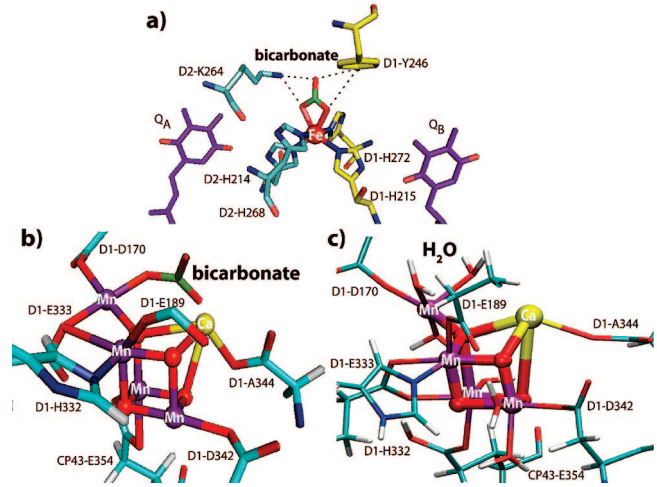

FIGURE 1: (a) Structure of the acceptor side of PSII showing a bicarbonate ion modeled as a bidentate ligand to the non-heme Fe. Taken from the 3.5 Å resolution X-ray crystal structure published by Ferreira et al. (*13*). Structures of the donor side of PSII (b) from the 3.5 Å resolution X-ray crystal structure published by Ferreira et al. (*13*) in which a bicarbonate ion was tentatively assigned and (c) from QM/MM calculations of Sproviero et al. (*15*) showing water molecules instead of a bicarbonate ion in the OEC.

In the 3.5 Å X-ray crystal of PSII (*13*), a bicarbonate ion was tentatively modeled in the OEC (Figure 1b), but the electron density attributed to bicarbonate was not found in the later 3.0 Å structure (*14*). Recently constructed OEC models using QM/MM calculations by Sproviero et al. (*15*) supported the presence of substrate water molecules rather than a bicarbonate ion (Figure 1c). However, structural analyses of the OEC remain equivocal because of radiation damage (*16*), and therefore, they cannot yet prove or disprove the presence of bicarbonate in the OEC.

Experiments comparing isotopic compositions of water, bicarbonate, and O_2 identified water as the natural substrate of PSII (*17–21*). However, ^{18}O-labeled bicarbonate could rapidly exchange with water in the presence of a carbonic anhydrase (CA) (*22*), which was discovered to be closely associated with PSII (*23, 24*). Following up on Metzner's hypothesis on bicarbonate acting as a water "shuttle" into the OEC (*25, 26*), Clausen et al. (*27*) and Hillier et al. (*28*) investigated the possibility of a bicarbonate that was in fast equilibrium with water, this time accounting for CA activity. Clausen et al. (*27*) explored the exchange of ^{18}O between labeled water and flash-induced O_2 and CO_2 using membrane inlet mass spectrometry (MIMS) as well as changes in absorption transients in the near UV due to the increased CO_2 pressure. Hillier et al. (*28*) used ^{18}O- and ^{13}C-labeled

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* To whom correspondence should be addressed. Phone: (203) 432-5202. Fax: (203) 432-6144. E-mail: gary.brudvig@yale.edu.

[‡] Department of Chemistry.

[§] Department of Geology and Geophysics.

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bicarbonate and MIMS to examine oxidation of bicarbonate to O_2 in different photosynthetic organisms. Both groups concluded that exchangeable bicarbonate was not the substrate of PSII. Shevela et al. (*29*) recently published results of their investigation of flash-induced oxygen evolution patterns in samples with reduced bicarbonate concentrations. Because bicarbonate was found not to affect redox potentials of the various S states of the Mn cluster, they ruled out the possibility of an exchangeable bicarbonate ion in the proximity of the OEC. However, these studies do not exclude the possibility that bicarbonate is associated with the OEC as a tightly bound structural component. Indeed, the possibility that a bicarbonate ion is trapped in the OEC during its assembly has been suggested but not yet investigated. Such a hypothesis is backed by studies suggesting that bicarbonate facilitates the reconstitution of the Mn cluster in apo-PSII (*8*).

In this communication, we test for a tightly bound bicarbonate ion in the OEC by using hydroxylamine (NH_2OH) to irreversibly disrupt the OEC or acetate (AcO^-) to displace bicarbonate from the non-heme iron and mass spectrometry to detect any CO_2 evolved. To release any possibly tightly bound bicarbonate ions in the OEC, solutions of active PSII were treated with excess NH_2OH, which is known to specifically target the OEC and reduce the Mn cluster (*30*), thereby releasing all bound ions into solution. At the pH of 5.0 used in our experiments, bicarbonate equilibrates in solution and is released as CO_2 into the headspace of a sealed vial. Equilibration of bicarbonate in solution is facilitated by additional CA (Worthington Biochemicals) in the sample. CO_2 could be detected in the headspace of airtight vials by gas chromatography–mass spectrometry (GC−MS). For each data set, NH_2OH was added to (1) a control that contains 50 mM MES (pH 5.0), 1 mM $CaCl_2$, 0.4 mM sucrose, and 0.01% (w/v) Triton X-100 (buffer A) and CA (20 mg/L), (2) a PSII sample in buffer A with CA, pretreated with excess NH_2OH, and thus with no intact OEC ("apo-PSII"), and (3) an active PSII sample in buffer A with CA ("active PSII"). Vial headspaces were all purged extensively with He before any measurements.

Data were collected by GC−MS while the vial headspaces were continuously purged with He with the needle used for sampling (*31*) (for a detailed description, see the Supporting Information). Each sampling results in two or three detectable peaks that appear at *m*/*z* 44, depending on the reagent used prior to incubation. The first one to appear (at 150 s) is from N_2O formed in the mass spectrometer from N_2 and/or O_2 associated with the air in the headspace, followed by CO_2 (at 180 s) and N_2O (at 190 s). The presence of N_2O in the sample is due to the reaction of NH_2OH with PSII, mostly with the Mn ions in the OEC. It is detected only after NH_2OH treatments of PSII-containing samples and never observed in the buffer. Identification of the peaks was confirmed by running standards of the assigned compound.

For each measurement, the sample vial was purged to give a level baseline of background CO_2. Then a solution of NH_2OH or AcO^- was added dropwise to a final concentration of 10 mM and the sample incubated with the reagent for 20 min while being stirred on ice. The first GC−MS measurement after incubation correlates to the amount of CO_2 that accumulated in the headspace after equilibration between solution and gas phases. Corrections for CO_2 as a result of leakage of air into the vial, which could be detected as an increase in the "air" peak, were performed for each sample (377.38 ppm CO_2 present in air in 2004) (*32*). Exchange of headspace CO_2 with buffer solution was investigated before CO_2 was quantified. Because of the volume of headspace gas, most of the CO_2 is in the gas phase: 97% of 1 nmol of CO_2 (0.3% in He) incubated for 20 min with 0.5 mL of buffer A and CA (prepared and purged following the same procedure as the experimental samples), while being stirred, remains in the headspace.

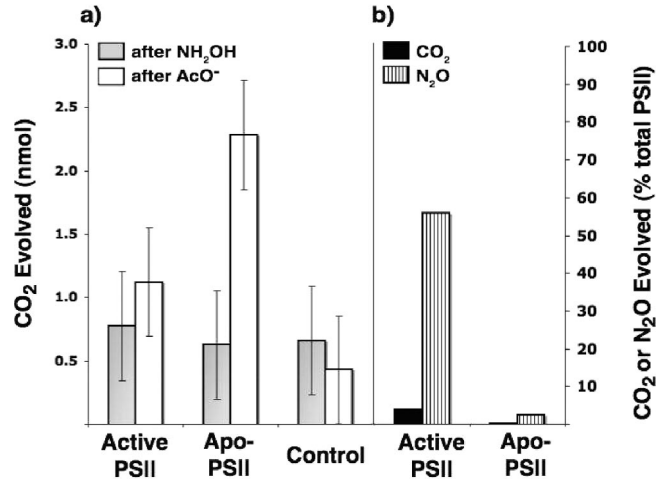

FIGURE 2: (a) Raw data in nanomoles, representing the evolution of CO_2 following NH_2OH and AcO^- treatments (not corrected for background). Active and apo-PSII samples were prepared as spinach PSII membranes (*34*). Variation of background CO_2 levels is marked with error bars calculated from three separate runs. For self-consistency, a single set of data is reported. The initial O_2 activity was 412 μmol of O_2 (mg of Chl)$^{-1}$ h^{-1}. O_2 activity measured after sample preparation and purging was 257 μmol of O_2 (mg of Chl)$^{-1}$ h^{-1}. Evolution of CO_2 after NH_2OH treatment: active PSII = 0.78 nmol, apo-PSII = 0.63 nmol, and control = 0.66 nmol. Evolution of CO_2 after AcO^- treatment: active PSII = 1.12 nmol, apo-PSII = 2.28 nmol, and control = 0.43 nmol. (b) Background-corrected data scaled for the concentration of PSII in the sample. The amount of CO_2 and N_2O evolved after the NH_2OH treatment is presented as the percentage of total moles of PSII. Total moles of PSII were calculated from the Chl concentration, assuming 200 mol of Chl/mol of PSII: active PSII = 1.07 mg/mL Chl (2.96 nmol of PSII), and apo-PSII = 2.61 mg/mL Chl (7.15 nmol of PSII). The very low CO_2 percentage (active PSII = 4%; apo-PSII = 0%) compared to that of N_2O [active PSII = 56% (1.65 nmol); apo-PSII = 2% (0.16 nmol)] shows that an increased number of dissociated Mn clusters does not affect the concentration of CO_2 released from samples.

Figure 2a shows results from a representative set of experimental samples. More than 10 sample sets were run as duplicates, which gave consistent results. Extensive purging was found to deplete bicarbonate from its binding niche in the acceptor side of PSII, and therefore, the level of CO_2 evolution obtained after AcO^- treatment was always much lower than the total amount of PSII in the sample and fluctuated according to the purging time. All duplicates followed the same trend; the amount of CO_2 released after NH_2OH treatment was remarkably much smaller than the expected value calculated for total moles of PSII. The data set in Figure 2 exhibits a ratio of 0.78 nmol of CO_2 (active PSII) to 2.96 nmol of total PSII, while the background CO_2 level (average of three buffer samples) is 0.66 $\pm$ 0.43 nmol. Similarly low CO_2 concentrations detected for active PSII samples, comparable to the background CO_2 concentration, were consistently obtained with each replicate measurement. Active PSII samples were expected to react more with

NH_2OH than apo-PSII samples. This hypothesis is supported by significantly greater release of N_2O [though not necessarily a stoichiometric reaction because of multiple products of NH_2OH oxidation such as N_2, which can contribute to the air peak (*33*)]. Figure 2b shows the distinct difference between N_2O detected in active (1.65 nmol) versus apo-PSII (0.16 nmol) samples, whereas the amount of CO_2 released from each sample is nearly the same and within the variation observed in background CO_2 levels as in Figure 2a.

We can, thus, conclude that there is evidence for the reaction of NH_2OH with the intact OEC; however, this reaction does not yield CO_2. These results strongly argue against any tightly bound bicarbonate ion that is trapped in the OEC of PSII.

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SUPPORTING INFORMATION AVAILABLE

Experimental methods for sample preparation, GC−MS procedures, sample GC−MS trace and detail, data analysis, and error analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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