Minireview

Role of bicarbonate in photosystem II, the water-plastoquinone oxido-reductase of plant photosynthesis

Jack J. S. van Rensen[a],*, Chunhe Xu[b] and Govindjee[c]

[a]*The Graduate School of Experimental Plant Sciences, Laboratory of Plant Physiology, Agricultural University, Arboretumlaan 4, 6703 BD Wageningen, The Netherlands*
[b]*Institute of Plant Physiology, Chinese Academy of Sciences, Shanghai, 200032, China*
[c]*Departments of Plant Biology and Biochemistry, and Center of Biophysics and Computational Biology, University of Illinois, 265 Morrill Hall, 505 S. Goodwin Ave, Urbana, IL 61801-3707, USA*
Corresponding author, e-mail: Jack.VanRensen@algem.PF.WAU.NL

Depletion of bicarbonate (carbon dioxide) from oxygenic cells or organelles not only causes cessation of carbon dioxide fixation, but also a strong decrease in the activity of photosystem II; the photosystem II activity can be restored by readdition of bicarbonate. Effects of bicarbonate exist on both the acceptor as well as on the donor side of photosystem II. The influence on the acceptor side is located between the primary and secondary quinone electron acceptor of photosystem II, and can be demonstrated in intact cells or leaves as well as in isolated thylakoids and reaction center preparations. At physiological pH, bicarbonate ions are suggested to form hydrogen bonds to several amino acids on both D1 and D2 proteins, the reaction center subunits of photosystem II, as well as to form ligands to the non-heme iron between the D1 and D2 proteins. Bicarbonate, at physiological pH, has an important role in the water-plastoquinone oxido-reductase: on the one hand it may stabilize, by conformational means, the reaction center protein of photosystem II that allows efficient electron flow and protonation of certain amino acids near the secondary quinone electron acceptor of photosystem II; and, on the other hand, it appears to play a significant role in the assembly or functioning of the manganese complex at the donor side. Functional roles of bicarbonate in vivo, including protection against photoinhibition, are also discussed.

Introduction

The primary photosynthetic reactions in plants, algae, prochlorophytes and cyanobacteria take place in the photosystem II (PSII) and photosystem I (PSI) complexes located within the thylakoid membrane (see Govindjee (1999) for a historical account). The reaction center of PSII carries out photochemical reactions, including the primary charge separation and the subsequent electron transfer from water to plastoquinone. Hence, this protein complex can be called water-plastoquinone oxido-reductase (Fig. 1). At the expense of light energy, water is split, and oxygen and plastoquinol are formed. The photochemically active PSII reaction center contains six polypeptides: D1, D2, a heterodimer of cytochrome b_{559} and the gene products of *psb*I and *psb*W. The central core of the PSII reaction center consists of the D1 and D2 proteins where all the redox active components are embedded. These components include a tetra-manganese cluster, two tyrosine residues, six chorophyll (Chl) *a* molecules, two pheophytins, the plastoquinones Q_A and Q_B and a non-heme iron, that does not directly participate in electron transfer, but is vital for the transfer process.

Carbon dioxide is required for photosynthesis. It is fixed by ribulose 1,5-bisphosphate carboxylase and further reduced to carbohydrate. However, CO_2 is also involved in the photosynthetic electron transport of plants, algae and cyanobacteria; it is called the 'bicarbonate effect'. This phenomenon includes decreased activity of PSII (any reaction between water oxidation and the reduction of plastoquinone) upon depletion from CO_2/bicarbonate, which is restored by addition of bicarbonate. Warburg and Krippahl (1958) were the first to show that CO_2 accelerates the production of oxygen on illumination of isolated chloroplasts in the presence of an electron acceptor like ferricyanide. This phenomenon was experimentally difficult to study. However, Stemler and Govindjee (1973) developed a dependable method (described in the next section) to investigate the bicarbonate effect. Since then, many research groups have investigated this phenomenon. It can be seen at the level of the intact leaf as well as in isolated reaction center preparations. The requirement for bicarbonate is located in PSII, both at the acceptor and the donor side. References to older literature can be found in the reviews by Govindjee and Van Rensen (1978), Blubaugh and Govindjee (1988) and Govindjee and Van Rensen (1993). This minireview mainly describes new developments since 1992.

The requirement of photosystem II for bicarbonate

The initial experiments of Warburg and Krippahl (1958) were difficult to reproduce until Stemler and Govindjee
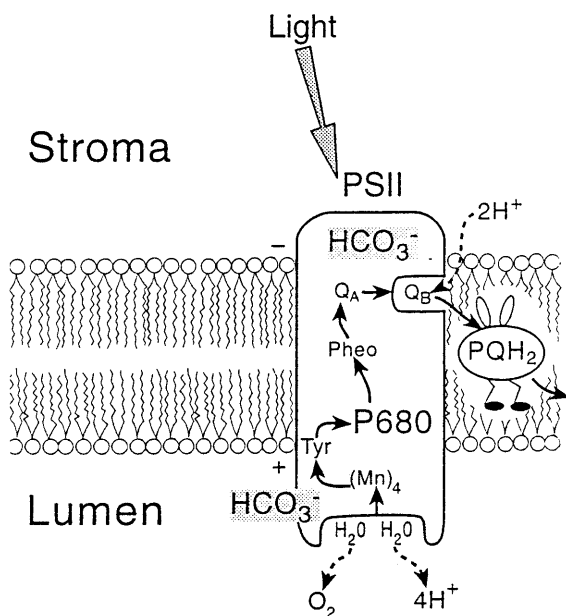

Fig. 1. A cartoon for photosystem II (PSII): it oxidizes water to oxygen, reduces a plastoquinone molecule and releases protons in the interior of the thylakoid membrane; it is also called water-plastoquinone oxido-reductase. P680 is the reaction center chlorophyll of PSII; Pheo is pheophytin; Q_A and Q_B are bound plastoquinones in PSII; PQH_2 is reduced plastoquinone (i.e. plastoquinol). Existence of bicarbonate effect in PSII is highlighted by showing it at two places: on the acceptor as well as the donor side. For a molecular model of PSII, see Xiong et al. (1998a).

(1973) described a method whereby reproducible and large increases in the rate of the Hill reaction in isolated chloroplasts could be observed upon the addition of bicarbonate to CO_2-depleted samples. The method depends on depletion of the chloroplasts from CO_2 by flushing the suspension in the dark with nitrogen gas (or CO_2-depleted air), while chloroplasts are suspended in a medium containing high concentrations of formate or acetate at a pH between 5.5 and 6.0. The resulting electron transport rate is then measured usually at a pH of 6.5. The electron transport rate in such samples is extremely low, but is restored to control values after incubation of about 1 min in the dark with bicarbonate, but nothing else. In all likelihood, CO_2 is the diffusing species, while bicarbonate is the binding species. Because the stimulation is evoked by the addition of a bicarbonate solution to CO_2-depleted thylakoids, the phenomenon usually is called the 'bicarbonate effect'. Included in this phenomenon are the stimulatory effects of bicarbonate when the inhibitor is nitric oxide (Petrouleas and Diner 1990) or other weak anions, as acetate, azide or nitrite (Stemler and Murphy 1985, Govindjee et al. 1997a), or even when no such inhibitors are used.

Stemler (1989) claimed to have observed normal electron flow activity in isolated thylakoids when the bicarbonate binding site at PSII was empty of all monovalent anions, including bicarbonate. Jursinic and Stemler (1992) discussed two hypotheses for the bicarbonate effect: (1) the 'bound bicarbonate' hypothesis: a high-affinity binding of bicarbonate is required for high rates of electron transport; and (2) the 'inhibitory-anion' hypothesis, also called the 'empty site' hypothesis: when the binding site is entirely free of any anion, high rates of electron flow may occur; weak anions like formate are inhibitory, and the only function of bicarbonate is to keep these inhibitory anions away. These two possibilities differ in the residency state of the binding site for bicarbonate when normal (rapid) electron flow occurs. Because Jursinic and Stemler (1992) did not find any CO_2 in CO_2-depleted thylakoids suspended in low (10 mM) NaCl medium showing high rates of electron flow, they concluded that the inhibitory-anion (or empty site) hypothesis is correct, implying that bicarbonate is not a requirement for PSII activity. Alternatively, Cao et al. (1992), using a kinetic model, were unable to distinguish between what they had called essential and nonessential activator models, equivalent to the 'bound bicarbonate' and the 'empty site' hypothesis, respectively. In addition, Govindjee et al. (1991) and Govindjee et al. (1997b) were able to detect the presence of CO_2 under conditions similar to those used by Jursinic and Stemler (1992). It thus appears that the 'bound bicarbonate' and not the 'empty site' hypothesis is correct. In normal systems, bicarbonate is bound to PSII and functions in the activity of PSII. However, under certain conditions, a water molecule itself may replace bicarbonate (Xiong et al. 1996).

While most research on the bicarbonate effect has been performed using isolated thylakoids, the requirement for bicarbonate has also been demonstrated in intact systems. For instance, using thermoluminescence, Garab et al. (1988) demonstrated the bicarbonate effect in intact leaves and Govindjee et al. (1993) monitored the requirement for bicar-

bonate in intact algal and cyanobacterial cells by measuring the antagonistic effects of light-I and light-II on the chlorophyll (Chl) a fluorescence yield and P700 turnover. However, no influence of bicarbonate has ever been demonstrated in PSI nor in anoxygenic photosynthetic bacteria (Shopes et al. 1989, Wang et al. 1992).

It is difficult to judge whether or not the activity of PSII in vivo is controlled by the bicarbonate concentration. The K_m of PSII for bicarbonate is 40–80 μM (Snel and Van Rensen 1984, Blubaugh and Govindjee 1988). According to Edwards and Walker (1983) (see pp. 414–415), the equilibration of CO_2 with bicarbonate in the leaf is very dependent on pH. In addition, the solubility of CO_2 in water is dependent on the temperature; solubility increases with lower temperatures. At 325 ppm atmospheric CO_2 and 30°C, the amount of dissolved CO_2 is 9.7 μM. Using the Henderson-Hasselbach equation, the bicarbonate concentration can be calculated to be 9.7 μM at a pH of 6.3. The bicarbonate concentration is much higher at higher pH values: 96.5 μM at pH 7.4 and 432 μM at pH 8.0. It should be mentioned that the presence of carbonic anhydrase allows a faster equilibration between CO_2 and $HCO_3^- + H^+$. Now the problem exists that during continuous light, the pH in the stroma may reach values as high as pH 8; in the thylakoid lumen it may become as low as 5, and the bicarbonate binding site is within the thylakoid membrane, most probably directed more to the stroma side. Thus, the actual pH at the bicarbonate binding site is not known. In the case that the pH at the bicarbonate binding site is 7 or higher, the bicarbonate concentration may not be apparently limiting for the activity of PSII. However, there are conditions during which the stomata close: drought, high temperature or high light intensity. Under those conditions, the CO_2 concentration in the chloroplast drops and the bicarbonate concentration may become low enough to decrease PSII activity.

Action of bicarbonate on the acceptor side of photosystem II

Warburg (1964) had argued that CO_2 must be the source of oxygen in photosynthesis, contrary to the generally accepted idea that water is the source of oxygen. Further, Stemler (1980) advocated the idea that the bicarbonate effect was mainly on water oxidation in PSII. However, it is now known that there is a major influence of bicarbonate on the acceptor side of PSII.

The first indication for a bicarbonate effect to be on the electron acceptor side of PSII was obtained by Wydrzynski and Govindjee (1975), who measured Chl a fluorescence induction kinetics in thylakoids after CO_2 depletion and after readdition of bicarbonate. Depletion of bicarbonate led to faster fluorescence rise, as is also the case when a herbicide, known to block electron flow at the PSII acceptor side, is added. However, the addition of bicarbonate restored normal fluorescence. Additional evidence for a requirement for bicarbonate at the reducing side of PSII came from experiments on the interaction between bicarbonate and PSII herbicides (that displace Q_B), studies on the chem-

ical modification of the amino acids on the acceptor side of PSII and from the use of herbicide-resistant mutants [details and references can be found in the review by Govindjee and Van Rensen (1993), also see Srivastava et al. (1995) and Vernotte et al. (1995)]. By the measurement of Chl a fluorescence yield decay, in the subms-ms range, after various single turnover preflashes, known to measure electron transfer from Q_A^- to Q_B, the requirement for bicarbonate was shown to be located between Q_A and Q_B (Eaton-Rye and Govindjee 1988, Xu et al. 1991). Because the largest slowing down of fluorescence decay was apparent after the second or the third flash in the CO_2-depleted samples, it was concluded that in the presence of formate (and thus, absence of bicarbonate) the protonation of Q_B^{2-} was prevented, because protonation occurs after the second, not the first, flash [for further details, see review by Govindjee and Van Rensen (1993)].

Vermaas and Rutherford (1984) demonstrated that formate addition to thylakoids increases the amplitude of the $g = 1.82$ EPR signal of $Q_A^- Fe^{2+}$ 10-fold [see also Nugent et al. (1988)]. A formate/bicarbonate effect has been established clearly by measurements of EPR spectra of the Q_A-Fe-Q_B complex with and without bicarbonate (Bowden et al. 1991). The Mössbauer spectrum of Fe is affected significantly by formate and is returned to its original on readdition of bicarbonate, indicating that Fe is a key element in the binding of the formate that is displaced by bicarbonate (Diner and Petrouleas 1987, Semin et al. 1990). A Fourier transform infrared (FTIR) difference spectroscopy study using ^{13}C-labeled bicarbonate has established that bicarbonate is a bidentate ligand of the non-heme iron (Hienerwadel and Berthomieu 1995). Examining the effects of a number of carboxylate anions on the EPR signals associated with the non-heme iron, Petrouleas et al. (1994) observed that glycolate, glyoxylate and oxalate compete with NO, formate and bicarbonate for binding to the iron. The anions inhibited diversely the electron flow rates from Q_A to Q_B (or to Q_B^-), which was measured by following the rates of relaxation, in the subms to ms time scale, of the chlorophyll fluorescence yield in isolated chloroplasts after single turnover saturating actinic flashes. Glycolate was the strongest inhibitor, glyoxylate being intermediate and oxalate being the weakest inhibitor of the PSII electron flow. It thus appears that many anions are able to bind as dissociable ligands to the non-heme iron of PSII.

Action of bicarbonate on the donor side of photosystem II

During the 1980s it was generally accepted that the major bicarbonate effect was located at the acceptor side of PSII. As described in the previous section, formate inhibits the electron flow on the acceptor side and bicarbonate relieves this inhibition. However, theories and experiments continued to be presented by Stemler and coworkers for a requirement of bicarbonate on the donor side of PSII [Stemler and Govindjee 1973, Stemler 1980, and a review by Stemler (1982)]. These were not generally accepted, mainly because of the large effects on the acceptor side of PSII, obtained at

high concentrations of formate. More recent developments indicate that Stemler's views were also correct; there is indeed an additional influence of bicarbonate on the donor side of PSII, which is described below.

Mende and Wiessner (1985) suggested that CO_2-depletion affects both the oxygen evolving and the electron accepting side of PSII in intact algal cells. El-Shintinawy and Govindjee (1990) established that short-term formate treatment of spinach leaf discs leads to a decrease in Chl a fluorescence yield instead of an increase as expected from an effect on the acceptor side of PSII. Govindjee et al. (1997b) presented data on chloroplast thylakoids for donor side effects, that is accompanied by acceptor side effects, from measurements on Chl a fluorescence yield changes after light flashes 1–6. Stemler and Lavergne (1997) reported evidence that formate destabilizes the S_1 state of the oxygen-evolving mechanism in photosystem II. Klimov et al. (1995a,b), Wincencjusz et al. (1996) and Hulsebosch et al. (1998) have demonstrated a strong suppression by formate of electron flow at the donor side, which is restored by the addition of bicarbonate. After depletion of PSII particles from manganese, bicarbonate was found to be essential for the early steps of photoactivation of the oxygen evolution complex when added together with manganese. Klimov et al. (1997a) suggested that bicarbonate may serve as a ligand to Mn. These results were obtained using specifically the so-called DT-20 PSII membrane fragments. Allakhverdiev et al. (1997) and Klimov et al. (1997b) reported an influence of bicarbonate on the water-oxidizing complex of PSII, not only in these DT-20 fragments, but also in the more often used Berthold-Babcock-Yocum (BBY) PSII particles (Berthold et al. 1981). However, Wincencjusz (1998) was unable to confirm the susceptibility to inhibition by formate in BBY particles and thus, a requirement of bicarbonate for the water-oxidizing complex of PSII may be exclusively present in the DT-20 particle preparations. It is possible that either the bicarbonate binding site becomes more accessible, as a result of the special isolation and/or storage conditions used, or that the isolation procedure induces a new bicarbonate effect that is absent in vivo. Alternatively, Yruela et al. (1998) studied modified BBY PSII membranes, which demonstrated an inhibition of 80% of their high rate of oxygen evolution after CO_2-depletion; this inhibition was partly restored upon addition of bicarbonate. Using these type of PSII membranes, these authors obtained a light-induced FTIR difference spectrum originating from the donor side of PSII. The main positive and negative bands disappeared after depletion from bicarbonate and were partly restored by the readdition of bicarbonate. It was suggested that bicarbonate can be a ligand to the Mn-containing water-oxidizing complex of PSII.

The influence of bicarbonate on the donor side occurs at lower concentrations or earlier in time than the effect on the acceptor side. Hutchison et al. (1996) and Xiong et al. (1997) have shown that a mutation of the arginine at D1-269 into a glycine at the acceptor side of PSII, significantly alters the structure and function of both the acceptor and the donor sides of the PSII complex, and may perturb the binding of bicarbonate and formate.

The bicarbonate binding niche

In both PSII and purple bacteria, a non-heme iron is located between Q_A and Q_B. In purple bacteria, the non-heme iron can be rather easily removed or replaced with other metal ions, and the resulting reaction centers still show almost normal electron tranport to Q_B, although with modified kinetics. In the bacterial reaction center, the non-heme iron is almost equidistant to the primary and secondary quinone electron acceptors. Four histidines from the polypeptides L and M and a bidentate glutamate provide the six ligands to the iron. On the basis that only reaction centers of PSII, not those of photosynthetic bacteria, show the bicarbonate effect, Michel and Deisenhofer (1988) and Van Rensen et al. (1988) suggested that glutamate 232 of the M subunit fulfills the role of bicarbonate in *Rhodopseudomonas viridis*. Because M-E232 forms a ligand to iron, bicarbonate was suggested to form a bidentate ligand to the iron in PSII. In addition to the bicarbonate, the non-heme iron is liganded by the four histidines D1-H215, D1-H272, D2-H214 and D2-H268. The binding site for bicarbonate at the non-heme iron was modeled by Xiong et al. (1996). The residues that form the binding pocket are positively charged and hydrophobic. They may include D1-L233, D1-V219, D2-N230, D2-T231, D2-F232, D2-R233, D2-A234, D2-P237 and D2-K264. Among these residues, D2-R233 was suggested earlier to be involved in binding and/or stabilizing bicarbonate and formate (Cao et al. 1991). The hydroxyl oxygen of the bicarbonate is separated from the main-chain hydrogen of D2-R233 by 4.8 Å. However, D2-K264 appears to be the most likely candidate to interact directly with the bicarbonate ion. The modeled distance from one of the ζ hydrogens of the lysine to the closest carbonyl oxygen of bicarbonate is 3.9 Å. In Fig. 2, a current model of the bicarbonate binding niche (Xiong et al. 1996, 1998a) is presented.

In addition to liganding to the iron, many experiments have suggested that bicarbonate may also function in promoting the protonation of Q_B^- or Q_B^{2-}. This second binding site is likely to exist in the Q_B niche. Characterization of a number of Q_B mutants that are also herbicide resistant implicated that the Q_B binding niche is involved in the binding of bicarbonate (Cao et al. 1992, Mäenpää et al. 1995, Vernotte et al. 1995). The tested residues involved were: D1-F211; D1-V219; D1-E242; D1-E243; D1-E244; D1-A251; D1-F255; D1-G256; D1-S264; D1-N266 and D1-L275 (Fig. 2). Because anionic bicarbonate/formate is very likely to be the active species involved, it may be expected that the binding would be electrostatic in nature and therefore positively charged amino acid residues are likely to participate in anion binding. Only three positively charged D1 residues, D1-H252, D1-R257 and D1-R269, are found near the putative Q_B binding niche based on homology modeling. Sequence analysis of the D1 protein indicates that D1-R257 is closer to the Q_B binding niche than D1-R269, making it a more likely residue to be involved in the protonation of Q_B. This residue is thought to be located on the stromal side between the putative transmembrane helices D and E of D1 and may be located within or close to the D1-de helix. Xiong et al. (1998b) constructed two site-directed mutants, D1-R257E and D1-R257M. The characteri-
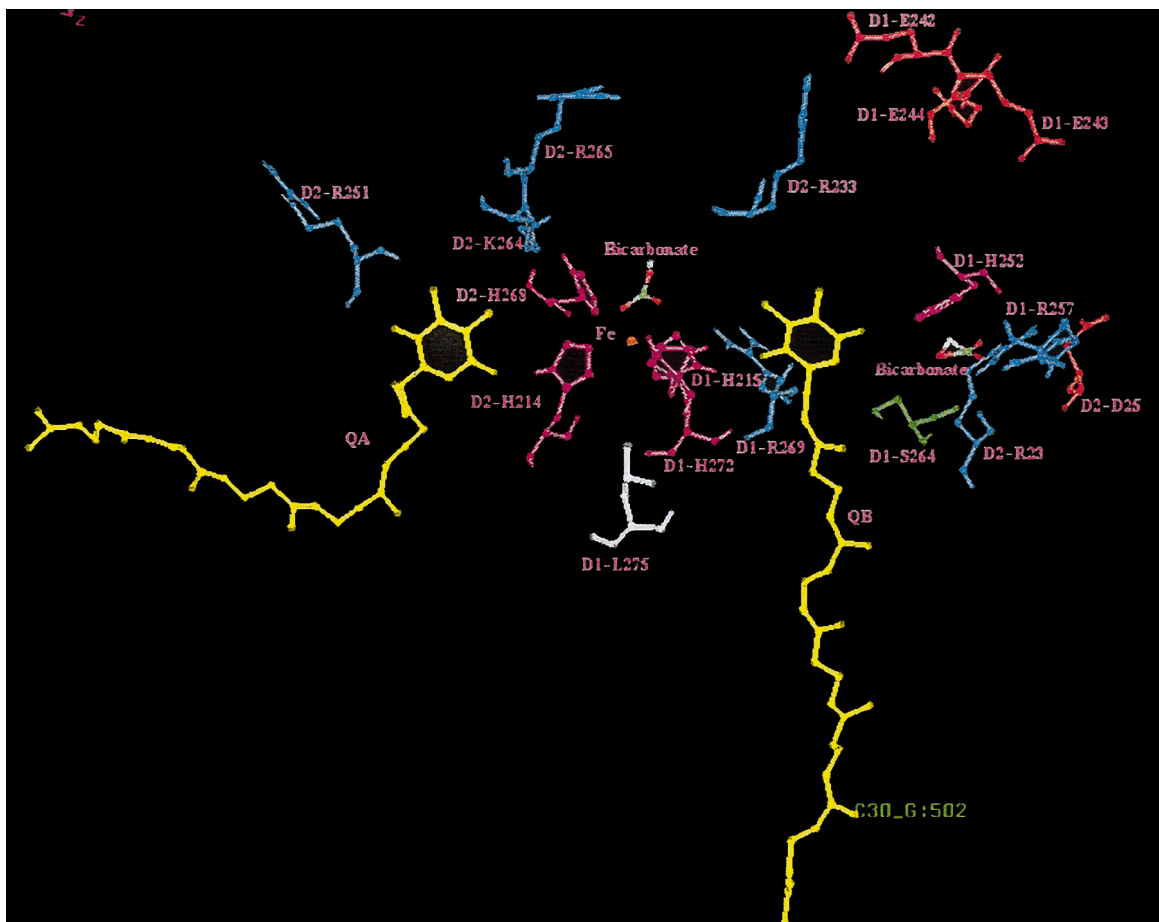
Fig. 2. A model of the bicarbonate binding niche. One bicarbonate ion is suggested to be bound to the non-heme iron center (Fe); and the other is shown to be close to D1-R257. Positively charged amino acids are in blue; and negatively charged amino acids are in reddish colors. See text for the description of the bicarbonate binding niche; most of the amino acids shown here had been either mutated or deleted to study the bicarbonate effect; others are expected to be mutated for further understanding of the bicarbonate binding niche. The photograph was constructed from the model of Xiong et al. (1998a). Also see the model on the cover.

zation of these two mutants leads to the conclusion that D1-R257 with its positively charged side chain is important for the binding of formate and also, in all likelihood, bicarbonate. Interestingly enough, the D1-R257E and D1-R257M mutants are not very sensitive to formate and have retained almost 70% of their electron transport and growth activities. Thus, assuming that not only formate but also bicarbonate binding is impaired in these mutants, the possibility can be raised that water molecules may substitute for bicarbonate in these mutants for their almost normal functioning.

A major question is concerned with the following. If there are only two bicarbonate binding sites and if both are on the acceptor side (one on the non-heme iron and the other near the D1-R257), then how does one explain the influence of bicarbonate on the binding of manganese in the oxygen evolving complex at the donor side of PSII? It seems that either the binding on the acceptor side affects the donor side through conformational changes, or a new (perhaps, a third) binding side remains to be discovered. A scissor-like model is envisioned, in which binding on one side opens or closes the other side as the case may be.

Functional role of bicarbonate

Because absence of bicarbonate drastically slows down the reduction of Q_B (electron flow from Q_A^- to Q_B^- is often measured by decay of Chl a yield in the subms to ms range, after the second actinic flash) and the pK_1 of $H_2O + CO_2/$ $HCO_3^- + H^+$ is about 6.3, it has been proposed several times that bicarbonate is involved in the protonation of Q_B^{2-} [for further methods and references, see Govindjee and Van Rensen (1993)]. Xiong et al. (1996) modeled a bicarbonate and a water molecule in the Q_B binding niche and proposed a hypothesis to explain the mechanism of Q_B protonation mediated by bicarbonate and water. The bicarbonate, stabilized by D1-R257, would donate a proton to Q_B^{2-} through the intermediate D1-H252 and a water molecule would donate another proton to Q_B^{2-}. Based on the discovery of a water transport channel in the bacterial reaction center, an analogous channel for transporting water and bicarbonate was proposed in the PSII model. The putative channel is primarily positively charged near Q_B and the non-heme iron. Because bicarbonate is liganded to the non-heme iron, an additional role of bicarbonate may be to

serve to stabilize the Q_A-Fe-Q_B structure. Upon the removal of bicarbonate, the distance between Q_A and Q_B may be altered, slowing the Q_A^- to Q_B electron transfer, although a larger effect is in the protonation of reduced Q_B (Q_A^- to Q_B^-; $Q_B^{2-} + 2H^+ \rightarrow Q_BH_2$).

The role of bicarbonate on the donor side of PSII is an active area of research and much remains to be examined and explored before a new picture is expected to emerge because most of the donor side effects have been shown in the DT-20 particles, but not in thylakoids. In fact, most of the earlier data on thylakoids contradict the proposed effects between water and the primary electron donor P680 (Jursinic et al. 1976, Govindjee et al. 1989) although an influence between Z and Q_A was suggested to occur in leaves and intact cells (El-Shintinawy and Govindjee 1990, El-Shintinawy et al. 1990). There is an urgent need of extensive investigations in the area of the requirement for bicarbonate on the donor side of thylakoids and cells.

Under conditions that photosynthesis can proceed well, enough bicarbonate is probably bound to PSII, in order for PSII to function normally. However, under stress conditions (e.g. drought, high light intensity, high temperature) the stomata may close, which leads to a decrease in internal CO_2 concentration resulting in a lowered bicarbonate concentration that may limit the activity of PSII. It has been suggested several times that bicarbonate may be involved in the process of photoinhibition (Sundby et al. 1992, Gong et al. 1993, Schansker and Van Rensen 1993, Mäenpää et al. 1995, Vass et al. 1995, Klimov et al. 1997b). Schansker (1996) proposed that loss of bicarbonate is an early event in the process leading to photoinhibition. As photoinhibition occurs at high light intensity, the acceptor side of PSII becomes highly reduced. This reduced state is probably related to a decreased affinity of PSII for bicarbonate (Van Rensen et al. 1988). This may lead to the diffusion from its binding site at the non-heme iron, resulting in an impaired rate of electron transfer from Q_A^- to Q_B or to Q_B^-. Finally, the reaction center may become overreduced and photooxidation may occur, leading to the degradation of the D1 protein.

Concluding remarks

There is no influence of bicarbonate in the reaction centers of anoxygenic photosynthetic bacteria (Shopes et al. 1989, Wang et al. 1992), and also no water splitting and no photoinhibition is present. However, in oxygenic higher plants, algae and cyanobacteria, bicarbonate is required for the functioning of PSII. There is not only an action site on the acceptor side of PSII, but also on the donor side. Bicarbonate is probably a ligand to the non-heme iron and, in addition, has a binding niche in the positively charged region of the Q_B binding site. It is suggested to function not only in the protonation of reduced Q_B, stabilization of the Q_A-Fe-Q_B structure, but also on the Mn cluster. Under normal conditions the bicarbonate concentration is probably not limiting for the activity of PSII. However, under stress conditions leading to low bicarbonate concentrations, PSII activity may be impaired. Bicarbonate may also be involved in the process of photoinhibition. The bicarbonate effect discovered by the great master Otto Warburg (Warburg and Krippahl 1958) remains to be investigated further to uncover its full meaning for the functioning of Photosystem II.

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