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Fifty Years of Research on the "Bicarbonate Effect" in Photosystem II: A Mini-Review

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ABSTRACT

Cyanobacteria, algae, and plants fix CO_2 in photosynthesis by utilizing the chemical energy generated in the light reaction. Photosystem II (PS II) plays a vital role in the photosynthetic energy fixation and oxygen evolution. Since the discovery of the stimulatory effect of CO_2 on the Hill reaction (non-cyclic electron transfer in the light reaction), researchers from different laboratories around the world have shared their perspectives on this unique role of CO_2. After approximately twenty-eight years of confusion regarding the role of CO_2 in photosynthesis (dating back to James Franck's 1945 finding of increased oxygen evolution in the presence of CO_2; Franck J (1945) Reviews of Modern Physics, 17:112-119), Alan Stemler and Govindjee from the University of Illinois at Urbana-Champaign (UIUC) established, in 1973, the effect of bicarbonate (HCO_3^-) on PS II but were unable to pinpoint the exact binding site. Ongoing research in Govindjee's laboratory and other research facilities worldwide (e.g., Canada, China, Israel, Finland, Switzerland, France, Germany, and The Netherlands) has predominantly focused on the effect of HCO_3^- on the (electron) acceptor side of PS II. However, key suggestions have been made regarding the effect of HCO_3^- on the electron donor side (of PS II) by Alan Stemler (USA) and Vyacheslav Klimov (Russia). Yanyou Wu (China) has also put forth an argument suggesting that bicarbonate may partly serve as a source of oxygen in the light reaction of photosynthesis. In this review, we provide a brief historical account of the conceptual progression of the "bicarbonate effect" and present current perspectives on both the (electron) acceptor and donor sides of PS II. Additionally, we briefly discuss the prevailing opinion on the carbonic anhydrase-like function of PS II for CO_2 hydration in oxygenic photosynthesis.

Keywords: Acceptor side of PS II, Bicarbonate effect, Carbonic anhydrase, Donor side of PS II, Non-heme iron, Photosystem II, Plastoquinone

INTRODUCTION

Plants, including algae and cyanobacteria, cannot perform photosynthesis without CO_2. These photosynthetic organisms fix atmospheric CO_2 using light energy to produce carbohydrates. CO_2 is absorbed by ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) and subsequently reduced to carbohydrates through various intermediates in the Calvin-Benson-Bassam Cycle (Bassham 2005; Benson 2005). To convert light energy into chemical energy, two photochemical reactions, working in series, are driven by two photosystems associated with the thylakoid membrane (Govindjee et al. 2017). Photosystem II (PS II) is a water-plastoquinone oxidoreductase (Shevela et

al. 2023; Wydrzynski and Satoh 2005) responsible for photochemical reactions, including primary charge separation and the subsequent transfer of electrons from water to plastoquinone. These electrons are further transferred through other intersystem components, ultimately reducing an oxidized electron acceptor in photosystem I (PS I). PS I, in turn, transfers these electrons to nicotinamide adenine dinucleotide phosphate ($NADP^+$), which, after reduction, participates in the Calvin-Benson-Bassham cycle. However, CO_2 serves not only to synthesize carbohydrates but also to regulate photosynthetic electron transport in PS II (Stemler and Govindjee 1974c; Stemler et al. 1974).

Due to its equilibrium with carbonic acid (H_2CO_3) and bicarbonate (HCO_3^-), CO_2 provides both the acidic (H^+ and CO_2) and basic (HCO_3^-) components for the bicarbonate buffering system. This buffering system maintains intracellular and extracellular pH levels. The interconversion of inorganic carbon allows rapid transport of its different forms ($CO_2/HCO_3^-/CO_3^{2-}$) within cells. While HCO_3^- has limited solubility in biological membranes, CO_2 can freely diffuse in and out of cells. Therefore, the interconversion of HCO_3^- to CO_2 facilitates the transport of inorganic carbon into intracellular spaces, while the conversion of CO_2 to HCO_3^- allows for trapping CO_2 within cells.

Although the role of HCO_3^- in the "light reaction" of photosynthesis was proposed as early as 1945 (Franck, 1945), its function remained unclear at the time. The discovery of the "bicarbonate effect" in the Hill reaction by Warburg and Krippahl (1958) highlighted its significance, but it remained dormant, so to speak, until Govindjee delved deeper into it. Working with numerous graduate students and research associates in his laboratory and collaborating with various laboratories in the USA and Europe, he provided valuable insights into one of the major functions of bicarbonate in the light reactions of photosynthesis, as well as the overall photosynthetic complex (see e.g., Govindjee 2019; Govindjee and Van Rensen 1978, 1993; Shevela et al. 2012; Vermaas and Govindjee 1982). Govindjee's perspective on the interaction of bicarbonate with the

electron acceptor side of PS II was visionary for scholars and colleagues alike. Subsequently, extensive studies on HCO_3^- and its role in the electron donor site of PS II were conducted by the laboratories of Alan Stemler (USA) and Vyacheslav Klimov (Russia), as well as Govindjee's own research group (see e.g., Banerjee et al. 2019; Brinkert et al. 2016; Fantuzzi et al. 2023; Shevela et al. 2008; Shevela et al. 2012; Shevela et al. 2013; Shevela et al. 2020; Shutova et al. 2008; Stemler, 1989; Stemler, 2002; Stemler and Murphy, 1983; Tikonov et al. 2018; Villarejo et al. 2002).

In this review, we summarize historical discoveries related to the "bicarbonate effect," particularly after its validation with reproducible results by Stemler and Govindjee (1973). We give special reference to the contributions of Govindjee and his coworkers and outline our current state of knowledge regarding the role of HCO_3^- in determining PS II activity. We have summarized the research of various laboratories in general, and of Govindjee and his group in particular, during the last 50 years (1973-2023), providing answers to many unresolved questions related to (1) the active species of molecules that regulate PS II activity; (2) the precise role(s) of bicarbonate in PS II function; (3) the binding niche of bicarbonate; (4) the functional details of bicarbonate in the protonation of the reduced form of the secondary bound plastoquinone Q_B^{2-}; (5) the donor side effect of bicarbonate, and finally, (6) the molecular mechanism of the bicarbonate effect on various PS II functions.

THE ORIGIN OF THE CONCEPT OF A NEW ROLE OF BICARBONATE

The role of CO_2, though not as HCO_3^-, in photosynthesis was clear to researchers from the very beginning of the history of photosynthesis. Some researchers had noticed the requirement for HCO_3^- long ago, without fully recognizing its importance for the activity of the photosystems. The experimental findings of Warburg and Krippahl (1958, 1960) established the need for CO_2 in the Hill reaction of chloroplasts. When Warburg and Krippahl (1958) measured this reaction in the presence

of 1.4% CO_2, using the grana of kohlrabi leaves, with quinone or ferricyanide as electron acceptor, they found much higher rates of oxygen evolution than without CO_2; they showed that the Hill reaction was inhibited by the removal and strongly stimulated by the addition of CO_2 at low partial pressure under conditions where CO_2 reduction did not occur! This CO_2 effect was shown to be a general phenomenon, observable with a wide variety of Hill reagents and a wide variety of plant species (Stern and Vennesland 1962). However, Izawa (1962) and Good (1963) argued against the scheme of Warburg and Krippahl regarding the stimulatory effect of CO_2 on the Hill reaction, as it was much reduced in weak light compared to strong light, suggesting that CO_2 was not involved in a photochemical reaction but in a non-photochemical step. Nevertheless, the correlation of CO_2 dependence with the presence of small anions weighed towards HCO_3^- as an important substance (Good 1963).

Various researchers until 1973 showed that it was bicarbonate rather than the CO_2 moiety that was the functional entity. Stemler and Govindjee (1973) were the first to note the enhancement in the Hill reaction after the addition of HCO_3^- at pH 6.5. They flushed the thylakoids with acetate or formate-containing suspensions (pH 5.6-6) in the dark with CO_2-free air or pure nitrogen gas and observed extremely low electron transport. However, the rate was restored to control levels upon the addition of bicarbonate (Figure 1). Furthermore, Stemler and Govindjee (1973) also noticed a fairly abrupt 2-fold increase in the rate of dichlorophenol indophenol (DCPIP) reduction as they increased the bicarbonate concentration from 5 to 20 mM at pH 5.8. Although they did not focus on the importance of this observation, they concluded that, in all probability, CO_2 may be the diffusing species, whereas HCO_3^- is the binding species. Since the stimulation is observed by the addition of a bicarbonate solution to anion-inhibited CO_2-depleted thylakoids, the phenomenon is called the "bicarbonate effect". Although the phenomenon was known earlier, the term "bicarbonate effect" was used for the first time by Govindjee and his coworkers. Then, in a series of experiments, Govindjee and his coworkers

(cited later) explained the effect of the presence and absence of bicarbonate on the reduction of secondary plastoquinone Q_B to Q_BH_2 (plastoquinol). These results suggested that HCO_3^- is a requirement for plastoquinol formation, confirming its involvement on the electron acceptor side of PS II.

EARLY ATTEMPTS TO LOCATE THE SITE OF ACTION OF BICARBONATE

The first attempt to locate the site of action of HCO_3^- was made by Punnett and Iyer (1964), who examined the effect of CO_2 on photophosphorylation. They observed that by adding relatively high concentrations of HCO_3^- to non-HCO_3^--depleted chloroplasts, they could accelerate the Hill reaction, as well as enhance the rate of phosphorylation. The ATP:2e$^-$ ratio also increased, particularly when the pH was above 7. Thus, one of the effects of added CO_2 appeared to be an improvement in the coupling between electron transport and phosphorylation. Punnett and Iyer (1964) proposed that CO_2 may increase the efficiency of the formation of a high-energy intermediate resulting from electron transport (now understood to be mainly pH). Batra and Jagendorf (1965) extended the observations of Punnett and Iyer (1964) and demonstrated that the effect observed by them is, in fact, different from the HCO_3^- dependence observed by Warburg and Krippahl (1958; 1960). Differences were noticed between the two observations: (i) the Punnett and Iyer effect required a relatively high [HCO_3^-], whereas the Franck/Warburg effect required much lower concentrations of HCO_3^- to be added to HCO_3^--depleted chloroplasts; (ii) uncouplers of phosphorylation eliminated the stimulation of the Hill reaction by HCO_3^- in non-depleted chloroplasts (Batra and Jagendorf 1965), but no such effect was observed in CO_2-depleted chloroplasts (Good 1963; Khanna et al. 1977; Stern and Vennesland 1962); (iii) added HCO_3^- stimulated phosphorylation under conditions of cyclic electron flow around PS I, whereas the removal of CO_2 by depletion had no effect on pyocyanin-supported phosphorylation (Batra and Jagendorf 1965); and (iv) the Franck/Warburg effect appears to represent a

requirement for HCO_3^-, whereas the Punnett and Iyer effect is simply a stimulation (Batra and Jagendorf 1965). Several other publications, most of them from Govindjee and his co-workers in the next decade, showed that a major site of HCO_3^- action is on the electron acceptor side of PS II, but there were/are arguments (and even some data) suggesting the existence of other site(s) for the ion to bind (Blubaugh and Govindjee 1984; El-Shintinawy et al. 1990; Khanna et al. 1977; Koroidov et al. 2014; Shevela et al. 2012; Shevela et al. 2020; Stemler 2002; Stemler and Govindjee 1973; Vermaas and van Rensen 1981; Wu 2021a; Wu 2021b; Wu 2022; Wu 2023).

A number of experiments were conducted in Govindjee's laboratory to precisely pinpoint the site of bicarbonate action and to demonstrate the effects of dark incubation and light pretreatment of chloroplasts suspended in varying concentrations of bicarbonate. Refer to Stemler and Govindjee (1973) for the results, where a bicarbonate
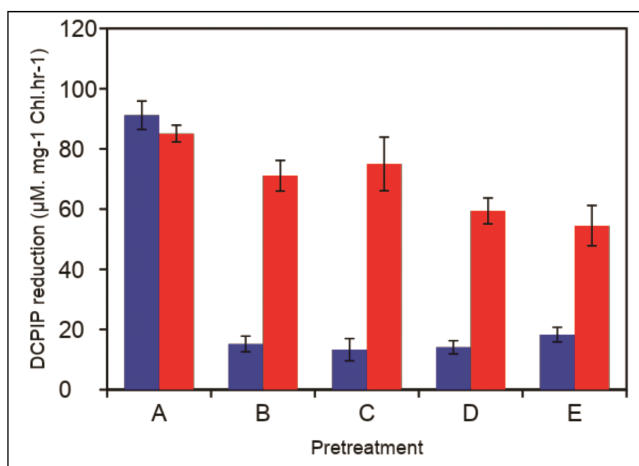

Figure 1. Initial rates of DCPIP reduction with normal (A) and HCO_3^--depleted (B-E) chloroplasts under various conditions. The blue and red columns represent conditions without and with HCO_3^- (20 μM) addition, respectively. [DCPIP] = 39 μM. Abbreviations: A - normal without pretreatment; B - HCO_3^--depleted without pretreatment; C - HCO_3^--depleted + 5 min dark + DCPIP; D - HCO_3^--depleted + 2 min saturating light + DCPIP; E - HCO_3^--depleted + 2 min saturating light + DCPIP + 5 min dark. The figure has been created using data from part of Table IV of Stemler and Govindjee (1973); reproduced with permission of the authors.

effect was suggested to occur on the water oxidation side of PS II.

THE BICARBONATE EFFECT

A series of experiments conducted by Govindjee and his colleagues at the University of Illinois at Urbana-Champaign aimed to precisely determine whether CO_2 or HCO_3^- was the species responsible for the effect on the "light reaction" phase of photosynthesis, thus justifying the use of the term "bicarbonate effect". However, experiments involving dark incubation and light pretreatment of chloroplasts under various concentrations of bicarbonate and CO_2 failed to establish a distinction between the two species, as comparable results were obtained under both conditions.

A clearer understanding of the actual role of CO_2 or HCO_3^- was achieved through the experiments conducted by Stemler and Govindjee (1974a-c) using oat (*Avena sativa* var. Cleland) chloroplasts. These experiments definitively established that HCO_3^- is involved in the early photochemical reactions of PS II, rather than in the dark enzymatic reactions, thus confirming the "bicarbonate effect" as a phenomenon occurring in PS II (Figure 2).

Jursinic et al. (1976) conducted new experiments to determine the exact site of the bicarbonate effect using different techniques, such as electron spin resonance (ESR) measurements of Signal IIvf, measurements of the rise and decay kinetics of chlorophyll a (Chl *a*) fluorescence yield, as well as delayed light emission (DLE) decay. Their observations included: (1) bicarbonate depletion causing a reversible inactivation of up to 40% of PS II reaction center activity, which closely aligned with the percentage of inactivated PS II centers reported by Stemler et al. (1974) from oxygen yield measurements; (2) bicarbonate having no significant effect on the electron flow from the charge-accumulating S state to the intermediate known as Z; (3) bicarbonate not affecting the rate of electron flow from the oxygen-evolving system "S" to Z, but reducing the formation of Z+ to some extent; (4) electron flow
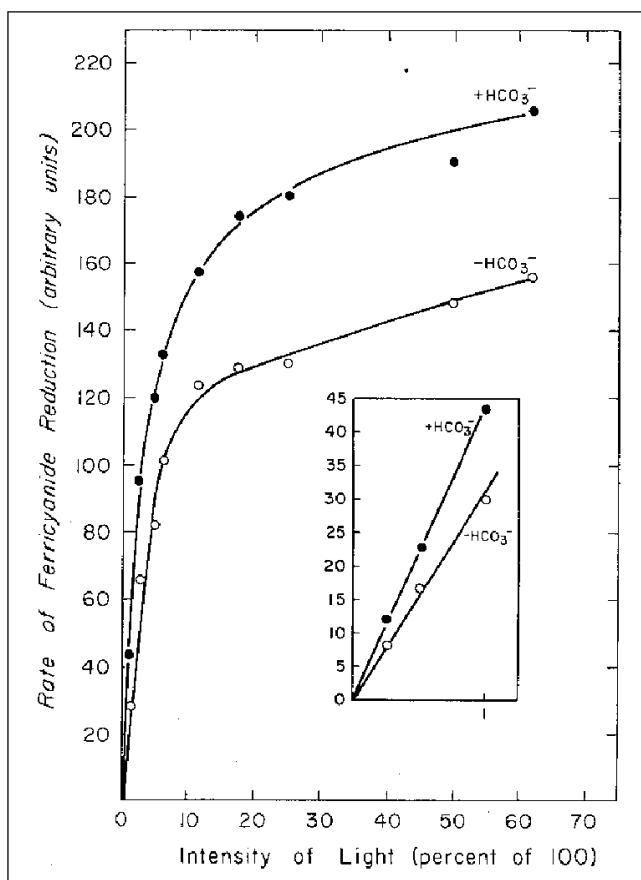
Figure 2. Ferricyanide reduction with and without 10 mM NaHCO$_3$ at different light intensities (percentage of saturating light). The insert shows the ferricyanide reduction at the lowest light intensity (reproduced with permission of the authors, without modification from Stemler and Govindjee 1974c).

from Z to P680+ being partially affected by the absence of bicarbonate, but the electron flow from the reduced Q$_A$ (Q$_A^-$) to the intersystem electron transport pool being drastically inhibited by 4 to 6 fold under bicarbonate-depleted conditions. These data suggested that bicarbonate primarily targeted the electron acceptor side of PS II, specifically between Q$_A$ (the quinone electron acceptor of PS II) and the intersystem electron carrier pool.

By considering the pH dependence of the equilibrium ratio of [CO$_2$] to [HCO$_3^-$], Blubaugh and Govindjee (1986) kept one component's concentration constant while altering the other. Their experiments, conducted with isolated chloroplasts depleted in a medium with a high anion content at pH below 6.0, showed the impact of bicarbonate. Since H$_2$O + CO$_2$ → H$_2$CO$_3$ has a pK of 6.37, an equilibrium at low pH favors CO$_2$, which disappears when nitrogen gas is flushed through the system. In these experiments, following the depletion of bicarbonate, chloroplasts were transferred to a medium at pH 6.5, and the Hill reaction was monitored after the addition of an electron acceptor. Initially, the rate of the Hill reaction was low, but upon the addition of bicarbonate, the rate significantly increased. Hence, it was concluded that HCO$_3^-$ is the binding species. Several experiments were then conducted to evaluate the site of inhibition caused by CO$_2$ depletion, which had previously been used to determine the binding sites of bicarbonate. In the presence of DBMIB (2,5-dibromo-6-isopropyl-3-methyl-1,4-benzoquinone), a significant bicarbonate effect was observed on the electron transport from water to oxidized diamino-durene, indicating an effect before the plastoquinone pool (Eaton-Rye and Govindjee 1984; Khanna et al. 1977). Since CO$_2$ depletion had no influence on the electron transport from water to silico-molybdate in the presence of DCMU, it was determined that the bicarbonate effect exists between Q$_A$ and the PQ pool. This site of inhibition due to the absence of bicarbonate was further inferred from the interaction of bicarbonate with various PS II-inhibiting herbicides (Khanna et al. 1981; Snel and van Rensen 1983; van Rensen and Vermaas 1981; Vermaas et al. 1982). By adding different concentrations of bicarbonate to CO$_2$-depleted thylakoids, various rates of restoration of the Hill reaction could be achieved. A typical Michaelis-Menten kinetics of the activity relationship was obtained between oxygen evolution and bicarbonate concentration (Figure 3; See McConnell et al. 2012). However, there is support for bicarbonate to function on both the electron acceptor and the electron donor sides of the PS II reaction center (Blubaugh and Govindjee 1984; Klimov et al. 1997a; Klimov et al. 1997b; Klimov et al. 1995a; Klimov et al. 1995b; Klimov et al. 2003; Koroidov et al. 2014; Kozlov et al. 2004; Stemler 2002; also see reviews of Govindjee and van Rensen 1993; McConnell et al. 2012; Shevela et al. 2012; Shevela et al. 2023). We begin with the acceptor side effect.
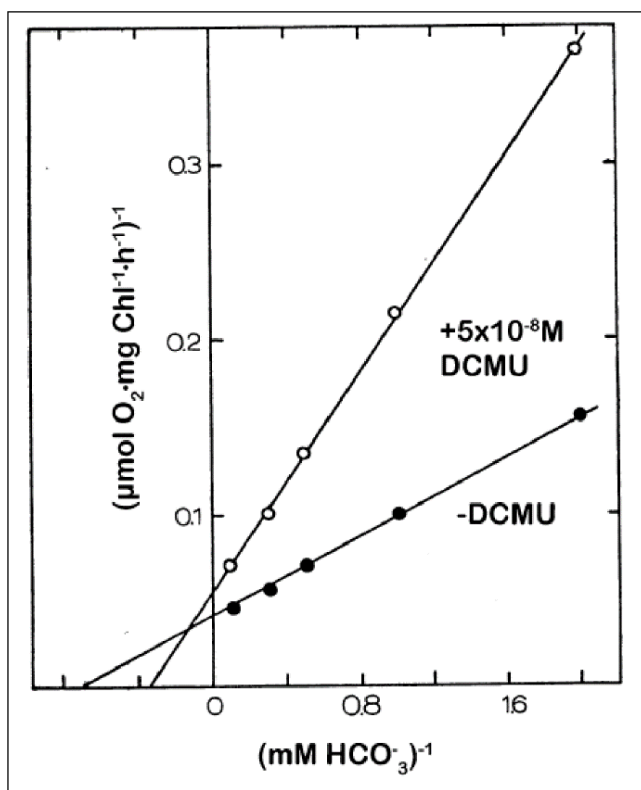
Figure 3. The double reciprocal plot of oxygen evolution as a function of bicarbonate concentration in bicarbonate-depleted pea chloroplasts in the absence and the presence of DCMU (0.05 µM) (reproduced without modification from McConnell et al. 2012).

EFFECTS ON THE ELECTRON ACCEPTOR SIDE OF PS II

During the early part of the 1970s, there was contradictory evidence concerning the location of bicarbonate in the photosynthetic electron transport chain. The very first evidence for the location of bicarbonate binding at the acceptor side of PS II, between Q_A and Q_B, was presented by Wydrzynski and Govindjee (1975). They observed that the absence of bicarbonate ions increased the turnover time of the PS II reaction center. They also noted that Chl *a* fluorescence transients measured as a function of decreasing bicarbonate concentrations were qualitatively similar to those observed with increasing concentrations of DCMU, which blocks the reducing (electron acceptor) side, rather than to transients observed with increasing

concentrations of NH_2OH or prolonged heat treatments, which impose a block on the oxidizing (electron donor) side. Di-phenyl-carbazide (DPC) as well as other artificial PS II donors restored electron flow in heat-treated and tris-treated chloroplasts (known to impair the electron donor side of PS II), but the effects of HCO_3^- depletion and restoration remained, even with these donor systems (Wydrzynski and Govindjee 1975). Subsequent experiments in Govindjee's laboratory provided further convincing evidence on the electron acceptor side effects of bicarbonate (Eaton Rye and Govindjee 1987; Govindjee et al. 1976; Govindjee and Khanna 1978; Govindjee and van Rensen 1978; Jursinic et al. 1976; Khanna et al. 1977; Khanna et al. 1981; for a review see Govindjee 1992). For example, the PS II electron transport prior to Q_A, as measured by O_2 evolution during electron transport from H_2O to silicomolybdate (SiMo), remained uninhibited by HCO_3^- depletion. However, the PS II reduction of oxidized di-amino-durene (DADox), which efficiently accepts electrons from the PQ pool, showed a strong HCO_3^- dependence (see below for discussion; for the use of SiMo, see Zilinskas and Govindjee 1975). These findings suggest that a major site of inhibition (by bicarbonate depletion) is after Q_A, but before the PQ pool. However, Graan (1986) challenged the generally accepted premise that SiMo accepts electrons from Q_A. He argued that all available evidence concerning SiMo involvement with PS II is also consistent with SiMo simply replacing DCMU from the Q_B binding site. Nevertheless, there remains convincing evidence on the involvement of HCO_3^- in electron transport between Q_A and the PQ pool. Using artificial electron donors (DPC, DAD, NH_2OH) and electron acceptors (MV, SiMo), as well as inhibitors (DCMU, DBMIB), it was earlier shown that the major HCO_3^- effect is on the $Q_A^- Q_B$ site of PS II, before the site of action of DBMIB (at the plastoquinone pool) (Khanna et al. 1977). The PS I electron transport, as measured by O_2 uptake during electron transport from reduced di-amino-durene (DADred) to MV, did not show any bicarbonate effect. Since the rates of electron flow were very high indeed, it was firmly established that HCO_3^- is not involved in these reactions.

Khanna et al. (1981) showed that HCO_3^- depletion decreases the binding affinity of atrazine. Similarly, a variety of atrazine-type herbicides have been shown to inhibit HCO_3^- binding (Snel and van Rensen 1983; van Rensen and Vermaas 1981; Vermaas et al. 1982). Most of these herbicides appear not to be competitive with HCO_3^- but bind close enough to be affected by it. Since these herbicides are believed to inhibit PS II by replacing PQ from the Q_B site (Oettmeier and Soll 1983), the binding of HCO_3^- at or near Q_B is a certainty. Eaton-Rye and Govindjee (1984) showed that when hydroxylamine is used to simultaneously inhibit O_2 evolution and to donate electrons to PS II, the reoxidation of Q_A is reversibly inhibited by HCO_3^- depletion. Thus, these experiments reaffirm the location of the HCO_3^- requirement to be after Q_A.

By monitoring the decay of Chl a fluorescence yield after an actinic flash, Jursinic et al. (1976) demonstrated that HCO_3^- depletion slows the oxidation of Q_A^-, and consequently, the reduction of Q_B, resulting in an increase in half-time from about 0.5 ms to approximately 2.6 ms. When the Chl a fluorescence decay was determined as a function of flash number (Govindjee et al. 1976), the oxidation of Q_A^- was even slower after the third and subsequent flashes, with a half-time of about 150 ms. Since Q_B acts as a "two-electron gate," this suggests that two electrons can still flow, albeit slowly, through Q_A to reduce Q_B to Q_B^{2-}, and that the reoxidation of the latter becomes rate-limiting. Thus, it appears that HCO_3^- depletion not only slows down the electron flow from the reduced Q_A to Q_B (especially to Q_B^-), but also leads to blocking the exchange of Q_B^{2-} with the PQ pool.

Inactivation of a portion of PS II also takes place due to HCO_3^- depletion (Jursinic et al. 1976; Siggel et al. 1976; Stemler et al. 1974), which has prompted the suggestion that HCO_3^- is essential for both the structural and functional integrity of PS II. In addition, Jursinic and Stemler (1984) found that a very slow component of the Chl a fluorescence decay, with a half-time of 1-2s, increases two-to-three-fold in HCO_3^- depleted samples, indicating that in a significant portion of the

reaction centers of HCO_3^- depleted chloroplasts, Q_B^{2-} was not re-oxidized in the dark time between flashes, thus keeping the reaction centers in a photosynthetically closed state. Since the increase of this very slow component occurred even after the first flash, Jursinic and Stemler (1984) concluded that it was a component of the electron transfer from the reduced Q_A to Q_B and suggested that HCO_3^- depletion may alter the redox potential of Q_A with respect to Q_B or reduce a local field that stabilizes Q_B. Furthermore, Eaton Rye and Govindjee (1984) observed a 6-7-fold increase in $H_2O \rightarrow MV$ reaction under aerobic conditions upon the addition of HCO_3^- to the HCO_3^--depleted samples. In these experiments, HCO_3^- depletion was shown to reduce the rate of oxidation of Q_A^- dramatically in the presence of artificial donors (such as hydroxylamine and benzidine). A fully reversible HCO_3^- effect on the oxidation of Q_A^- was observed even when the formate ion, previously regarded as an essential factor for the HCO_3^- effect, was absent both in the depleted and enriched samples. These results clearly indicate that the acceptor side of PS II is a major site for the HCO_3^- effect.

It is pertinent to note that Vermaas and Govindjee (1982) did not find any effect of HCO_3^- on the redox potential of Q_A/Q_A^-. However, HCO_3^- depletion seems to have destabilized Q_A by preventing the protonation of a nearby protein group and causing a slow rate of Q_A^- oxidation (Eaton-Rye and Govindjee 1988a). It has been proposed that this slow component is due to the presence of some inactive PS II centers since they don't have bound HCO_3^- (Eaton-Rye and Govindjee 1988a; Garab et al. 1988), and that HCO_3^- depletion somehow increases the number of such centers, perhaps by inhibiting the binding of PQ (Blubaugh 1987). In normal active centers, PQ binding and its release must occur with a half-time of less than 1 ms (Crofts et al. 1984). Robinson et al. (1984) substantiated the above concept through their observation of a slower chlorophyll fluorescence decay of HCO_3^- depleted thylakoids but had obtained much faster rates than were reported by Govindjee et al. (1976), which was attributed to a slower flash frequency

(1 Hz, instead of 33 Hz) that permitted most of the very slow component to decay between the flashes in their experiments.

Interaction of Bicarbonate and Non-heme Iron

The first indication that non-heme iron (NHI) may be involved in bicarbonate action was reported by Vermaas and Rutherford (1984). They showed that the addition of formate (HCO_2^-) to thylakoids increased the amplitude of the electron paramagnetic resonance (EPR) signal (g = 1.82) of Q_A–Fe^{2+} by ten-fold. Bicarbonate drastically decreased the rate of reduction of Q_B by Q_A^-, suggesting its involvement in the protonation of Q_B^{2-} (Eaton-Rye et al. 1986; Govindjee and Eaton-Rye, 1986). Formate is unable to function as bicarbonate since its pKa is 3.8. Indirect evidence for such a function of bicarbonate, in thylakoid membranes, was reported by Eaton-Rye and Govindjee (1987, 1988a, 1988b), who had noted the pH dependence of Q_A^- oxidation after one or two actinic flashes in membranes, with and without bicarbonate. Between pH 6.5 and pH 7.75, both the rate and the amplitude of the initial first-order component of the kinetics of Q_A^- oxidation were found to be pH-dependent. A similar, although quantitatively different, pH dependence was observed for the slow Q_A^- oxidation, by a back reaction with the S2 state, in the presence of DCMU. The replacement of HCO_3^- by HCO_2^- introduced a conformational change in the PS II quinone acceptor complex that is pH-dependent, resulting in a decreased protonation of Q_B^{2-}. All of the above, taken together, agrees with the concept that HCO_3^- is a ligand to Fe^{2+}, while the hydroxyl group of the bound HCO_3^- protonates a dissociable protein group that is functional in the protonation of Q_B^{2-} (Blubaugh and Govindjee 1986; Blubaugh and Govindjee 1988; Crofts et al. 1984; Eaton-Rye and Govindjee 1988a).

Quite remarkably, when Michel and Deisenhofer (1988) compared the primary structure of the L and M polypeptides of the bacterial reaction centers with the D1 and D2 polypeptides of PS II, they suggested that bicarbonate may serve as a functional homologue to the glutamate residue (M232 in *Rps. viridis*) in the bacterial

reaction center that provides ligands to the NHI. There is no homologous glutamate residue in the D1 and D2 sequences, and there is no bicarbonate stimulatory effect in the bacterial system (Shopes et al. 1989). Furthermore, EPR experiments with PS II membranes confirmed the binding of bicarbonate to the non-heme iron (Diner and Petrouleas 1990), although the involvement of M232 as a substitute for bicarbonate could not be confirmed by site-directed mutagenesis at that time (Wang et al. 1992).

Van Rensen et al. (1988) showed that the kinetics of bicarbonate binding to thylakoids are influenced by the redox state of the NHI. Nitric Oxide (NO) has been shown to be able to ligate to the NHI (Diner and Petrouleas 1990). Kinetic measurements of electron transport from reduced Q_A to Q_B indicated that NO treatment shows the same effect of slowing down electron transport as does formate; this effect is completely reversed by the addition of bicarbonate, indicating that it is a ligand to the NHI. Diner et al. (1991) suggested two different patterns for the bicarbonate-NHI binding, in which bicarbonate either binds to the iron as a mono- or a bidentate ligand; these authors suggested that iron-bound bicarbonate may be one of the pathways for the protonation of reduced Q_B. Different ways of binding (ligand formation) of bicarbonate to the NHI were also discussed by Govindjee and van Rensen (1993), in which bicarbonate is stabilized by hydrogen bonding interactions with lysine 265 (numbering from *Pisum sativum*) in the D2 protein (Figure 4). The direct involvement of bicarbonate in binding to the iron is supported by several lines of evidence. For example, Mössbauer spectrum of Fe signal, indicative of the inner-coordination sphere of iron, was found to be significantly affected by the addition of formate, and it was fully restored upon the re-addition of bicarbonate (Diner and Petrouleas, 1987; Govindjee et al. 1997; Semin et al. 1990; van Rensen et al. 1999). Fourier transform infrared (FTIR) difference spectroscopy study, using 14C-bicarbonate, has further indicated that bicarbonate is a bidentate ligand of the NHI in PS II (Hienerwadel and Berthomieu 1995); in addition, the bicarbonate ion was shown to switch from
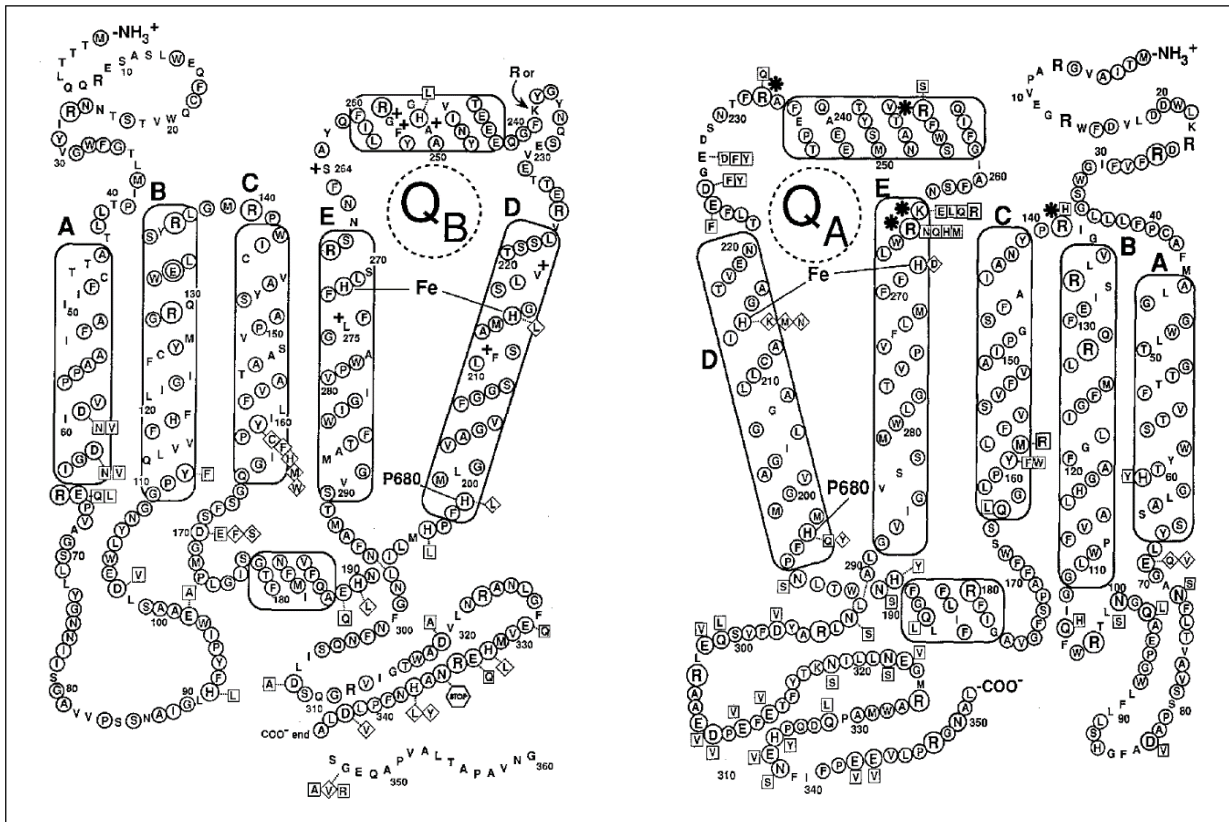
Figure 4. The protein folding model of D1 (left) and D2 (right) polypeptides of *Synechocystis* sp. PCC 6803 showing the amino acids in the vicinity of the bicarbonate binding site at the acceptor side of PS II. The residues indicated by asterisks are associated with the bicarbonate effect (Reproduced with modification from Govindjee and van Rensen, 1993; with permission of the authors).

a chelating to a monodentate binding mode when the iron is oxidized.

Furthermore, Xiong et al. (1996), from Govindjee's laboratory, constructed a model with a bicarbonate and a water molecule positioned in the Q_B binding pocket. They proposed a hypothesis for the role of bicarbonate in the protonation of Q_B^{2-}. In this model, bicarbonate, stabilized by D1-Arg257, could donate a proton to Q_B^{2-} through D1-His252. Additionally, a nearby water molecule could donate another proton to Q_B^{2-}, resulting in the formation of Q_BH_2 (plastoquinol). The residues that form the binding pocket are positively charged and hydrophobic (Xiong et al. 1998a; Xiong et al. 1998b). Furthermore, HCO_3^- is suggested to stabilize the Q_A-Fe-Q_B structure. The available crystal structures of PS II indeed demonstrate HCO_3^- as a ligand of the NHI positioned between the two-electron acceptor side quinones Q_A and Q_B (Ferreira et al.

2004; Loll et al. 2005). X-ray crystallographic and cryo-EM studies have firmly established that HCO_3^- binds as a bidentate ligand to the NHI (Fe^{2+}; NHI) between Q_A and Q_B in cyanobacteria, algae, as well as higher plants (Ago et al. 2016; Guskov et al. 2010; Umena et al. 2011; Wei et al. 2016). The removal of bicarbonate is expected to alter the distance between Q_A and Q_B, slowing down the rate of electron transport from Q_A^- to Q_B, although a more significant effect is seen on the protonation of reduced Q_B^{2-}. In addition to bicarbonate, the NHI appears to be liganded by four histidines of D1 and D2 proteins: D1-His215, D1-His272, D2-His214, and D2-His268 (Figure 5).

Takahashi et al. (2009) proposed, based on FTIR measurements of PS II core complexes, that D1-Tyr246 (or D2-Tyr244) provides a hydrogen bond to the oxygen of the bicarbonate ligand. These authors further suggest
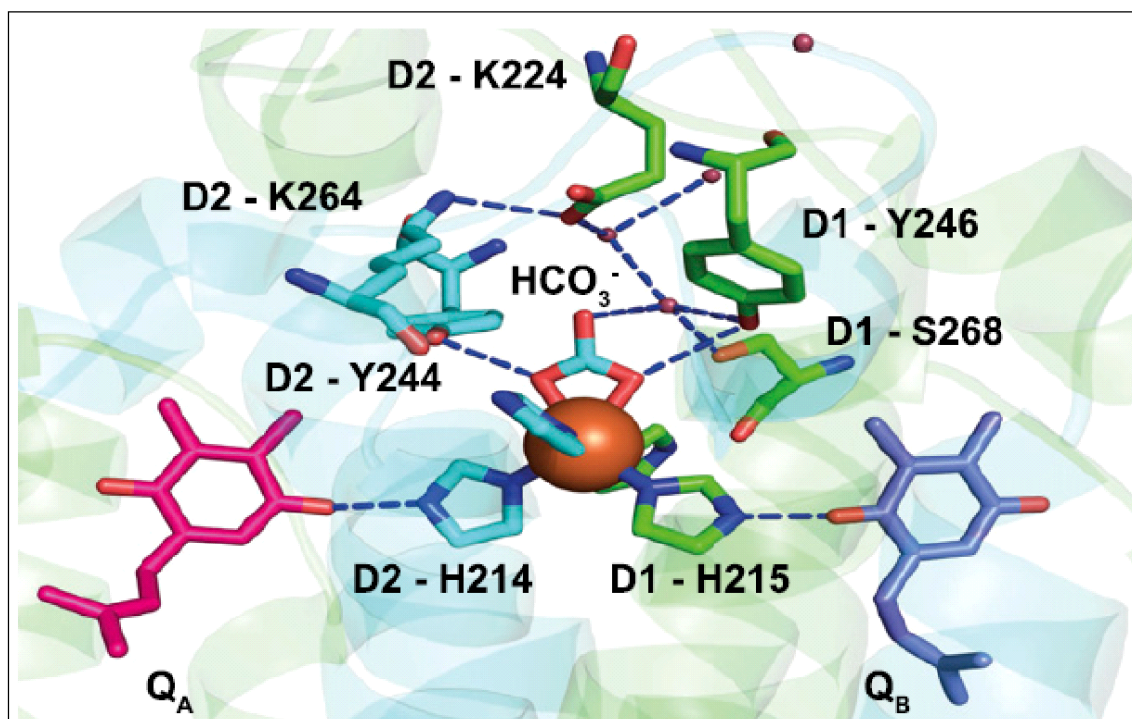
Figure 5. Structure of PS II in the region of the quinones Q_A and Q_B, and the NHI, showing the position of bicarbonate on the electron acceptor side. The H-bond network (represented by broken lines) illustrates the interaction among the relevant amino acids of D1 and D2 proteins in the vicinity of the bicarbonate site. The red beads represent water molecules near the HCO_3^- distal to the Fe^{2+} (represented by the rust-red sphere at the center) (reproduced without modification from Brinkert et al. 2016).

that "the Tyr residue coupled to the non-heme iron may play a key role in the regulatory function of the iron-bicarbonate center by stabilizing the bicarbonate ligand and forming a rigid hydrogen bond network around the non-heme ion." The atomic-level structure, at 1.9 Å resolution of PS II, has provided the final picture (Umena et al. 2011); the closest amino acids to the bicarbonate ion are D2-Tyr244 and D1-Tyr246.

While the heterogeneity of PS II has been linked to differences in the binding of bicarbonate to the NHI, the connectivity of photosystems appears to have a negligible effect on the regulatory role of bicarbonate. It's important to note that the addition of bicarbonate has been shown to block Q_A^- reoxidation by O_2 in the presence of herbicides (Fantuzzi et al. 2023). Dissociation of bicarbonate leads to an increase in the redox potential of Q_A/Q_A^-, and consequently, the presence of Q_A^- decreases the bicarbonate affinity for its binding site on the NHI (Brinkert et al. 2016). Furthermore, these

authors proposed that when the intracellular CO_2 concentration is low, resulting in CO_2 fixation limitation, there is over-reduction of the electron transfer chain and accumulation of a long-lived Q_A^-. This is suggested to trigger the dissociation of bicarbonate by lowering its affinity for the NHI, and the loss of bicarbonate increases the energy gap between the Q_A/Q_A^- and PheoD1/PheoD1-redox couples (Brinkert et al. 2016). This leads to the inhibition of back-reaction, i.e., the formation of P680+Pheo-. Under these conditions, O_2 can bind to the Fe^{2+} and then be reduced by Q_A^-, forming Q_A and O_2^-. Thus, the role of HCO_3^- in PS II also involves a regulatory/protective redox-tuning, linking PS II function to CO_2 concentration.

Chlorophyll *a* fluorescence changes as evidence of the bicarbonate effect

Govindjee and his colleagues were the first to use Chl *a* fluorescence as a tool not only to gather evidence for

the existence of the HCO_3^- effect in PSII but also to discover the major site of binding of HCO_3^- on it (Stemler and Govindjee 1974b). Many previously published results by that time had suggested that HCO_3^- depletion imposed an inhibition on the PS II functions, but the site of binding was not known (Batra and Jagendorf 1965; Punnett and Iyer 1964; Stemler and Govindjee 1973; Stern and Vennesland 1962; Warburg and Krippahl 1958; Warburg and Krippahl 1960), which was later confirmed by measuring Chl a fluorescence transient. Stemler and Govindjee (1974a) reported that F_0 (the initial "O" level fluorescence) and F_M (the maximum "P" level fluorescence) were not affected, but the intermediate inflection showed a rapid rise with HCO_3^- depletion. To explain this fluorescence rise, the authors reasoned that HCO_3^- depletion may block electron flow either before or after Q_B. Since variable Chl a fluorescence ($F_V = F_M - F_0$) remains almost unaffected by HCO_3^- depletion, the authors concluded that the effect is presumably on the oxygen-evolving (electron donor) side of PS II. Further evidence that HCO_3^- is not acting on the reducing (electron acceptor) side of PS II was provided by using long-term delayed light emission, which reflects back reactions in PSII after light-induced charge separation (Stemler et al. 1974). The redox state of Q_A could be assessed by Chl a fluorescence since Q_A is a quencher of fluorescence, not Q_A^- (Duysens and Sweers 1963). Fluorescence induction measurements helped detect a rapid accumulation of Q_A^- due to an inhibition of electron transport beyond Q_A. The first indication for a bicarbonate effect on the electron acceptor side of PS II was deduced through Chl a fluorescence induction kinetics in maize chloroplast fragments after CO_2 depletion and after the re-addition of bicarbonate (Wydrzynski and Govindjee 1975). HCO_3^- depletion accelerated the rise of the Chl a fluorescence transient in a manner similar to the herbicide, DCMU.

As mentioned above, Govindjee et al. (1976) measured Chl a fluorescence to assess the consequences of bicarbonate depletion on the electron transport from the primary electron acceptor, Q_A, to the plastoquinone pool; they concluded that the reoxidation of the reduced form of the electron acceptor Q_A was hampered. The slower decay rate in the absence of HCO_3^- decreased the Hill reaction by 5-10 times under saturating light conditions. Under HCO_3^- depleted conditions, Q_AQ_B remained in the reduced state $Q_A^-Q_B^{2-}$. This conclusion was in agreement with the results on the DCMU-induced Chl a fluorescence rise in the presence of bicarbonate. Similarly, Eaton-Rye and Govindjee (1984) showed that when hydroxylamine is used to simultaneously inhibit O_2 evolution and to donate electrons to PS II, the decay of Chl a fluorescence after a flash, which monitors the reoxidation of Q_A^-, was reversibly inhibited by HCO_3^- depletion. The accelerated rise from F_0 to F_M was due to the faster accumulation of Q_A^-, while the observed slower rise from F_I to F_M represents the filling of the plastoquinone (PQ) pool; only when the PQ pool is fully reduced can $[Q_A^-]$ accumulate to its maximum level (Vermaas and Govindjee 1981). Thorough HCO_3^- depletion causes a complete, or nearly complete, blockage of electron flow from QB to the PQ pool (Vermaas and Govindjee 1982).

Additional evidence for the requirement of bicarbonate on the electron acceptor side of PS II was obtained from comparative measurements on Chl a fluorescence transients of bicarbonate-depleted and PS II herbicide (which displaces Q_B)-treated samples, from studies on the chemical modification of the amino acids on the (electron) acceptor side of PS II, as well as from the use of herbicide-resistant mutants (Govindjee and Van Rensen 1993; Srivastava et al. 1995; Vernotte et al. 1995). Enhanced variable Chl a fluorescence of DCMU-treated (10 µM) thylakoids was observed both in the absence and at high concentration (60 mM) of HCO_3^- (in HCO_3^- - depleted thylakoids). In non-depleted thylakoids, the F_V was independent of the order in which DCMU and HCO_3^- were added, but in HCO_3^- - depleted thylakoids, the effect was seen only when HCO_3^- was added before DCMU (Blubaugh and Govindjee 1984). With this experiment, the effect of HCO_3^- between Q_A and PQ was confirmed. Furthermore, by adding bicarbonate after bathocuproine, Blubaugh and Govindjee (1984) observed a heterotropic binding of these two

compounds and concluded that this effect requires light. They proposed two binding sites for HCO_3^- around PS II: (1) a light-independent high-affinity binding site near the site of DCMU where bicarbonate exerts its major effect and its depletion causes enhancement of Chl *a* fluorescence; and (2) a light-dependent low-affinity binding site (Blubaugh and Govindjee 1984; El-Shintinawy et al. 1990), elsewhere. However, no clear explanation for light-dependent binding of bicarbonate was given at this time. By giving saturating actinic flashes to HCO_3^- - depleted thylakoids of *Synechocystis* sp. 6803, Cao and Govindjee (1988) observed Chl *a* fluorescence changes similar to those observed in DCMU-treated thylakoids. Similarly, by measuring Chl *a* fluorescence yield decay, in the sub-ms range, after various single turnover pre-flashes, the largest slowing down of fluorescence decay was observed after the second or the third flash in the CO_2-depleted samples. Protonation of Q_B^{2-}, mediated by HCO_3^-, occurred after the second flash (Eaton-Rye and Govindjee 1988a; Eaton-Rye and Govindjee 1988b; Govindjee and Van Rensen 1993; Xu et al. 1991). All of the above is consistent with the mechanism of bicarbonate action on the QA-QB site(s), as discussed above.

THE BICARBONATE EFFECT ON THE ELECTRON DONOR SIDE OF PS II

In the early 1970s, the electron donor side of PS II was considered as a possible site for bicarbonate (Stemler and Govindjee 1973; Stemler et al. 1974; see above). Several researchers have suggested that HCO_3^- may act as a substrate or an intermediate in photosynthetic O_2 evolution, possibly coupled with carbonic anhydrase (CA) activity (Kreutz 1974; Lu and Stemler 2002; Lu and Stemler 2005; Metzner 1978; Stemler 1980; Wu 2021a; Wu 2021b; Wu 2022; Wu 2023). Stemler and his collaborators have continued to investigate the possible involvement of HCO_3^- ions in the mechanism of O_2 evolution on the oxidizing (electron donor) side of PS II (see Castelfranco et al. 2007; Li et al. 2023; Lu and Stemler 2002; Lu et al. 2005; Stemler 1980; Stemler 1998; Stemler 2002; Stemler and Castelfranco 2023).

Since the mid-1990s, the idea of an additional role of HCO_3^- on the electron donor side of PS II has been revived through a series of experiments by Slava Klimov and his coworkers (Klimov and Baranov 2001; Klimov et al. 1995a; Klimov et al. 1995b; Klimov et al. 1997a; Klimov et al. 1997b; Klimov et al. 2003). Other research groups have also indicated a requirement for HCO_3^- on the water-splitting side of PS II (Ananyev et al. 2005; Baranov et al. 2004; Kalman et al. 2011; Shutova et al. 2008; Ulas and Brudvig 2010). On the other hand, several experiments in the past, under different experimental conditions, did not show the involvement of HCO_3^- on the electron donor side of PS II (Jursinic et al. 1976; Khanna et al. 1977; Khanna et al. 1981; van Rensen and Vermaas 1981).

Initially, there were contradictions regarding the effect of bicarbonate on oxygen evolution and CO_2 fixation. The stimulation of oxygen evolution by HCO_3^- was observed at low light intensity by some researchers and was found to be enhanced with irradiance (Good 1963; Izawa 1962). Similarly, the enhancement of light-intensity-dependent carbon fixation by the presence of bicarbonate was proposed at that time. However, these results contradicted the findings of West and Hill (1967) and of Stemler and Govindjee (1974c), who showed the HCO_3^- effect to be independent of light intensity, although later Govindjee and his coworkers (Blubaugh and Govindjee 1984; Blubaugh and Govindjee 1988; Govindjee et al. 1983; Govindjee et al. 1985) observed both light-dependent and light-independent effects. It was also proposed that a light-intensity-dependent effect implies that HCO_3^- is acting on enzymatic carbon fixation, while a light-intensity-independent effect implies that the HCO_3^- effect is on the photochemical processes. Stemler and Govindjee (1974b) observed that under HCO_3^--depleted conditions, maize chloroplast fragments lost their oxygen-evolving ability, as well as their capacity to reduce ferricyanide. Furthermore, with these observations on the Hill reaction (DCPIP reduction), they concluded that at least one site of action of bicarbonate is at, or very near, the oxygen-evolving center. They suggested that there is an endogenous

donor that donates electrons to PS II and reduces ferricyanide without the liberation of molecular O_2. However, if HCO_3^- was supplied to the medium, it acted as an electron donor with a proportionate increase in O_2 evolution. In the presence of HCO_3^-, the O_2 evolution is elevated by ~15 fold, and there is a 4-5-fold increase in ferricyanide reduction in maize chloroplasts. The S-state kinetic model for oxygen evolution by Kok et al. (1970) was considered to support this result of Stemler and Govindjee (1974c), as HCO_3^- was suggested to maintain a high oxidation state of the primary electron donor of PS II. However, the observations of Wydrzynski and Govindjee (1975), mentioned above, initiated the idea of the acceptor side effect of HCO_3^-, which was confirmed with many subsequent experiments (see the section above). When chloroplasts were heat-treated to inactivate the oxygen-evolving system, HCO_3^- produced no effect on the partial Hill reaction from diphenyl carbazide (DPC) to dichlorophenol indophenol. In addition, HCO_3^- depleted conditions decreased the S-state transitions in the oxygen-evolving complex, implying that there is a possible site of action of bicarbonate on the electron donor side of PS II (Jursinic et al. 1976; Govindjee and Khanna 1978). Studies by El-Shintinawy et al. (1990); Jursinic and Dennenberg (1990); Stemler and Jursinic (1993); Klimov et al. (1995a,b); Wincencjusz et al. (1996) have also shown that bicarbonate has an effect on the electron donor side function of PS II, in addition to its established effect on the electron acceptor side (see above).

Studies on the time of release of oxygen in single flash exposure to the thylakoid membrane in the presence of formate have shown that it can be restored by the addition of bicarbonate as it causes rapid S state transitions on the initial flash, and the rates of both $S0^* \rightarrow S1$ and $S1^* \rightarrow S2$ become equal (Jursinic and Dennenberg 1990; Stemler 1982; Stemler 1998; Stemler 2002). On the other hand, Govindjee et al. (1989), who did repetitive flash measurements to determine the half-time of decay of the ESR signal II, observed that HCO_3^- depletion did not affect this part of electron flow to PS II. Thus, they suggested that electron flow from "Z"

(Yz, a tyrosine) to the oxidized reaction center of PS II (P680+) was independent of bicarbonate.

However, from 1995, the hypothesis for an additional role of HCO_3^- on the electron donor side of PS II has been revived by experiments showing that HCO_3^- is required for both the maximal activity and the stability of the OEC (Allakhverdiev et al. 1997; Klimov et al. 1995a; Klimov et al. 1995b; Klimov et al. 1997a; Klimov et al. 1997b). The stimulating effects of HCO_3^- ions are especially pronounced during the photoactivation steps (Baranov et al. 2000; Baranov et al. 2004). Furthermore, reconstitution of the Mn cluster after a complete removal of manganese and calcium from PS II preparations was shown to be enhanced by bicarbonate (Ananyev and Dismukes 1996a; Ananyev and Dismukes 1996b; Ananyev et al. 1999; Chen et al. 1995; Miller and Brudvig 1990; Noriaki and Cheniae 1987; Shafiev et al. 1988; Zaltsman et al. 1997). However, it was not clear as to which specific step(s) during the reconstitution of the Mn4 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 PS II protein subunits (photoactivation). This process involves multiple steps that require both light-induced Mn^{2+} oxidation and the binding of a Ca^{2+} ion in the dark for the reactivation of O_2 evolution (Allakhverdiev et al. 1997; Boranov et al. 2000; Klimov et al. 1995a; Klimov et al. 1995b; Klimov et al. 1997a; Klimov et al. 1997b). For the assembly of the functional inorganic core (Mn_4CaO_5Cl) starting from the cofactor-depleted apo-OEC- PS II center and free Mn^{2+}, Ca^{2+}, and Cl^-, two binding sites for bicarbonate were found that stimulate photoactivation by accelerating the formation and suppression of the decay, respectively, of the first light-induced assembly intermediate, apo-OEC-Mn(OH)$_2^+$ (Baranov et al. 2000):

A high-affinity bicarbonate binding site ($Kd \leq 10$ µM) was shown to stimulate the rate of recovery of O_2-evolving centers (Figure 6). This stimulation involves enhanced binding of the initial Mn^{2+} and occurs only at concentrations of Mn^{2+} at or below the stoichiometric requirements for water oxidation (≤ 4 Mn/PS II) and
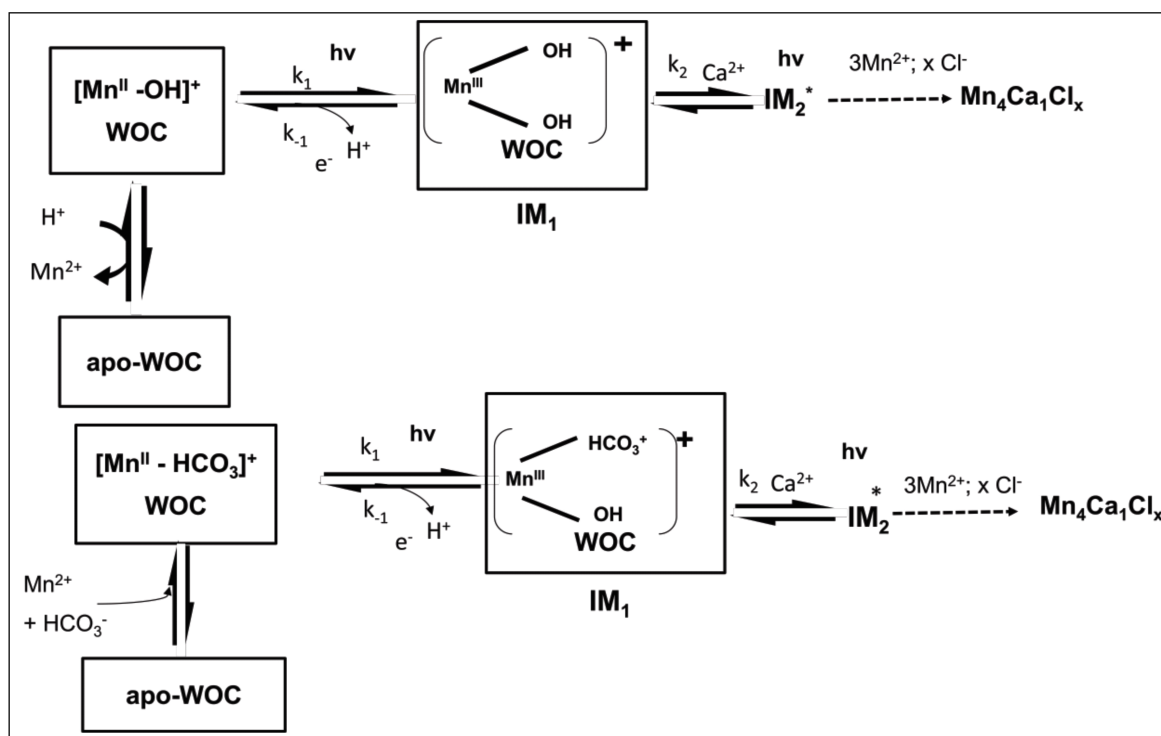
Figure 6. Bicarbonate involvement in the reassembly of the active water oxidizing complex ($Mn_4CaO_5Cl_x$). The model above is based on the photoactivation steps in the formation of Apo-WOC-PS II (reproduced without modification from Baranov et al. 2000).

disappears above 4 Mn/PS II. The absence of an effect by added bicarbonate on photoactivation kinetics and yield at saturating concentrations of Mn^{2+} and Ca^{2+} has been attributed to the availability of atmospheric bicarbonate (~4 μM at pH 6.0), which is sufficient for the photoactivation step.

A second low-affinity bicarbonate site has also been observed; it has been shown to stimulate the rate of formation of IM1[apo-WOC-Mn(OH)$_2^+$], but with much lower affinity (Kd at millimolar level); further, it becomes observable only at low concentrations of Ca^{2+} that are limiting for photoactivation.

Baranov et al. (2000) presented four interpretations of the high-affinity bicarbonate effect: (i) it might act as an integral cofactor within the OEC (possibly serving as a ligand to the first Mn); (ii) it functions as a Bronsted base, accelerating proton release during the formation of either the dark precursor [apo-OEC-Mn(OH)$^+$] or IM1 [apo-OEC-Mn(OH)$_2^+$]; (iii) it directly supplies one or more hydroxide ions during the formation of the latter two

species (with the release of CO_2); or (iv) it acts as a membrane-soluble anion, thereby electro-statically elevating the local concentration of Mn^{2+} in PS II.

Electrochemical and EPR characterizations of HCO_3^- complexes with MnII and MnIII ions indicate that these ions form electro-neutral complexes. The dissociation constant (Kd) of the MnIII-HCO_3 complex is nearly 10 orders lower than that of the MnII-HCO_3 complex (Kozlov et al. 2004). These properties of MnII-HCO_3 complexes may facilitate the photo-induced assembly of the inorganic core of the OEC (Dismukes et al. 2001; Kozlov et al. 2004). These findings align with proposals by Klimnov and his associates (Klimov and Baranov 2001; van Rensen and Klimov 2005): (a) HCO_3^- is bound to or is a structural component of the assembled Mn_4CaO_x cluster; (b) HCO_3^- remains bound in the vicinity of the Mn_4CaO_5 cluster; or (c) HCO_3^- is required during photoactivation and subsequently leaves the site.

Klimov and his coworkers have established, through numerous experiments, the functional role of HCO_3^- as a

ligand to the Mn_4CaO_5 cluster, serving as an essential cofactor in stabilizing the water-oxidizing complex (Klimov and Baranov 2001). Ferreira et al. (2004) found, at 3.5 Å resolution, that HCO_3^- (or CO_3^{2-}) may be involved as a ligand bridging Mn and Ca ions within the OEC. However, higher-resolution X-ray crystallography studies of PS II seemed to reject this notion, instead showing HCO_3^- as a ligand between Q_A and Q_B. Various techniques, including UV spectro-photometry under high backpressure of CO_2, mass spectrometry (MS) with ^{18}O-labeling of H_2O and HCO_3^-, GC-MS, light-induced FT-IR difference spectroscopic analysis, high-resolution crystallography, computational models based on density functional theory (DFT), and quantum mechanics/molecular mechanics studies, have not confirmed the presence of HCO_3^- as a significant intermediate substrate (ligand) for photosynthetic water oxidation. Thus, there is no conclusive support for the concept of water being transported to the Mn_4O_5Ca cluster in the form of HCO_3^- (or peroxydicarbonic acid; $H_2C_2O_6$) (Castelfranco et al. 2007; Shevela et al. 2012; Stemler and Castelfranco 2023). FT-IR spectroscopy, which examined the structural coupling of HCO_3^- to the OEC, has not indicated any HCO_3^- band from the OEC during the S-state transitions (Aoyama et al. 2008). This is consistent with results obtained by flash-induced O_2 evolution pattern (FIOP) studies, where the redox potentials of the S states of the OEC were unaffected by HCO_3^- depletion via washing with CO_2/HCO_3^--free buffer (Shevela et al. 2007).

Clausen et al. (2005) studied possible product inhibition of electron transfer into the catalytic Mn_4CaO_5 complex during the oxygen-evolving reaction by significantly increasing CO_2 pressure. They found 50% inhibition by raising the O_2 pressure only tenfold over ambient, excluding the idea that exchangeable bicarbonate is the substrate for (and CO_2 an intermediate product of) oxygen evolution by photosynthesis. However, they support the involvement of firmly bound or sequestered bicarbonate in water oxidation, consistent with the idea of Stemler and Castelfranco (2023). It remains conceivable that bound HCO_3^- may (i) be part of a deprotonation pathway;

(ii) alter the redox properties of the Mn_4CaO_5 complex; (iii) stabilize the metal-cluster as a ligand to manganese and/or calcium; or (iv) provide a binding site for substrate water (also, see: Klimov et al. 1995a; Klimov et al. 1995b).

Shevela et al. (2006) demonstrated that the hydrazine-induced transition of the OEC to super-reduced S-states depends on the presence of bicarbonate in the medium. After a 20-minute treatment of isolated spinach thylakoids with 3 mM NH_2NH_2 at 20°C in the CO_2/HCO_3^--depleted buffer, the S-state population is high (42%) in the S3 state, but the S4 state is reached easily in the presence of 2 mM $NaHCO_3$. However, the same treatment produces less (30%) S3 state and no S4 state when bicarbonate is reduced. The bicarbonate requirement for oxygen-evolving activity is low in untreated thylakoids but considerably increases during the transition of the OEC to the super-reduced S-states. However, the bicarbonate requirement becomes low again when the OEC returns to the normal S-states after pre-illumination, suggesting that bicarbonate is associated with manganese ions within the OEC (Shevela et al. 2006).

Carrieri et al. (2007) reported an in vivo requirement for bicarbonate that is both reversible and selective for efficient water oxidation activity in a hyper-carbonate-requiring cyanobacterium *Arthrospira maxima*. Using F_V, Carrieri and co-workers observed a very large reversible bicarbonate effect on the PS II activity, indicating the requirement for bicarbonate on the water-oxidizing complex. Ananayev et al. (2005) interpreted their results on a mutant of CP43-arginine-357 to serine in *Synechocystis* sp. 6803 to imply that arginine R357 functions in binding a (bi)carbonate ion, essential for the normal catalytic turnover of the water-oxidizing complex. They postulated that bicarbonate, through hydrogen bonds with R357, abstracts protons from oxidized water molecules (Ananyev et al. 2005; cf. McEvoy and Brudvig 2004). On the other hand, Villarejo et al. (2002) proposed that bicarbonate may act as the endogenous base for protons released into the lumen upon water oxidation. All these ideas warrant serious

consideration, and future research should aim to precisely determine how bicarbonate functions on the water oxidation side of PS II.

Yruela et al. (1998) suggested that bicarbonate (rather than a carboxylic group of amino acid residues ligating the inorganic core of the OEC; cf. Noguchi et al. 1995) acts as a bridging ligand between a Mn-ion and a Ca^{2+} within the OEC. Later, from the X-ray analysis of the OEC structure (Ferreira et al. 2004), a similar suggestion was made, where a bicarbonate (or carbonate) anion was "predicted" to be located between Ca^{2+} and Mn. However, the 3.5 Å resolution may not be high enough to confirm this conclusion. At the 3.0 Å resolution, bicarbonate, as a ligand to Mn, was not observed by Loll et al. (2005). Further research is necessary, at different pH levels, as there may have been a loss of bicarbonate from the OEC due to the reduction of MnIII ions to MnII caused by X-ray irradiation and the treatment required during X-ray measurements.

It has already been shown that bicarbonate ions are required for both the maximal activity and the stability of the OEC in PS II (Allakhverdiev 1997; Klimov et al. 1995a; Klimov et al. 1995b; Klimov et al. 1997a; Klimov et al. 1997b). The stimulating effects of bicarbonate are especially pronounced during the reactivation of the electron donor side of PS II with MnII ions added to Mn-depleted PS II preparations (Baranov et al. 2000; Baranov et al. 2004). Various suggestions have been made regarding the possible role of bicarbonate within the OEC of PS II (e.g., Klimov and Baranov 2001; Stemler and Castelfranco 2023; van Rensen and Klimov 2005). Further exploration is needed to ascertain whether bicarbonate can indeed be considered a direct ligand to the Mn_4CaO_5-cluster and whether its removal from the OEC makes the Mn_4CaO_5-cluster unstable. Shevela et al. (2006) demonstrated that reducing the bicarbonate concentration in photosynthetic samples by 5-fold relative to air-saturated buffers did not affect the redox potential of the OEC in PS II. Even at ~50-fold reduced bicarbonate levels, the rate of reduction of the OEC by NH_2OH remained unchanged. Therefore, it appears likely

that bicarbonate, after its possible involvement in the assembly of the Mn_4CaO_5 cluster, leaves the OEC. Alternatively, the ion could remain so tightly bound to the OEC that no one has been able to remove it by washing with HCO_3^-/CO_2-depleted buffer. However, no clear evidence of such tightly bound bicarbonate is yet available, with the only definite site being on the electron acceptor side of PS II. An open mind is needed. HCO_3^- was shown to be a transient ligand to Mn ions during the photo-assembly process of the Mn_4O_5Ca cluster in the OEC-depleted PS II centers (Baranov et al. 2004; Dasgupta et al. 2007; Kozlov et al. 2010). Furthermore, Klimov and Baranov (2001) demonstrated a pronounced stimulating effect of HCO_3^- ions on electron donation from exogenous Mn^{2+} ions to Mn-depleted PS II and the photo-induced reconstitution of the functional OEC (Allakhverdiev et al. 1997; Allakhverdiev et al. 2011; Hulsebosch et al. 1998; Klimov et al. 1995a; Klimov et al. 1995b). We await future research in this area.

PS II-DONOR-SIDE-ASSOCIATED CARBONIC ANHYDRASE (CA) ACTIVITY

The CA-type action of PS II was proposed as early as 1980 by Alan Stemler (Stemler 1980). Since then, several reports have shown that easily exchangeable HCO_3^- ions improve water oxidation by acting as specific acceptors of protons during this process (Ananyev et al. 2005; Koroidov et al. 2014; Shevela et al. 2013; Shutova et al. 2008; Villarejo et al. 2002). This process is coupled with the PS II-donor-side-associated carbonic anhydrase (CA). Deprotonation reactions and the removal of protons away from the OEC are thought to have a significant impact on the thermodynamics of the water-splitting process. Ananyev et al. (2005) proposed that HCO_3^- may play an indirect role in water splitting as a proton transfer mediator, and some results support this assumption (Shutova et al. 2008; Ulas and Brudvig 2010; Ulas et al. 2008). For example, Stemler (1985, 1997) suggested that a thylakoid CA might be involved in the 'donor-side' effects of HCO_3^- (also see: Moubarak-Malid and Stemler 1994; Lu and Stemler 2002; Lu and Stemler 2005). Shutova et al. (2008) showed that in

Chlamydomonas reinhardtii, both HCO_3^- and Cah3 (the CA protein in *C. reinhardtii* associated with the PS II donor side) have specific 'donor-side' effects on the proton release steps but not on the electron transfer per se. Furthermore, Shutova et al. (2008) suggested that a CA/HCO_3^- system in *C. reinhardtii* may facilitate proton removal away from the OEC during water splitting by accelerating interconversion between HCO_3^- and CO_2. Additionally, it was suggested that HCO_3^- may stabilize the OEC via binding to the extrinsic proteins, specifically to the manganese-stabilizing PsbO protein (Pobeguts et al. 2007; Pobeguts et al. 2010). However, Tikonov (2018) presented a new approach for the quantification of bicarbonate (HCO_3^-) molecules bound to PS II, where he used a combination of membrane-inlet mass spectrometry (MIMS) and ^{18}O-labeling. This approach excludes the possibility of "non-accounted" HCO_3^- by avoiding the use of formate to remove HCO_3^- from PS II and by employing extremely low concentrations of HCO_3^-/CO_2 during online MIMS measurements. In spinach PS II membrane fragments, Tikonov (2018) observed that 1.1 ± 0.1 HCO_3^- is bound per PS II reaction center, while none is bound to the isolated PsbO protein, suggesting that PS II binds only one HCO_3^- molecule as a ligand to the NHI of PS II, while unbound HCO_3^- optimizes the water-splitting reactions by acting as a mobile proton shuttle. However, this experiment needs to be redone, particularly at different pH levels. A photoprotective role of HCO_3^-, which controls chlorophyll triplet state-mediated singlet oxygen formation, has been suggested by Brinkert et al. (2016). Fantuzzi et al. (2023) reported that PS II monomers from the stromal lamellae contain PsbS, which limits HCO_3^- binding, whereas those of the granal lamellae are activated by HCO_3^- binding.

The possibility of bicarbonate functioning as a ligand to the OEC or a substrate in the oxygen-evolution reaction has been excluded by many researchers. However, experiments utilizing bicarbonate as a mobile proton carrier to probe the proton-transfer pathway on the electron donor side of PS II have been conducted (Banerjee et al. 2019; Debus 2015; Ho 2012; Pokhrel et al. 2013). Analysis of several single-point mutations D1-D61A, D2-K317A, D1-E65A, D1-R334A, using FT-IR studies and flash-induced polarographic measurements, has been instrumental in tracing the proton-transfer pathway on the electron donor side of PS II (Ho, 2012; Pokhrel et al. 2013; Debus, 2015). Computational analyses have also designated the above-mentioned residues as part of the proton-transfer channel (Ho, 2012). Banerjee et al. (2019) used bicarbonate as a mobile exogenous proton-transfer reagent to recover the activity lost by the above-mentioned site-directed mutations to identify amino acid residues participating in the proton-transfer pathway. Banerjee and coworkers found that bicarbonate restores efficient S-state cycling in D2-K317A PS II core complexes but not in D1-D61A and CP43-R357K PS II core complexes, indicating that chemical rescue by bicarbonate can be used to differentiate single-point mutations affecting the pathways of proton transfer from mutations that affect other aspects of the water-oxidation mechanism. It is interesting to note that perturbations in water oxidation by D1-S169A substitution have also been reported (Ghosh et al. 2019); thus, the future of understanding how bicarbonate plays a key role on the electron donor (the water oxidation) side is not far from us – whereas that for its action on the electron acceptor side has already been revealed, mainly pioneered by Govindjee and his research students.

Contradicting the conclusions of many scientists (See Govindjee and van Rensen 1993; Govindjee et al. 2006), Hiller et al. (2006) had suggested the presence of a CA type activity of PS II but concluded that bicarbonate is not a physiologically significant substrate and is not directly a source for photosynthetic oxygen evolution; nevertheless, PS II CA activity is a determinant for the rate of oxygen evolution. On the other hand, using labeled $HC^{18}O_3^-$, Delsome and Joliot (2002) found that PS II, like CA, has a long-lasting catalytic activity (more than a second), which almost leads to full exchange of heavy oxygen in CO_2 with oxygen in H_2O, resulting in a minimal amount of heavy O_2 in $HC^{18}O_3^-$. Therefore, if the photolysis of $HC^{18}O_3^-$ (if at all present) occurs in HCO_3^--depleted maize chloroplast fragments, the oxygen evolved would be (almost) entirely of the normal type.

Figure 7. A scheme showing a combined pathway of bicarbonate and water photolysis in photosynthetic oxygen evolution. The CA (carbonic anhydrase) activity converts CO_2 to bicarbonate. Bicarbonate photolysis and water photolysis work together and release oxygen and carbon dioxide in a 1:1 (mol/mol) stoichiometry; in the scheme, Calvin cycle should be read as the Calvin-Benson-Bassham cycle (reproduced without modification from Wu 2023).
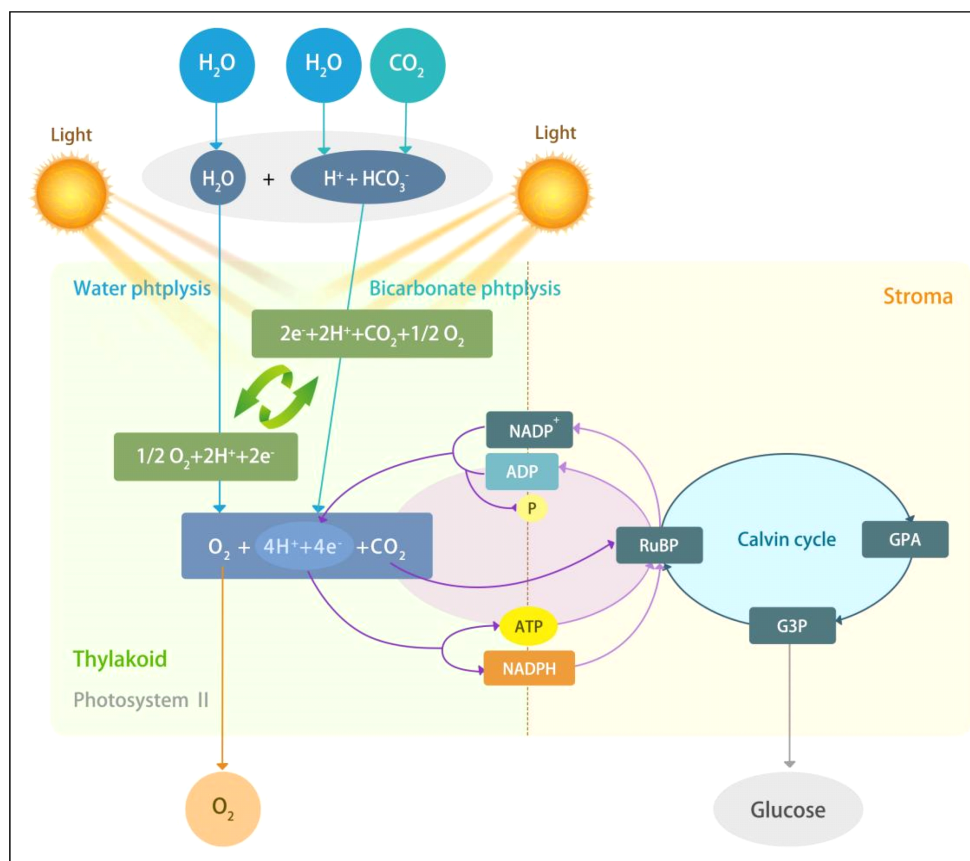
However, Wu (2021a) has argued that HCO_3^- is a direct substrate in photosynthetic oxygen evolution at PS II. He has observed that HCO_3^- would exchange with almost all oxygen in water molecules, and therefore it is difficult to compartmentalize whether oxygen has come from water only or a combination of the O_2 evolution from HCO_3^- and water. By arguments from geochemistry, bicarbonate photolysis and water photolysis as well as their possible roles in photosynthesis, Wu (2021a, 2021b, 2022, 2023) has suggested a synthetic formula for oxygen evolution as: $2H_2O + CO_2 \rightarrow H_2O + H^+ + HCO_3^- \rightarrow O_2 + 4e^- + 4H^+ + CO_2$ (Figure 7). He has also suggested that PS II functions as CA to catalyze the reaction of CO_2 hydration under physiological conditions, and CO_2 hydration coupled with chemical equilibrium, $H^+ + HCO_3^- \rightarrow 1/2O_2 + 2e^- + 2H^+ + CO_2$, occurs in a PS II core complex. Thus, water photolysis and bicarbonate photolysis account for half of the oxygen evolution, respectively, by PS II (Wu 2023). However, it is necessary to question and to develop and optimize experimental protocols for obtaining reproducible results to confirm the derived assumptions on such CA type activity of PS II and O_2 evolution from water via HCO_3^- as a catalyst.

CONCLUDING REMARK

The extensive research on the "bicarbonate effect" on PS II activity, particularly the pioneering work by Govindjee and his colleagues at UIUC starting in 1973, has significantly advanced our understanding of the role of bicarbonate in photosynthesis. This research has delved into the mechanisms and sites of action of bicarbonate on both sides of PS II.

The evidence supporting bicarbonate as a ligand to the quinone-NHI complex at the acceptor side of PS II demonstrates a crucial role for HCO_3^- in facilitating and regulating electron transfer from PS II to PS I,

both in isolated systems and in living organisms. The presence of HCO_3^- as a bidentate ligand bridging Q_A and Q_B and its involvement in Q_B^{2-} protonation have been convincingly established. It's noteworthy that the absence of HCO_3^- leads to a down-regulation of this electron transfer step. Given the universality of HCO_3^-'s action in all oxygenic photosynthetic organisms, it is evident that this ligand's role evolved very early in the evolution of oxygenic photosynthesis.

Quantitative membrane-inlet mass spectroscopic studies have indicated that there is typically only one bound HCO_3^- per PS II. However, there is still experimental evidence pointing to a potential role for this ligand on the electron donor side of PS II, which requires further investigation. Some researchers have proposed an indirect role for bicarbonate in water splitting and as a mediator of proton transfer (Ananyev et al. 2005; Shutova et al. 2008; Ulas and Brudvig 2010). Additionally, it has been suggested that bicarbonate may stabilize the OEC through its binding to the PsbO protein (Pobeguts et al. 2007; 2010). Recent findings have indicated that a PS II monomer with PsbS and Psb27 as additional subunits, while inactive when isolated, becomes activated in the presence of bicarbonate, representing a late-stage intermediate in the photo-assembly of PS II (Fantuzzi et al. 2023). However, as of now, no conclusive evidence has been obtained for the presence of bound bicarbonate on the donor side of PS II. It is hypothesized that bicarbonate is firmly bound to the acceptor side while acting as a mobile proton shuttle on the donor side of PS II (Debus 2015; Banerjee et al. 2019). Nevertheless, further research is necessary to pinpoint any potential binding sites for bicarbonate on the waterside of PS II. Additionally, ongoing investigations are required to explore the CA-type action of PS II and to validate the assumption that bicarbonate serves as a direct substrate for a portion of photosynthetic oxygen evolution.

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