Bicarbonate requirement for the donor side of photosystem II

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Abstract **Suppression of electron flow (and its subsequent restoration with 3–10 mM $NaHCO_3$) on the donor side of photosystem II is shown upon either a partial depletion of pea subchloroplast membranes in bicarbonate or the addition of 5–20 μM formate. At higher concentrations (5 mM) formate induces the known 'bicarbonate effect' on the acceptor side of photosystem II. In preparations depleted of manganese the restoration of electron flow with 0.1–0.2 μM $MnCl_2$ (2–4 Mn per photosystem II reaction center) occurs only in the presence of bicarbonate and it is accompanied by an increased functional binding of manganese. Restoration of electron flow with diphenylcarbazide or NH_2OH does not require the addition of $NaHCO_3$. It is suggested that bicarbonate participates in the formation of the Mn-cluster capable of water oxidation or serves as a substrate for the water-oxidizing center.**

Key words: Photosystem II; Bicarbonate; Donor side; Manganese

1. Introduction

Bicarbonate (BC) is known to be necessary for maximal activity of photosystem II (PS-II) [1–3]. There is at least two pools of BC with different affinity of its binding to PS-II, and evidently only the high-affinity one (1 molecule per 400–600 Chl molecules or per one PS-II reaction center) controls PS-II activity while the lower-affinity pool (close to Chl concentration) can be removed without essential consequence for PS-II [4,5]. Interpretation of the stimulating effect of BC on PS-II activities (the 'BC effect') remains controversial. In the early 1970s the high-affinity pool had been ascribed to the donor side of PS-II [2,5,6] and a model including BC as a mediator for photosynthetic water oxidation had been suggested [5,6] which however was in contradiction with the results of isotopic experiments [7]. Wydrzynski and Govindjee [3] observed an accelerated rise of Chl fluorescence in BC-depleted chloroplasts which demonstrated that reoxidation of the first plastoquinone acceptor, Q_A, by the plastoquinone pool has been impaired by the removal of BC which was supported by a number of data ([8–11] and references therein). The non-heme Fe between Q_A and Q_B has been shown to play an essential role in BC binding [8]. However, there are data showing that BC-depletion affects both the electron acceptor and the donor side of PS-II [9]. Govindjee and co-workers [10–11] (using formate for accentuating the BC effect) confirmed that BC evidently has two sites of action: the first accelerates the electron flow beyond Q_A, and the other stimulates it between the secondary electron donor, Y_Z, and Q_A (the site between the primary electron acceptor, pheophytin, and Q_A was suggested for the latter case [10]). In this paper we present evidence for a BC requirement on the donor side of PS-II and for involvement of BC in reconstitution of the Mn cluster in Tris-washed PS-II membranes.

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Abbreviations: BC, bicarbonate; DCMU, 3-(3,4-dichlorphenyl)-1,1-dimethylurea; DCPIP, 2,6-dichlorophenol-indophenol; DPC, diphenylcarbazide; EDTA, ethylenediaminetetraacetic acid.

2. Materials and methods

Subchloroplast PS-II membrane fragments designated here as DT-20 were isolated from pea chloroplasts using digitonin and Triton X-100 [12]. A complete (>95%) removal of Mn from the membrane fragments was carried out using 1 M Tris-HCl (pH 8.0) plus 0.5 M $MgCl_2$ [13]. The treatment by 1 M $CaCl_2$ was done as described earlier [14]. A partial removal of BC from DT-20 preparation was reached by a 200-fold dilution of concentrated (2 mg/ml) PS-II membrane fragments in the medium depleted of the endogenous BC by means of 60-min flushing with air (which was freed from CO_2 by passage through a solution of 50% NaOH and 20 cm layer of ascarite) and subsequent 15-min incubation at 20°C. The kinetics of photoinduced absorbance changes (ΔA) of chlorophyll P_{680} and DCPIP in the Hill reaction as well as the changes of chlorophyll fluorescence yield (ΔF) were measured in a 10-mm cuvette at 20°C using a phosphoroscopic set-up [13]. The rate of oxygen evolution was measured in a 2-ml cell using a Clark type electrode under illumination with red light (55 W/m^2) in the presence of 50 μM 2,6-dichloro-*p*-benzoquinone.

3. Results

Partial depletion of the initial PS-II membrane fragments (DT-20) in BC by flushing the medium with air freed of CO_2 leads to a significant (1.5–2 times) decrease of both the rate and the amplitude of photoinduced ΔF related to photoreduction of Q_A (Fig. 1A). Subsequent addition of 6 mM $NaHCO_3$ results in complete reactivation of ΔF. The level of F_0 is not changed in these experiments. The BC effect becomes much more accentuated when the sample depleted of BC is illuminated a few times by actinic light. The addition of 6 mM $NaHCO_3$ to the preilluminated sample also leads to practically complete reactivation of ΔF (Fig. 1A). Similar effects of the inhibition of ΔF and their subsequent reactivation by $NaHCO_3$ without a change in F_0 is also observed upon the addition of formate at low (5–20 μM) concentration (Fig. 1A). In contrast, upon the addition of formate at higher (≥ 1 mM) concentration the F_0 level as well as the sum $F_0 + \Delta F$ are increased. The latter effect was quite similar to that observed upon the addition of 1 μM DCMU (data omitted) blocking electron transfer beyond Q_A. The addition of 6 mM $NaHCO_3$ reverses the effect: F_0 as well as the sum $F_0 + \Delta F$ return to their initial levels (Fig. 1A).

The requirement for BC is especially pronounced in experiments showing the reactivation of electron transfer with exogenic Mn^{2+} in DT-20 deprived of Mn (Fig. 1B). The addition

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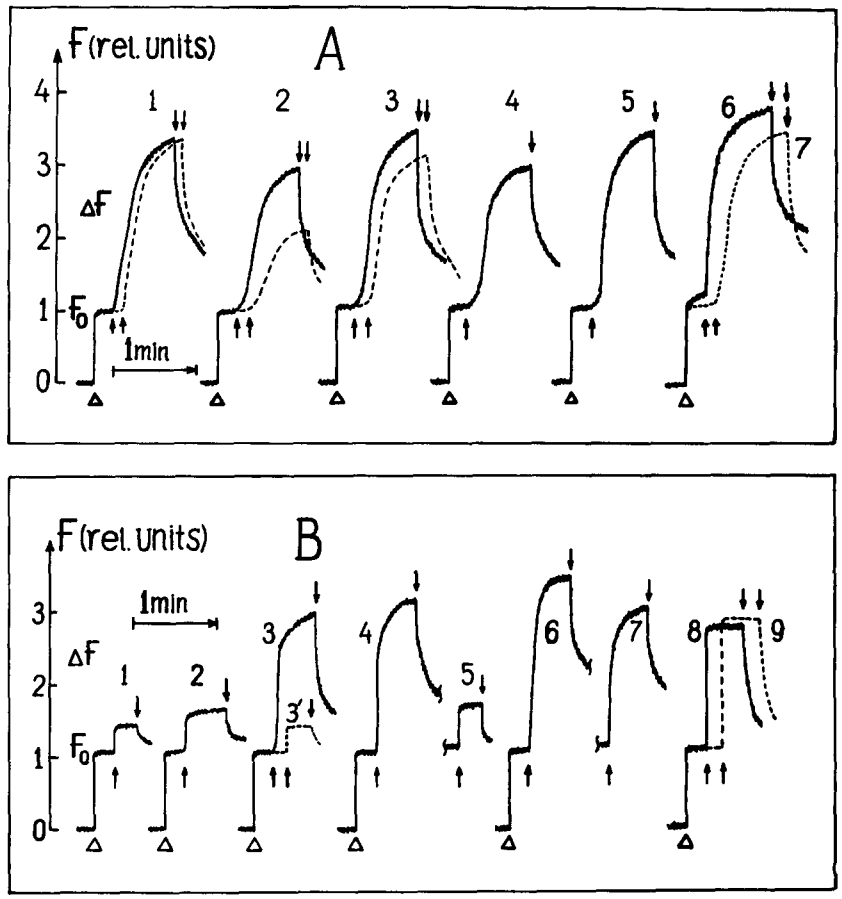

Fig. 1. Effect of the removal and re-addition of BC on photoinduced changes of chlorophyll fluorescence yield (ΔF) related to photoreduction of Q_A in PS-II membrane fragments (DT-20): (**A**), untreated DT-20; **1**, in the medium non-depleted of the BC; **2**, after the depletion of BC in the medium by a 60-min flushing with CO_2-free air; **3**, '**2**' plus 6 mM $NaHCO_3$; **4**, after the addition of 10μM $NaHCO_2$ to sample '**1**'; **5**, '**4**' plus 6 mM $NaHCO_3$; **6**, after the addition of 5 mM $NaHCO_2$ to sample '**1**'; **7**, '**6**' plus 6 mM $NaHCO_3$. Dashed traces **1**, **2** and **3** are same as solid but after 3 cycles of 1-min illumination separated by 1-min dark incubation at 20°C; (**B**), Tris-treated (Mn-depleted) DT-20 in the BC depleted medium with no additions (**1**) and after the addition of: 0.2 μM $MnCl_2$ (**2**); 0.2 μM $MnCl_2$ plus 5 mM $NaHCO_3$ (**3**); 5 mM $NaHCO_3$ (**3'**); 20 μM $MnCl_2$ (**4**); 20 μM $MnCl_2$ plus 40 μM EDTA (**5**); 20 μM $MnCl_2$ plus 10 mM $NaHCO_3$ (**6**); 20 μM $MnCl_2$ plus 10 mM $NaHCO_3$ plus 40 μM EDTA (**7**); 1 μM DPC (**8**); 1 μM DPC plus 10 mM $NaHCO_3$ (**9**). The medium contains 50 mM MES-NaOH buffer, pH 6.2 and 35 mM NaCl; $[C]_{Chl}$ = 10 μg/ml; Δ, switching on the measuring light (480 nm, 0.15 W/m^2); ↑ and ↓ actinic light (λ > 600 nm, 100 W/m^2) on and off, respectively.

of 0.2 μM $MnCl_2$ (which corresponds to 4 Mn atoms per one PS-II reaction center) alone does not restore photoinduced ΔF in the medium depleted of BC while a joint addition of 0.2 μM $MnCl_2$ and 6 mM $NaHCO_3$ leads to a complete reactivation of ΔF. On the other hand, 6 mM $NaHCO_3$ alone does not restore the activity. Fig. 2 shows that the BC addition is also required for maximum reactivation of ΔF with Mn^{2+} in the medium non-depleted of BC although the effect is much less than that after preliminary removal of BC. The activation effects of $NaHCO_3$ were saturated at 5–10 mM which was used in most of our experiments. Reactivation of PS-II with $MnCl_2$ in the presence of BC was accompanied by an increased binding of Mn^{2+} to PS-II which was revealed in experiments on removal of the added Mn^{2+} from PS-II membranes by means of their

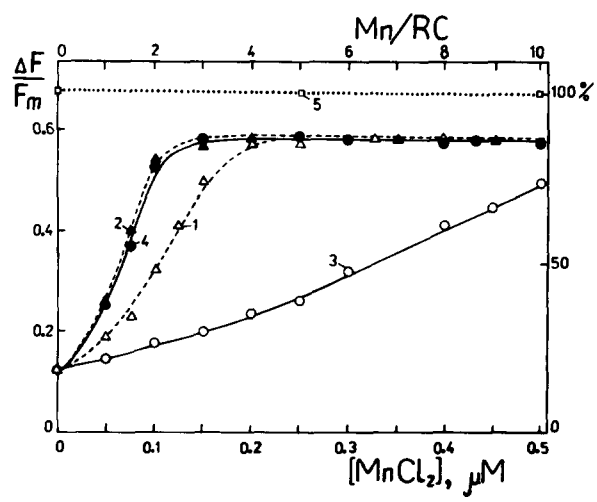

Fig. 2. Magnitude of photoinduced ΔF in DT-20 depleted of manganese as a function of added $MnCl_2$: **1**, in the medium non-depleted of BC; **2**, '**1**' plus 6 mM $NaHCO_3$; **3**, in the medium depleted of BC; **4**, '**3**' plus 6 mM $NaHCO_3$. Line 5 is DT-20 non-depleted of Mn in the medium non-depleted of BC. The experimental conditions are same as in Fig. 1.

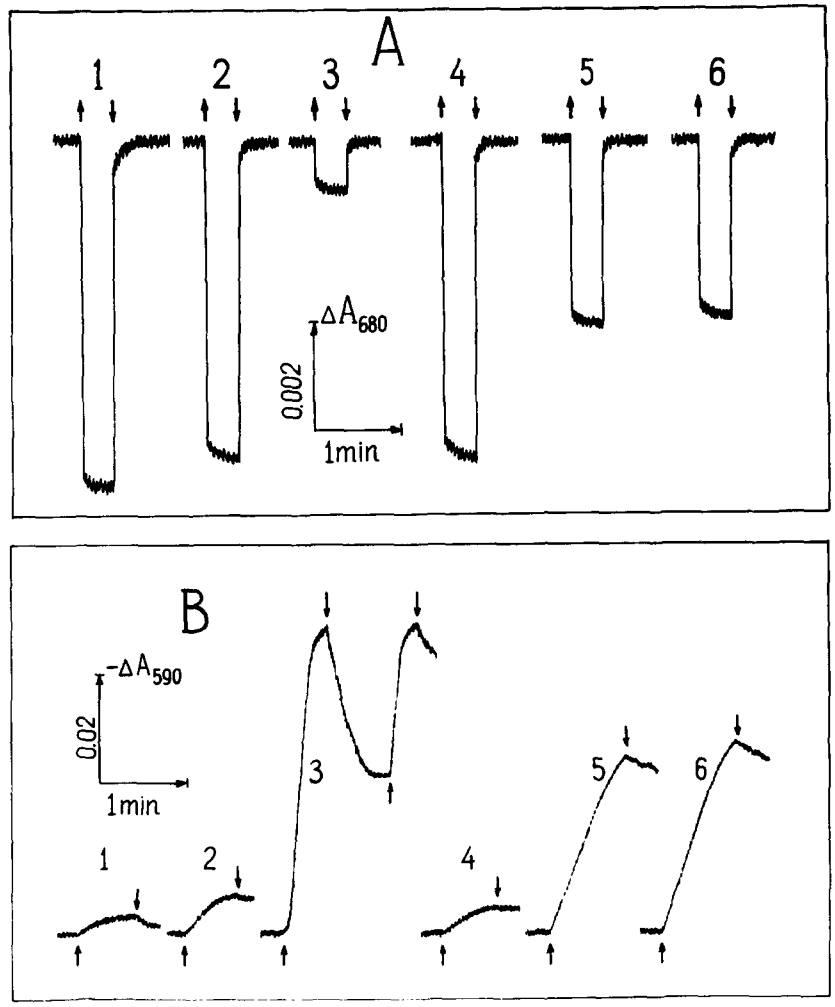

Fig. 3. Kinetics of photoinduced ΔA at 680 nm related to photooxidation of P_{680} in the presence of 0.1 mM SiMo (**A**) and ΔA at 590 nm related to photoreduction of DCPIP (**B**) in Mn-depleted DT-20 in the medium depleted of BC: with no addition (**1**) and after the addition of: 0.2 μM $MnCl_2$ (**2**); 0.2 μM $MnCl_2$ plus 5 mM $NaHCO_3$ (**3**); 5 mM $NaHCO_3$ (**4**); 1 μM DPC (**5**); 1 μM DPC plus 5 mM $NaHCO_3$ (**6**). The experiments conditions are same as in Fig. 1 except pH 7.2 is used instead of pH 6.2.

centrifugation and resuspending of the pellet in the medium free of Mn^{2+}. Photoinduced ΔF was conserved to a much higher extent (2–3 times) if centrifugation of PS-II membranes was done in the presence of 6 mM $NaHCO_3$ (data omitted). Similarly, the addition of 40 μM EDTA (chelating Mn^{2+} and other bivalent cations) to DT-20 reactivated by 20 μM $MnCl_2$ led to considerable (>80%) inhibition of ΔF in the medium depleted of BC while in the presence of 6 mM $NaHCO_3$ the inhibitory effect of 40 μM EDTA was much less, near 20% (Fig. 1B). It is important that the restoration of ΔF in Mn-depleted DT-20 by 2 mM DPC (Fig. 1B) or by 2 mM NH_2OH (in contrast to $MnCl_2$) does not depend on the presence of BC in the medium. On the other hand, BC is needed for the restoration of other partial PS-II reactions with Mn^{2+} (Fig. 3). It is known that continuous illumination of PS-II preparations in the presence of silicomolibdate (SiMo) induces reversible absorbance changes with a maximum around 680 nm (ΔA_{680}) related to photooxidation of the primary electron donor of PS-II, chlorophyll P_{680} [15]. The ΔA are considerably increased upon removal of Mn and inhibited by re-addition of low ($\leq$1 μM) concentration of $MnCl_2$ [13] due to the reactivation of electron donation to PS-II. Fig. 3 shows that the inhibitory effect of 0.2 μM Mn^{2+} on ΔA_{680} is observed only in the presence of 6 mM $NaHCO_3$. On the other hand, the inhibitory effect of DPC (Fig. 3) or NH_2OH (data not shown) on this ΔA does not require BC. Similarly, photoreduction of DCPIP from either DPC or NH_2OH is equally restored both in the presence and absence of BC while restoration of this photoreaction by 0.2 μM $MnCl_2$ takes place only if BC is present in the medium (Fig. 3). Fig. 4 shows the BC effect in DT-20 treated with 1 M $CaCl_2$ (to remove the water-soluble proteins with m.w. 17, 23 and 33 kDa without extraction of Mn [14]). It is clearly seen that the depletion of BC in the medium affects both ΔF and oxygen evolution. The rate of oxygen evolution and ΔF are increased more than two times upon subsequent addition of 6 mM $NaHCO_3$ (Fig. 4). It is important that in all experiments described above the replacement of BC by other anions (sulfate, phosphate, acetate) or an increase of NaCl concentration up to 200 mM did not lead to reactivation of PS-II activities lowered by the BC depletion. On the other hand, the reactivation effects were observed if the BC free medium was exposed to air for two hours (instead of the addition of $NaHCO_3$).

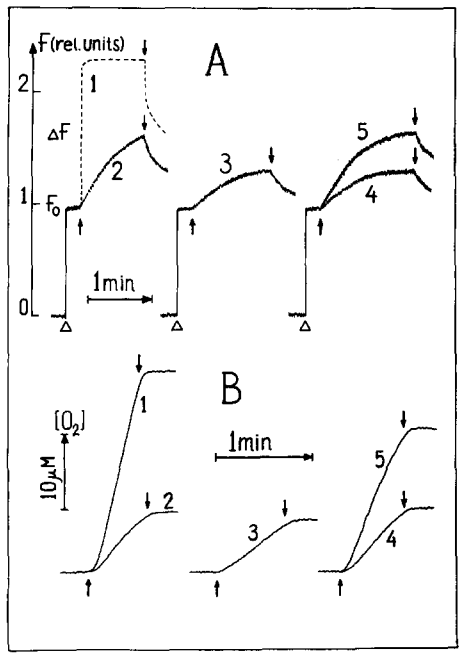

Fig. 4. Kinetics of ΔF (**A**) and oxygen evolution (**B**) in DT-20 treated with 1 M $CaCl_2$ in the medium non-depleted of BC (**2**) and after the depletion of BC in the medium by a 60-min flushing with CO_2-free air (**3–5**) with no additions (**3**) and after the addition of 25 mM $CaCl_2$ (**4**), 25 mM $CaCl_2$ plus 10 mM $NaHCO_3$ (**5**). Intensity of actinic light ($\lambda > 600$ nm) is 20 W/m^2 for **A** and 55 W/m^2 for **B**. Curve **1** (dashed line) on Fig. **A** is ΔF in $CaCl_2$-treated membrane fragments under illumination by saturating light (10^2 W/m^2), curve **1** on Fig. **B** is oxygen evolution in untreated DT-20. The medium is same as in Fig. 1 plus 0.3 M sucrose.

4. Discussion

Inhibition of the photoinduced ΔF without an increase in the level of F_0 upon depletion of BC in the medium and subsequent reactivation of the ΔF with $NaHCO_3$ (Fig. 1) is a characteristic of a reversible blocking of electron transfer on the donor side of PS-II [13]. A typical BC effect on the acceptor side of PS-II is revealed upon the addition of 5 mM formate when F_0as well as the sum F_0 plus ΔF are increased (Fig. 1) while $NaHCO_3$ reverses this effect. This effect was investigated in detail earlier [3,10,11] and it was ascribed to binding of BC to the non-heme iron acting between Q_A and Q_B [8]. The quite opposite effect with low (5–20 μM) concentrations of formate on ΔF (Fig. 1) clearly demonstrates that there is another site of formate action on PS-II. The evident similarity of this effect with that of BC removal from the medium shows that it is also located on the donor side of PS-II. These data also show that the BC binding site on the donor side is characterized by a much lower affinity than that on the acceptor side. Therefore, in all our experiments on partial removal of BC by means of flushing of the medium with air free of CO_2 (without additional removal of BC from the concentrated PS-II membranes) we deal only with inhibition of the low-affinity binding site on the donor side and do not disturb the known high-affinity site(s) on the acceptor side of PS-II. Govindjee and co-workers [10,11] showed that the second binding site of BC revealed by a short-term BC depletion in the presence of formate is located between Y_z and Q_A; however the site between pheophytin and Q_A rather than the donor side of PS-II was suggested [10]. The BC requirement for the donor side of PS-II is strongly supported by the experiments on the restoration of PS-II activities with $MnCl_2$ in DT-20 depleted of Mn (Figs. 1–3). They demonstrate that the restoration effects of Mn^{2+} added at catalytic (less than 1 μM) concentrations are revealed only in the presence of BC. These data as well as the absence of the stimulatory effect of BC on restoration of the electron flow by DPC or NH_2OH (Figs. 1 and 3) show that BC is required for electron donation from the Mn center rather than for the electron flow beyond Y_z. Our results show that one can suggest many possible ways of involvement of BC in the events on the donor side of PS-II.

1. BC 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 O_2-evolving center. This idea of the involvement of BC in the chemistry of photosynthetic oxygen evolution was worked out earlier [5,6]. However, it was not confirmed by experiments on O_2-evolution in the presence of $HC^{18}O_3^-$ or $H_2{}^{18}O$ which showed that oxygen from HCO_3^- was evidently not included in O_2 molecules evolved in PS-II [7]. On the other hand, carboanhydrase activity of PS-II [16] as well as possible discrimination against heavier isotopes in the O_2-evolving process could be responsible for the effects. Our data on restoration of electron transfer with BC in PS-II preparations after re-addition of Mn^{2+} and the absence of the reactivation in the absence of Mn^{2+} do not contradict the idea about direct oxidation of BC with Mn-atoms added to PS-II.

2. BC converts the aqua-ions of Mn^{2+} (non-oxidizable by PS-II) into an easily oxidizable form $Mn(HCO_3)^+$ or $Mn(HCO_3)_2$. The decrease (or the loss) of the positive charge(s) can be favorable for the accessibility of Mn^{2+} to the donor side of PS-II. Besides, our recent experiments (Kozlov et al., in preparation) have shown that the redox potential of Mn^{2+} is lowered from 1.2 V to 0.7 V upon the addition of HCO_3^-. So, thermodynamically $Mn(HCO_3)_2$ must be easier oxidizable by PS-II then the aqua-ion of Mn^{2+}.

3. BC is an essential constituent of the water-oxidizing Mn cluster. BC can be involved in ligation of the Mn atoms which is important for the assembling of the Mn-cluster capable of water oxidation or for regulation of its redox capabilities. Reactivation of O_2-evolution along with reactivation of ΔF (Fig. 4) shows that BC is required for restoration of the water-oxidizing activity (rather than only for reactivation of the electron flow if $Mn(HCO_3)_2$ would be just a good electron donor for PS-II). Data on the increased functional binding of Mn^{2+} in the presence of BC confirm the idea that BC participates in the assembling of the Mn-cluster. It is known that BC complexes of Mn are characterized by extremely high catalase activity [17]. One can suggest that carboxyl group(s) taking part in the formation of the Mn-containing water-oxidizing complex [18] belong to BC rather than to aminoacid residues. On the other hand, enhancement of the BC effect upon repetitive illumination of the sample in the medium depleted of BC (Fig. 1) reveals 'exhaustion' of a bound BC and/or its release as a result of photochemical PS-II reactions (probably due to a dynamic formation of the Mn–BC complex).

4. BC increases the binding of Mn^{2+} and the formation of the cluster capable of water oxidation indirectly, through its binding to PS-II protein(s) thus increasing the capability of the PS-II complex to bind Mn^{2+}. The relatively high concentrations of BC required for the reactivation effects (mM) seem to confirm the idea on involvement of BC in large scale structural changes

rather than in the direct ligation of Mn^{2+}. However, taking into account [17] that the stability constants K_1 and K_2 for the complexes $[Mn(HCO_3)^+]$ and $[Mn(HCO_3)_2]$ are equal to 11 and 3.7, respectively, their formation at concentration of 0.1 μM (2 Mn per one PS-II reaction center) in the presence of 0.1 μM $MnCl_2$ requires the addition of 0.1 M and 0.15 M $NaHCO_3$. This can explain the requirement for a rather high concentration of BC. The BC effect on the acceptor side of PS-II also requires a high (0.1–10 mM) concentration of BC despite the very high binding constant of BC on this site [1–4, 9–11]. The formation of $Mn(HCO_3)^+$ or $Mn(HCO_3)_2$ is the most probable explanation of the BC requirement on the donor side of PS-II. HCO_3^- can be considered as an essential constituent of the Mn-containing water-oxidizing cluster (although a dynamic formation of such a complex as a way of involvement of water molecules in the oxidizing process as well as indirect effects of BC through its binding to PS-II protein(s) can not be excluded).

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