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Effect of bicarbonate on the S_2 multiline EPR signal of the oxygen-evolving complex in photosystem II membrane fragments

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Abstract Removal of bicarbonate from spinach photosystem II BBY particles by means of washing in a CO_2-free medium results in the loss of their capability to accumulate the S_2 multiline EPR signal upon continuous illumination at 190 K. Addition of 1 mM $NaHCO_3$ before illumination leads to a 50–60% restoration of the multiline signal. Similarly, in BBY particles depleted of Mn by treatment with 1 M Tris-HCl (pH 8.0) and 0.5 M $MgCl_2$, re-addition of $MnCl_2$ in the presence of 1 mM $NaHCO_3$ results in a partial restoration (~30%) of the S_2 multiline EPR signal of the Mn cluster, while in the absence of $NaHCO_3$ no restoration is observed. The results provide further evidence that bicarbonate is essential for maintaining the Mn-containing oxygen-evolving complex of PS II in a functionally active form.

Key words: Bicarbonate; Donor side; Manganese; Multiline EPR signal; Photosystem II; S_2 state

1. Introduction

It is well-established that bicarbonate is required for functional activity at the acceptor side of photosystem II (PS II), providing efficient re-oxidation of the first plastoquinone electron acceptor, Q_A ([1] and references therein). The non-heme Fe between Q_A and the secondary electron acceptor, Q_B, has been shown to play an essential role in bicarbonate binding [2]. On the other hand, recently, bicarbonate requirement for the donor side of PS II has been clearly demonstrated [3–7]. It has been shown that bicarbonate removal (using 0.1 mM formate) in so-called DT-20 PS II particles perturbs the Mn cluster of the oxygen-evolving complex (OEC) and the electron transfer from Mn to the oxidized tyrosine $Y_z^{\cdot}$ [7]. Evidence was found that addition of bicarbonate and Mn^{2+} to Mn-depleted PS II membrane fragments suspended in a bicarbonate-free medium considerably advanced the restoration of photoinduced electron flow and oxygen evolution [6].

The S_2 oxidized state of the OEC Mn complex forms a useful and direct probe to extend the investigations of the effect of bicarbonate at the donor side. In this communication we show that bicarbonate is strictly required for manifesting the multiline EPR signal that is attributed to the S_2 state of the OEC [8,9].

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Abbreviations: PS II, photosystem II; OEC, oxygen-evolving complex

2. Materials and methods

The photosystem II BBY membranes were isolated from spinach leaves as described [10]. The BBY particles were stored in liquid nitrogen at a chlorophyll (Chl) concentration of 2 mg/ml. In order to remove the bicarbonate from the preparations, the BBY particles, at a Chl concentration of 100 μg/ml, were incubated for 2 h at 4°C in the dark in a buffer (100 mM KCl/100 mM MES-NaOH, pH 6.2) deprived of endogenous bicarbonate by means of flushing with N_2 gas [3,4,6,7]. Flushing with N_2 gas reduces the CO_2 concentration in the medium and thus indirectly reduces the HCO_3^- concentration in the samples to a value lower than the dissociation constant of HCO_3^- at the donor side (20–34 μM [6]). The suspension was pelleted immediately by centrifugation at $20\,000 \times g$ for 20 min or was pelleted after addition of 1 mM $NaHCO_3$ and subsequent incubation for several minutes. After resuspension in the same bicarbonate-free buffer, with or without additional 1 mM $NaHCO_3$, the samples were photoactivated. All samples were photoactivated by continuous light for 5 min at an intensity of about 150 μE/m^2/s at room temperature. After illumination EDTA was added to bind the free Mn^{2+} and the samples were pelleted by centrifugation and resuspended to a final Chl concentration of 4 mg/ml for use in the EPR experiments.

Complete (>95%) removal of Mn from the membrane fragments was achieved using a 1 M Tris-HCl (pH 8.0) and 0.5 M $MgCl_2$ buffer [11]. BBY particles at a Chl concentration of 100 μg/ml were incubated in this buffer in the dark at 4°C for 30 min. Then, the suspension was pelleted by centrifugation at $20\,000 \times g$ for 20 min. In order to remove all free Mn, the pellet was resuspended in a 0.8 M Tris-HCl buffer (pH 8.0) and incubated in the dark at 4°C for 10 min. Again, the suspension was pelleted by centrifugation and for storage was resuspended in a final 100 mM KCl/100 mM MES-NaOH (pH 6.2) medium to a Chl concentration of 2 mg/ml. Prior to bicarbonate/Mn^{2+} addition, the Mn-depleted BBY particles were suspended in 0.3 M sucrose, 5 mM $CaCl_2$, 100 mM KCl and 100 mM MES-NaOH (pH 6.2) to a Chl concentration of 250 μg/ml. Then suspension (15–20 ml) plus 0.3 mM PPBQ in the presence of either 10 μM $MnCl_2$, 1 mM $NaHCO_3$, or both was preincubated in the dark at 4°C for 20 min before photoactivation.

EPR measurements were performed with a Varian E-9 spectrometer, equipped with an Oxford helium flow cryostat, using 100 kHz field modulation at a microwave frequency of 9.15 GHz, modulation amplitude of 1.0 mT and a microwave power of 1 mW. All EPR spectra were recorded at 10 K.

Photoaccumulation of the S_2 state multiline EPR signal was induced by continuous illumination with white light provided by a 300 W xenon lamp filtered by 3 cm water at 190 K.

3. Results

Fig. 1A shows the characteristic S_2 state multiline EPR spectrum of the OEC in untreated BBY membrane fragments, generated upon illumination by continuous white light at 190 K. When the BBY particles are washed in a buffer deprived of bicarbonate by flushing with N_2 gas, virtually no S_2 state multiline formation is observed after photoactivation attempts (Fig. 1B). However, if $NaHCO_3$ is added to this buffer after the washing period, photoactivation of the sample partially (~60%) restores the S_2 multiline EPR spectrum (Fig. 1C).

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The absence of the S_2 multiline signal upon removal of bicarbonate can be due to blocking of electron transfer through PS II (thus preventing photooxidation of the Mn cluster of the OEC), or to a modification of the Mn cluster itself or of its ligand environment. Note that the S_2 state may instead of the multiline signal exhibit a $g = 4.1$ signal [12]; in our samples this signal was obscured by a strong $g = 4.3$ signal (adventitious rhombic iron). We can therefore not exclude a conversion of the multiline signal to the $g = 4.1$ signal.

In Fig. 2 the effect of bicarbonate on Mn-depleted BBY particles is demonstrated. The upper trace (A) in this figure shows that the S_2 multiline signal is absent in Mn-depleted BBY particles. The relatively low pH (6.2) of the sample medium prevents high activity of endogenous bicarbonate since the p*K* value of its dissociation is 6.4. Addition of excess bicarbonate or Mn^{2+} to the Mn-depleted sample does not lead to formation of the multiline signal upon illumination (see Fig. 2B,C). After an incubation period and photoactivation of the samples, EDTA was added to reduce the six-line EPR signal of the excess unbound Mn^{2+}. On the other hand, the S_2 multiline signal is observed when both bicarbonate and Mn^{2+} are added to the sample (Fig. 2D), indicating a partial restoration of photoinduced electron flow. Although the intensity of the S_2 state multiline EPR spectrum from the photoactivated sample is only 30% of the signal from the untreated sample (Fig. 1A), probably due to incomplete reconstitution, its shape is identical.

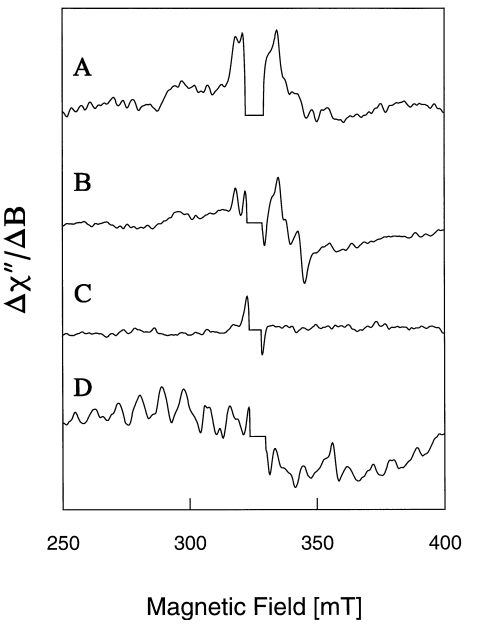

Fig. 2. The S_2 state multiline EPR signal in Mn-depleted BBY particles after a photoactivation procedure. A: Control. B: In the presence of 1 mM $NaHCO_3$. C: In the presence of 5 μM $MnCl_2$. D: In the presence of 5 μM $MnCl_2$ and 1 mM $NaHCO_3$. 10 mM EDTA was added to C and D after the photoactivation procedure to bind the free Mn^{2+} in the EPR sample. Photoaccumulation of the S_2 state was induced by continuous illumination for 10 min at 190 K. EPR conditions: microwave frequency, 9.15 GHz; temperature, 10 K; modulation amplitude, 1.0 mT; microwave power, 1 mW; sample Chl concentration, 4 mg/ml. Dark signals were subtracted from the light-induced signals.

4. Discussion

Recently, it has been shown that removal of bicarbonate from PS II membrane fragments, using formate for replacement of bicarbonate in its binding site(s), seriously disturbs the OEC resulting in both inhibition of electron transfer from Mn to the oxidized electron donor Y_Z, and a release of Mn from its functional binding site [7]. When bicarbonate is present in excess over formate no such effects are observed [7]. Removal of bicarbonate using dilution of PS II membrane fragments in a medium depleted of CO_2 causes, similar to the addition of formate, a bicarbonate-reversible inhibition of electron transfer on the donor side of PS II [3–6]. It has also been shown that in pea thylakoids in the absence of bicarbonate fast one-electron donation to S_2 does not occur [13]. Thus, the suppression of the S_2 multiline signal (Fig. 1) is evidently caused by a perturbation of the donor side of PS II, most probably the OEC. The inhibition of the S_2 multiline signal upon bicarbonate removal is probably not induced by blocking of electron acceptors (due to possible bicarbonate release from its binding site on the non-heme Fe [2]), since it is known that even more efficient blocking of electron transfer from Q_A^- to Q_B by means of addition of a highly specific inhibitor, DCMU, results in only partial inhibition of photoaccumulation of the S_2 multiline signal (probably due to acceleration of charge recombination in PS II) [14], while we observe a complete disappearance of the signal (Fig. 1B). One could also suggest that in the bicarbonate depleted samples, in the dark the S_0 state is accumulated instead of the S_1 state so that the one-electron transfer upon illumination at 190 K results in photoaccumulation of the S_1 state (instead of the S_2 state), resulting in the inhibition of the S_2 multiline EPR signal. However, it has been shown earlier that, upon

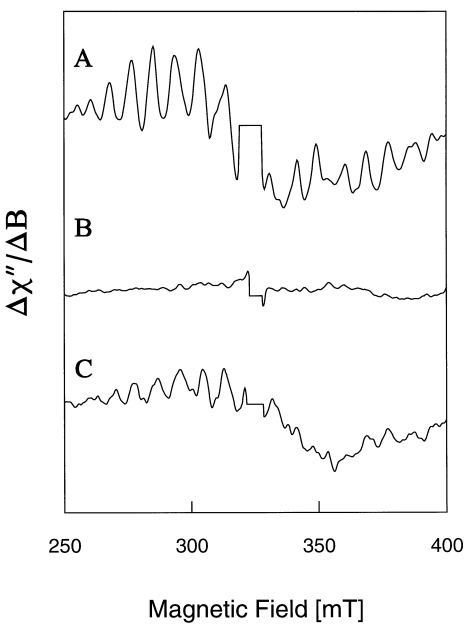

Fig. 1. Effect of removal and re-addition of bicarbonate on the S_2 state multiline EPR signal in untreated BBY particles induced by 10 min continuous illumination at 190 K. A: Control. B: After washing in a CO_2-free buffer and photoactivation at room temperature. C: Same as B plus 1 mM $NaHCO_3$. EPR conditions: microwave frequency, 9.15 GHz; temperature, 10 K; modulation amplitude, 1.0 mT; microwave power, 1 mW; sample Chl concentration, 4 mg/ml. Dark signals were subtracted from the light-induced signals.

partial removal of bicarbonate from the donor side of PS II (using 10 μM formate), the period-four oscillation of the absorbance changes at 295 nm, which is related to the oxidation cycle of the OEC, retains its original shape and phase while the overall amplitude of the absorbance oscillation is lower, indicating that the number of active centers is decreased [5]. It should be mentioned, however, that these experiments were performed on so-called DT-20 PS II membrane fragments and that attempts to repeat the same experiments on 'BBY' particles failed. On the other hand, Allakhverdiev et al. showed considerable effects of bicarbonate on the rates of electron flow and oxygen evolution for Mn-depleted BBY particles [6].

An explanation for the observed effects is found in the concept that bicarbonate may be required for effective ligation of the manganese in the OEC [7]. By decreasing the natural amount of bicarbonate in the sample medium, thereby perturbing its equilibrium with bicarbonate in the BBY reaction center, part of the bicarbonate may leave the reaction center and as a result the manganese cluster of the OEC may become disconnected from the reaction center or may change its configuration.

Fig. 2 shows that nearly 30% of the original S_2 state multiline signal can be restored in Mn-depleted PS II membrane fragments if Mn^{2+} is added jointly with bicarbonate, while in the absence of bicarbonate there is no restoration of the signal. These data are in a good agreement with the earlier results showing that reconstitution of both electron transfer from added Mn^{2+} [3,4,6,7] and oxygen evolution [6] in Mn-depleted PS II preparations required the presence of bicarbonate in the medium. Though photoactivated functional restoration of 30–40% of the S_2 state multiline signal after a complete removal of Mn has been demonstrated earlier [15], our data show that only in the presence of bicarbonate is the OEC able to reassemble in a form capable of photoaccumulation of the characteristic S_2 state multiline EPR signal. This observation strongly supports the conclusion that bicarbonate is an essential constituent of the OEC [3–7]. Moreover, since the shape of the S_2 state multiline EPR spectrum from the Mn-reconstituted BBY sample (Fig. 2D) resembles the control spectrum for untreated BBYs (Fig. 1C), we may conclude that the structure of the reconstituted Mn-complex is similar to the structure before depletion of the Mn.

The bicarbonate effects described here are not due to the known requirement of Cl^- [16,17], and Ca^{2+} [18], for PS II activities including photoaccumulation of the S_2 state, since all the experiments were done in the presence of 100 mM KCl and 5 mM CaCl.

5. Conclusions

By monitoring the multiline EPR signal we have shown that bicarbonate is required to reactivate the S_2 state in BBY and Mn-depleted BBY particles. The results provide strong evidence for previous suggestions that bicarbonate is an essential component for reconstitution of the Mn cluster at the donor side of PS II [3–7]. Further studies of the effect of bicarbonate on the $g = 4.1$ signal are in progress.

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