Abstract

It is well established that bicarbonate stimulates electron transfer between the primary and secondary electron acceptors, Qₐ and Qₐ⁺, in formate-inhibited photosystem II; the non-heme Fe between Qₐ and Qₐ⁺ plays an essential role in the bicarbonate binding. Strong evidence of a bicarbonate requirement for the water-oxidizing complex (WOC), both O₂ evolving and assembling from apo-WOC and Mn²⁺, of photosystem II (PSII) preparations has been presented in a number of publications during the last 5 years. The following explanations for the involvement of bicarbonate in the events on the donor side of PSII are considered: (1) bicarbonate serves as an electron donor (alternative to water or as a way of involvement of water molecules in the oxidative reactions) to the Mn-containing O₂ center; (2) bicarbonate facilitates reassembly of the WOC from apo-WOC and Mn²⁺ due to formation of the complexes Mn(HCO₃)⁺ and Mn(HCO₃)₂ leading to an easier oxidation of Mn²⁺ with PSII; (3) bicarbonate is an integral component of the WOC essential for its function and stability; it may be considered a direct ligand to the Mn cluster; (4) the WOC is stabilized by bicarbonate through its binding to other components of PSII. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Photosystem II; Water-oxidizing complex; Manganese; Bicarbonate; Formate

1. Introduction

Bicarbonate was discovered to stimulate electron flow in the Hill reaction by Warburg and Krippahl [1]. Now it is well established that bicarbonate ions are required for the maximum activity of photosystem II (PSII) (for recent review see [2,3] and references therein). However, the interpretation of the stimulating effect of bicarbonate (‘bicarbonate effect’) on PSII activities remains controversial. Initially the site of action was thought to be in the water-oxidizing complex (WOC) [4,5], and a model including bicarbonate as a mediator for the photosynthetic water oxidation had been suggested [5,6] which, however, seemed to be in contradiction with the results of isotopic experiments [7]. On the other hand, strong evidence for the action of bicarbonate on the electron acceptor side of PSII, providing efficient reoxidation of the first plastoquinone electron acceptor Qₐ, has been presented [8], and the idea was supported by a number of data (for review see [2,3]).
The non-heme Fe between QA and the secondary plastoquinone electron acceptor QB has been shown \[9,10\] to play an essential role in bicarbonate binding (Fig. 1).

It was shown that bicarbonate depletion may affect both the electron acceptor and donor sides of PSII [12]. On the other hand, El-Shintinawy and Govindjee [13] found that bicarbonate has two sites of action: the first accelerates the electron flow beyond QA, and the other stimulates it between QA and QB (the site between the primary electron acceptor, pheophytin (Pheo), and QA was speculated for the latter case). So, the idea of a bicarbonate requirement for the acceptor side of PSII began to dominate.

However, strong evidence for bicarbonate requirement for the WOC of PSII (Fig. 1) recently has been provided [14–23]. The following approaches were used in those experiments for removing bicarbonate from PSII preparations to reveal and augment the bicarbonate effects: (1) replacement of bicarbonate in its possible binding site(s) by another anion, formate [14–17,23]; (2) a 100–1000 dilution (and washing in some cases) of concentrated (2–5 mg Chl/ml) PSII preparations into the medium depleted of endogenous bicarbonate by means of both flushing with CO₂-depleted air and/or boiling [14,15,17–21]; (3) a shift of the medium pH to a value lower than the pK for H₂CO₃ dissociation (pH 6.4) [14,15,17–20,22].

2. Bicarbonate effect in untreated oxygen-evolving PSII membrane fragments

It was clearly demonstrated that formate induces bicarbonate-reversible inhibition of electron transfer in both the electron acceptor and donor sides of PSII [14–17]. Upon the addition of formate at a concentration of 5 mM or higher, a typical bicarbonate effect on the acceptor side of PSII (described earlier in the classical work by Wydrzynski and Govindjee [8]) was revealed by means of measurements of Chl fluorescence [14,15]. The initial level of fluorescence \(F₀\) as well as the sum \(F₀ + \Delta F\) was increased while the addition of bicarbonate reversed the effects. The effect of formate was similar to that induced by 1 \(\text{mM} 3-(3,4\text{-dichlorophenyl})-1,1\text{-dimethylurea (DCMU)}\) and it was related to the blocking electron transfer between QA and QB (due to removal of bicarbonate with binding of formate to the non-heme Fe [8,9]).

In contrast, at 100–1000 times lower concentrations, formate induced a decrease (reversed by addition of bicarbonate) of \(\Delta F\) without changing the \(F₀\) level (Fig. 2A) which is characteristic of reversible inhibition of electron transfer on the donor side of PSII. Similar effects of the \(\Delta F\) inhibition and its subsequent reactivation with bicarbonate without a change in \(F₀\) was observed upon placing the PSII preparation into a medium depleted of CO₂ (Fig. 2A) which demonstrated that in both cases the inhibition of electron transfer on the donor side of PSII was related to removal of bicarbonate from its binding site [14,15,18].

The effects on the donor side of PSII observed upon either partial removal of bicarbonate or the
addition of 10^100 M formate were also revealed under the conditions when the known bicarbonate-dependent step of electron transfer between QA and QB was not operable [14, 15]. Photoaccumulation of the long-lived state of PSII reaction center with the reduced primary electron acceptor, Pheo^3, (that occurs due to competition of electron donation to the reaction center with charge recombination in the ion-radical pair [P^‡_680 Pheo^-]) is observed after preduction of QA and QB [24]. It was shown recently [15] that the rate of photoaccumulation of Pheo^- was considerably inhibited by bicarbonate depletion or addition of formate and the photoreaction was restored with bicarbonate. Similarly, photoaccumulation of the oxidized primary electron donor, P^+_680, in the presence of SiMo (taking electron from Pheo and, probably, from QA [25], i.e., before the electron transfer between QA and QB) also depended on the presence of formate and bicarbonate in the medium [14, 15].

Flash-induced absorbance changes at 295 nm oscillating with a period of four due to accumulation of charges in the WOC of PSII preparations were sufficiently inhibited upon the addition of formate at concentration of 10–100 μM and they were restored with bicarbonate [16]. In the same range of formate concentration a considerable suppression of photo-reduction of dichloro-p-benzoquinone (DCBQ) from water was observed while in the presence of tetraphenylboron, which replaces water as the electron donor to the PSII reaction center, inhibition of photoreduction of exogenous quinones took place only at formate concentration higher than 10 mM (Fig. 3). These results strongly support the conclusion that formate inhibits photochemical reactions of PSII due to its binding to two independent sites. One of them (with a higher formate binding) is associated with the WOC since it is revealed only when the addition of 10–100 μM formate were also revealed under the conditions when the known bicarbonate-dependent step of electron transfer between QA and QB was not operable [14, 15].
WOC is operable while the other one (with a 1000 times lower formate binding) inhibits the electron transfer on the acceptor side of PSII [14–16].

The importance of bicarbonate for both functional and structural manifestation of the WOC was convincingly shown using electron paramagnetic resonance (EPR) measurements [17,19]. The light-induced EPR signal II related to photooxidation of the secondary electron donor of PSII, Y_Z (tyrosyl residue 161 of the D1 protein [27]), was activated upon the addition of 0.1 mM formate that was consistent with the idea that formate inhibits the electron donation from the WOC to Y_Z, while photooxidation of Y_Z was not impaired. Elimination of the formate effect with bicarbonate suggests that the electron donation to Y_Z is bicarbonate-dependent. This conclusion was strongly supported by the kinetic measurements of the flash-induced EPR signal II (Fig. 4): in the presence of 0.1 mM formate, the reduction of Y_Z from the WOC greatly slowed down and it occurred largely by back reaction with reduced electron acceptors. Bicarbonate was shown to prevent the loss of the fast electron donation from the WOC to Y_Z.

The functional modification of the WOC with formate was accompanied by considerable structural rearrangements in the Mn cluster that led to the release of one or two free Mn^{2+} atoms per PSII reaction center as revealed by the appearance of the 6-line EPR signal of Mn^{2+} (Fig. 4). Protective effect of bicarbonate against the formate-induced modifications of the WOC (Fig. 4) suggested that formate might act by replacing bicarbonate that is essential for Mn binding. The data on bicarbonate-reversible loss of the S_2 multiline EPR signal upon removal of bicarbonate from spinach PSII particles by means of washing in CO_2-free medium [20] are in line with the conclusion. Similar effects of formate on EPR signals of PSII preparations were reported in a recent paper [23]. Recently Stemler and Lavergne have shown that formate modifies the S_1 state of the WOC so that it becomes reducible to the S_0 state [28].

An attempt to find direct evidence for possible ligation of bicarbonate to WOC components was made in a recent work using Fourier transform infrared spectroscopy (FT-IR) [21]. It was shown that the light-induced FT-IR difference spectrum originating from the donor side of PSII was considerably modified upon washing a PSII preparation with the medium depleted of bicarbonate: the main negative bands at 1560, 1541, 1522 and 1507 cm^{-1} and positive bands at 1589 and 1365 cm^{-1} disappeared upon bicarbonate removal and they were partially restored by bicarbonate addition. Similar ^13C-labeling FT-IR measurements led to the conclusion that the negative band at 1560 cm^{-1} and positive bands at 1589 cm^{-1} and 1365 cm^{-1} could be assigned to COO^- stretching modes from bicarbonate. The first two bands corresponded to U_{as} (COO^-) while the latter one could be ascribed to U_{s} (COO^-). The results were
consistent with the suggestion that bicarbonate could be a ligand to the WOC. A disadvantage of those experiments was that they were done under continuous illumination. Similar measurements of flash-induced FT-IR spectrum are needed in order to assign them to specific S state transitions and to reveal possible ligation of bicarbonate to the Mn cluster.

3. Stimulating effect of bicarbonate during reconstitution of the WOC

Bicarbonate requirement for the donor side of PSII was especially evident during reconstitution of the Mn cluster of the WOC in Mn-depleted PSII preparations (apo-WOC-PSII) [14,15,17–20,22]. An efficient restoration of electron donation from the added Mn\(^{2+}\) to the PSII reaction center (lost due to removal of Mn) could be reached only in the presence of bicarbonate in the medium [14,15,17,18]. That effect of bicarbonate was clearly shown for reactivation of photoinduced \(\Delta F\) (Fig. 2B), photoreduction of 2,6-dichlorophenolindophenol (DCPIP) [14,15] and photooxidation of \(P_{680}\) [14,15]. It was important that the bicarbonate requirement was not revealed if Mn\(^{2+}\) was substituted for other exogenous electron donors (NH\(_2\)OH, diphenylcarbazide) [14,15]. The data clearly demonstrated that the observed stimulatory effects of bicarbonate were associated with reconstitution of a Mn center rather that with the electron transfer on the acceptor side of PSII.

The increased restoration of PSII activities with Mn\(^{2+}\) in the presence of bicarbonate was accompanied by an increased functional binding of Mn\(^{2+}\) to PSII which was revealed in the experiments on removal of the added Mn\(^{2+}\) from PSII membranes by centrifugation and suspension of the pellet in a medium freed of Mn\(^{2+}\) or by the addition of EDTA [14,15]. In both cases the effect of bicarbonate leading to an increased binding of Mn\(^{2+}\) was especially clear when the experiments were done under illumination [15].

It was shown that the formation of bicarbonate complexes with Mn\(^{2+}\) considerably changed the redox properties of the metal: the redox potential of its oxidation was shifted from +1.2 V (aqua ion Mn\(^{2+}\)) to +0.92 V for Mn\(^{2+}(\text{HCO}_3^-)^+\) and to +0.63 V for Mn\(^{2+}(\text{HCO}_3^-)^2\) [29] which, of course, is important for redox interaction of Mn\(^{2+}\) with apo-WOC-PSII.

Measurements of flash-induced EPR signal II demonstrated that bicarbonate is probably not needed for photooxidation of \(Y_Z\) in Mn-depleted PSII while the fast electron donation to \(Y_Z^+\) from the added Mn\(^{2+}\) requires bicarbonate [17]. The fast phase of rereduction of \(Y_Z^+\) (characteristic of the functionally competent WOC) could be restored only upon joint addition of bicarbonate and Mn\(^{2+}\) (and it was not seen if one of the two was absent) (Fig. 5).

Bicarbonate was also required for structural rearrangements of Mn\(^{2+}\) leading to formation of a functionally active Mn center in the apo-WOC-PSII [17]. The characteristic 6-line EPR signal of added Mn\(^{2+}\) (2–4 Mn per one PSII reaction center) was diminished upon illumination (evidently due to photooxidation of Mn\(^{2+}\) to Mn\(^{3+}\)). However, the signal completely recovered if bicarbonate was not added to the medium (Fig. 6). Upon the addition of bicarbonate, a part of the signal was eliminated already in the dark and it practically disappeared upon illumination and, what was especially important, the signal did not recover after the actinic light was switched off [17]. So, the EPR-silent (at room temperature) form of manganese (which is also characteristic of manga-
The conclusion on creation of a functionally (and structurally) competent form of the WOC in the presence of bicarbonate was strongly supported by the appearance of the characteristic, period four, oscillations of the flash-induced absorbance changes at 295 nm after photoactivation of apo-WOC-PSII in the presence of both Mn$^{2+}$ and bicarbonate. In the

![Image](image_url)

Fig. 6. Effect of bicarbonate and illumination by continuous light on the EPR signal of Mn$^{2+}$ added to Mn-depleted DT-20 membrane fragments at concentrations of (A,B) 28 μM MnCl$_2$ (2 Mn per one PSII) and (C,D) 56 μM MnCl$_2$ (4 Mn per one PSII), before (A,C) and after (B,D) the addition of 1 mM NaHCO$_3$. Spectra 1, 2, and 3 were measured in dark-adapted samples, during illumination and after 5 min incubation of the illuminated sample in the dark, respectively. For details, see [17].

![Image](image_url)

Fig. 7. Effect of removal and re-addition of bicarbonate on the rate of O$_2$ evolution in Mn-restored PSII membrane fragments as a function of pH. BBY membranes were depleted of Mn by TEMED treatment. In A, 0.4 μM MnCl$_2$ (2 Mn per one PSII) was added. In B, 0.8 μM MnCl$_2$ (4 Mn per one PSII) was added. All samples were given 0.5 mM DCBQ before illumination. Curves: 1 and 2, bicarbonate-non-depleted medium, before and after addition of 0.4 mM NaHCO$_3$; 3 and 4, bicarbonate-depleted medium, before and after addition of 0.4 mM NaHCO$_3$. [Chl]=40 μg/ml; 20°C. The rate of oxygen evolution in untreated preparations at pH 6.5 (400 μmol/mg Chl/h) is taken as 100%. The rate of O$_2$ evolution in the Mn-depleted preparations was near zero if MnCl$_2$ was not added to the medium. (C) The same pH dependence for oxygen evolution rate in untreated (Mn-containing) BBY membrane fragments. For details, see [18].
absence of bicarbonate, the oscillations were not restored [17]. Besides, the S 2 multiline EPR signal also could be restored in those samples only if Mn 2+ was added jointly with bicarbonate [20].

What was most important, bicarbonate was required for photoreactivation of the WOC competent in oxygen evolution (Fig. 7). In Mn-depleted PSII preparation, photoactivation of both photoinduced ΔF and oxygen evolution with added Mn 2+ was very low if the experiments were done in bicarbonate-depleted medium while both of the activities were activated after the addition of bicarbonate [18]. The bicarbonate requirement could be revealed without a special procedure for bicarbonate depletion if the pH of the medium used for photoactivation was shifted to pH 6.5 (lower than the pK for H 2CO 3 dissociation) leading to a sufficient lowering of HCO 3− concentration in the medium (Fig. 7). (A similar stimulatory effect of bicarbonate on oxygen evolution was observed in an untreated O 2-evolving PSII preparation [18].)

From 2 to 4 Mn per PSII reaction center were enough for maximum photoactivation of the oxygen-evolving function if the photoactivation was performed in the presence of bicarbonate [18].

From the analysis of the concentration dependence of the stimulating effects of bicarbonate it was found that the dissociation constant (Kd) for bicarbonate bound to the WOC is equal to 20–34 μM [18]. In addition, a Kd lower than 10 μM was found although its correct value was not determined [18].

Much progress has been made recently in the investigation of the mechanism of photoactivation of the WOC in Mn-depleted PSII preparations due to new experimental developments which have enabled kinetic resolution of the first three intermediates formed during assembly of the inorganic core of the WOC [30]. The first light-dependent step can be related to the formation of MnIII(OH) 2−-WOC-PSII intermediate, whose concentration defined the yield of the process of the WOC assembly [30]. Using this photoactivation approach it was shown [22] that at pH 6.0 bicarbonate considerably (1.2–3 times) accelerated photoassembly of the WOC from apo-WOC-PSII and Mn 2+ added in stoichiometry between 2 and 4 Mn per PSII reaction center. Nearly 50% of the bicarbonate effect was observed at 15–25 μM of added bicarbonate. At pH 6.0 that corresponded to approx. 10 μM of HCO 3−, and that was close to the concentration of added Mn 2+. Analysis of the data suggested that bicarbonate stimulated the first light-dependent step of apo-WOC-PSII photoactivation increasing, probably, the quantity of the first intermediate through the formation of MnIII(OH)(HCO 3−)-WOC-PSII intermediate [22].

4. Stabilizing effect of bicarbonate during photo- and thermoinactivation of PSII

Experiments on PSII photoinhibition in CO 2/bicarbonate-depleted samples provided contradictory results. There are data showing that in thylakoids bicarbonate protects the PSII machinery against photoinhibition [31,32]. According to other publications, depletion of bicarbonate in thylakoid membranes (by formate treatment [31,32]) or in green algae (using bicarbonate-depleted medium [33,34]) resulted in a lower susceptibility of PSII to photoinhibition which was consistent with the idea of blocking the electron transfer between QA and QB in bicarbonate-depleted samples since similar protection against photoinhibition was reached upon DCMU addition.

It has been shown recently that in PSII membranes, the rate of photoinhibition of PSII activities (photoinduced ΔF and photoreduction of DCPIP) in the medium depleted of CO 2/bicarbonate was considerably decreased upon addition of 5 mM NaHCO 3 [19]. A similar protecting effect was revealed when 100 μM MnCl 2 was added instead of bicarbonate. In PSII membrane fragments depleted of Mn, the photoinhibition led to irreversible loss of the capability of PSII to be reactivated by Mn 2+, and the rate of photoinhibition was decreased by a factor of 2 or 5 if the preillumination was done in the presence of 0.2 μM MnCl 2 (4 Mn per PSII reaction center) added alone or in combination with 5 mM NaHCO 3, respectively. A similar protective effect of bicarbonate was also revealed in the dark, during thermoinactivation of O 2-evolving PSII preparations at 40°C: the rate of thermoinactivation was decreased by a factor 3 if bicarbonate was added to the medium [19]. The results are consistent with the idea that bicarbonate is an essential component of the WOC,
and it is required for both functioning and stability of the Mn-containing enzyme.

5. Possible ways of involvement of bicarbonate in the events on the donor side of PSII

The following explanations of the bicarbonate requirement for the donor side of PSII have been suggested.

(1) Bicarbonate serves as an electron donor (alternative to water or as a way of involvement of water molecules in the oxidative reactions) to the Mn-containing WOC [14]. This hypothesis of the involvement of bicarbonate in the chemistry of photosynthetic oxygen evolution was worked out earlier [5,6]. However, it was not confirmed by experiments on O2 evolution in the presence of HC\(^{18}O_3\) or H\(^{18}O\) that showed that oxygen atoms from HC\(^{18}O_3\) were evidently not included in O2 molecules evolved in PSII [7]. On the other hand, carbonic anhydrase activity of PSII has been revealed [35,36] and it considerably increased upon isolation of the O2-evolving core complexes from PSII membrane fragments [37]. Besides, a novel carbonic anhydrase associated with PSII has been discovered recently in *Chlamydomonas reinhardtii* [38]. One can suggest that due to multiple exchange of HCO\(_3^-\)/CO2 species on the carbonic anhydrase enzymatic center, the labeled \(^{18}O\) could ‘leak’ to water (the concentration of which is a few orders higher than that of added bicarbonate) that could be responsible for the lack of \(^{18}O_2\) evolution from HC\(^{18}O_3\) [14,39]. Most of the data on restoration of electron transfer with bicarbonate in apo-WOC-PSII in the presence of Mn\(^2+\) and the absence of its slow oxidation [29] or the decrease (or loss) of positive charge(s) that is favorable for the accessibility of Mn\(^2+\) to its specific binding site(s) [14]. According to our recent publication [22] bicarbonate may: (1) act as an integral cofactor within the WOC (possible ligand to the first Mn), (2) act as a Bronsted base to accelerate proton release during formation of either the dark precursor (apo-WOC-Mn(OH)\(^3+\)) or IM\(_1\) (apo-WOC-Mn(OH)\(^3+\)), (3) directly deliver one or more hydroxide ions during formation of the latter two species (with release of CO2), (4) act as a membrane soluble anion which electrostatically elevates the local concentration of Mn\(^2+\) in PSII.

(2) Bicarbonate is required only for the process of assembly of the functionally competent WOC from the cofactor-depleted apo-WOC-PSII centers (appearing as a result of disassembling of the WOC under stress conditions or when newly synthesized). There are many possible ways of bicarbonate involvement in photoactivation of the WOC [14, 15]. Photooxidation of Mn\(^2+\) to Mn\(^3+\) (as an important step of the WOC photoactivation) is facilitated upon formation of complexes Mn(HCO\(_3\))^\(-\) and Mn(HCO\(_3\)\(_2\)) due to either lowering the redox potential of Mn\(^2+\) oxidation [29] or the decrease (or loss) of positive charge(s) that is favorable for the accessibility of Mn\(^2+\) to its specific binding site(s) [14]. Recently [41,42] it has been shown that an arginine residue of the D1 protein of PSII of *C. reinhardtii* (D1-R269) may be an additional binding site for bicarbonate on the acceptor side of PSII. It has been suggested that this possible binding site for bicarbonate is important for both the acceptor and the donor side of PSII since a mutation of the arginine into a glycine was accompanied by the loss of the tetranuclear Mn assembly [42,43]. However, this ‘scissor-like’ model needs additional reliable confirmation since the mutation of the D1-R269 itself can induce considerable structural changes in the PSII complex regardless of the possible participation of the arginine residue in bicarbonate binding. Besides, a reversed version of the ‘scissor-like’ model could be suggested: bicarbonate bound to the donor side could indirectly account for the acceptor side effects as well.
Bicarbonate is an integral cofactor of the WOC (Fig. 1). It can be involved in direct ligation of the Mn atoms that is important for the assembly of the functionally competent Mn cluster or for modulation of its redox capabilities. Reactivation of O₂ evolution along with reactivation of electron donation to the PSII reaction center in Mn-depleted PSII preparations [18,22] shows that bicarbonate is required for reconstitution of the WOC rather than only for reactivation of the electron flow (if Mn(HCO₃)₂ would be just a good electron donor for PSII). Data on the increased functional binding of Mn²⁺ in the presence of bicarbonate [15], the release of Mn²⁺ from the WOC in the presence of formate [17] and the protecting effect of bicarbonate against thermal disassembly of the WOC [19] confirm the idea that bicarbonate participates in the assembling of the Mn cluster.

One can suggest that some of the carboxyl group(s) taking part in the formation of Mn-containing WOC [43,44] belong to bicarbonate rather than to amino acid residues. The results of recent FT-IR measurements [21] are consistent with this idea although more reliable experiments dealing with difference absorption FT-IR spectra of specific S transitions of the WOC are needed.

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