Bicarbonate May Be Required for Ligation of Manganese in the Oxygen-Evolving Complex of Photosystem II†

V. V. Klimov,*,‡ R. J. Hulsebosch,§ S. I. Allakhverdiev,‡ H. Wincencjusz,§ H. J. van Gorkom,§ and A. J. Hoff§

Institute of Soil Science and Photosynthesis, Russian Academy of Sciences, Pushchino, Moscow Region, 142292, Russian Federation, and Department of Biophysics, Huygens Laboratory, Leiden University, P.O. Box 9504, 2300 RA Leiden, The Netherlands

Received July 21, 1997; Revised Manuscript Received October 13, 1997⊗

ABSTRACT: It was previously shown in the photosystem II membrane preparation DT-20 that photoxidation of the oxygen-evolving manganese cluster was blocked by 0.1 mM formate, unless 0.2 mM bicarbonate was present as well [Wincencjusz, H., Allakhverdiev, S. I., Klimov, V. V., and Van Gorkom, H. J. (1996) *Biochim. Biophys. Acta 1273*, 1−3]. Here it is shown by measurements of EPR signal II that oxidation of the secondary electron donor, Y_Z, is not inhibited. However, the reduction of Y_Z^+ is greatly slowed and occurs largely by back reaction with reduced acceptors. Bicarbonate is shown to prevent the loss of fast electron donation to Y_Z^+. The release of about one or two free Mn^{2+} per photosystem II during formate treatment, and the fact that these effects are mimicked by Mn-depletion, suggests that formate may act by replacing a bicarbonate which is essential for Mn binding. Irreversible light-induced rebinding in an EPR-silent form of Mn^{2+} that was added to Mn-depleted DT-20 was indeed found to depend on the presence of bicarbonate, as did the reconstitution in such material of both the fast electron donation to Y_Z^+ and the UV absorbance changes characteristic of a functional oxygen-evolving complex. It is concluded that bicarbonate may be an essential ligand of the functional Mn cluster.

Bicarbonate is known to be necessary for the maximal activity of photosystem II (PS II)[1] (reviewed in 1). Initially, its site of action was thought to be in the oxygen-evolving system (*2*, *3*). However, later strong evidence for the action of bicarbonate on the acceptor side of PS II, providing efficient re-oxidation of the first plastoquinone electron acceptor, Q_A, has been presented (*4*), and the idea was supported by a number of data (for review see 1). The non-heme Fe between Q_A and the secondary plastoquinone electron acceptor, Q_B, have been shown to play an essential role in bicarbonate binding (*5*). On the other hand, recently a bicarbonate requirement also at the donor side of PS II in so-called DT-20 membrane fragments has been clearly demonstrated (*6*−*8*), and has now also been reported for "BBY" membrane fragments (*9*). In this work we present evidence indicating that bicarbonate is required to maintain the Mn center in a functionally active state capable of efficient electron donation to the oxidized secondary electron donor, Y_Z^+ [tyrosine 161 of the D1 protein of PS II (*10*)].

MATERIALS AND METHODS

Subchloroplast PS II membrane fragments designated here as DT-20 were isolated from spinach chloroplasts using 0.4% digitonin and 0.15% Triton X-100 as described (*11*), with some modifications (*12*, *13*) (except that pH 6.5 was used instead of pH 7.8). Under illumination in the presence of 0.3 mM phenyl-*p*-benzoquinone (PPBQ) the DT-20 fragments evolved 140−200 μmol of O_2/mg of Chl/h. DT-20 fragments were earlier shown to contain one PS II reaction center per 200−220 Chl molecules and only one molecule of P700 impurity per 10 000 Chl molecules (*11*−*13*). 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*). Restoration of the Mn cluster in Mn-depleted DT-20 (Figure 4) was carried out as follows: the Tris-treated DT-20 particles, suspended in 0.3 M sucrose, 5 mM $CaCl_2$, 100 mM KCl, and 100 mM MES-NaOH (pH 6.2) at a chlorophyll concentration of 250 μg/mL, were put in a flat glass tray of 30 mm in diameter. The suspension (10 mL), in the presence of 2.5 μM (2 Mn/PS II) or 5.0 μM (4 Mn/PS II) $MnCl_2$, was preincubated in the dark at 4 °C for 10 min before photoactivation. Photoactivation was carried out at room temperature, in the "absence" of bicarbonate (HCO_3^- concentration in the medium equilibrated with atmosphere at pH 6.2 is less than 20 μM) or after the addition of 1 mM $NaHCO_3$, by continuous light with an intensity of 100 μE m^{-2} s^{-1} for 5 min, in the presence of 0.3 mM PPBQ. The suspension was gently shaken during illumination and tightly sealed. After illumination, the suspension was centrifuged at 20000*g* for 20 min and the pellet was suspended in 100 mM KCl/100 mM MES-NaOH (pH 6.2). The preparations were stored in liquid nitrogen in a medium containing 0.1 M MES-NaOH buffer, pH 6.2,

† This work was supported by the Life Sciences Foundation (SLW) and the Netherlands Foundation for Chemical Research (SON), and was financed by the Netherlands Organization for Scientific Research (NWO), the INTAS Foundation (Grant 93-2849-Ext), and the Russian Foundation of Basic Research (Grant 96-04-50394). S.A. is grateful to the Ministerie de Education y Ciencia of Spain for financial support through the sabbatical program.

* Corresponding author. Fax: +77 (0967) 790 532. E-mail: Klimov@issp.serpukhov.su.

‡ Russian Academy of Sciences.

§ Leiden University.

⊗ Abstract published in *Advance ACS Abstracts,* December 1, 1997.

[1] Abbreviations: PS II, photosystem II; OEC, oxygen-evolving complex of PS II; Y_Z, redox-active tyrosine residue acting as an electron donor to $P680^+$; P680, PS II primary electron donor chlorophyll; Chl, chlorophyll; PPBQ, phenyl-*p*-benzoquinone; DCBQ, 2,6-dichloro-*p*-benzoquinone.

S0006-2960(97)01768-6 CCC: $14.00

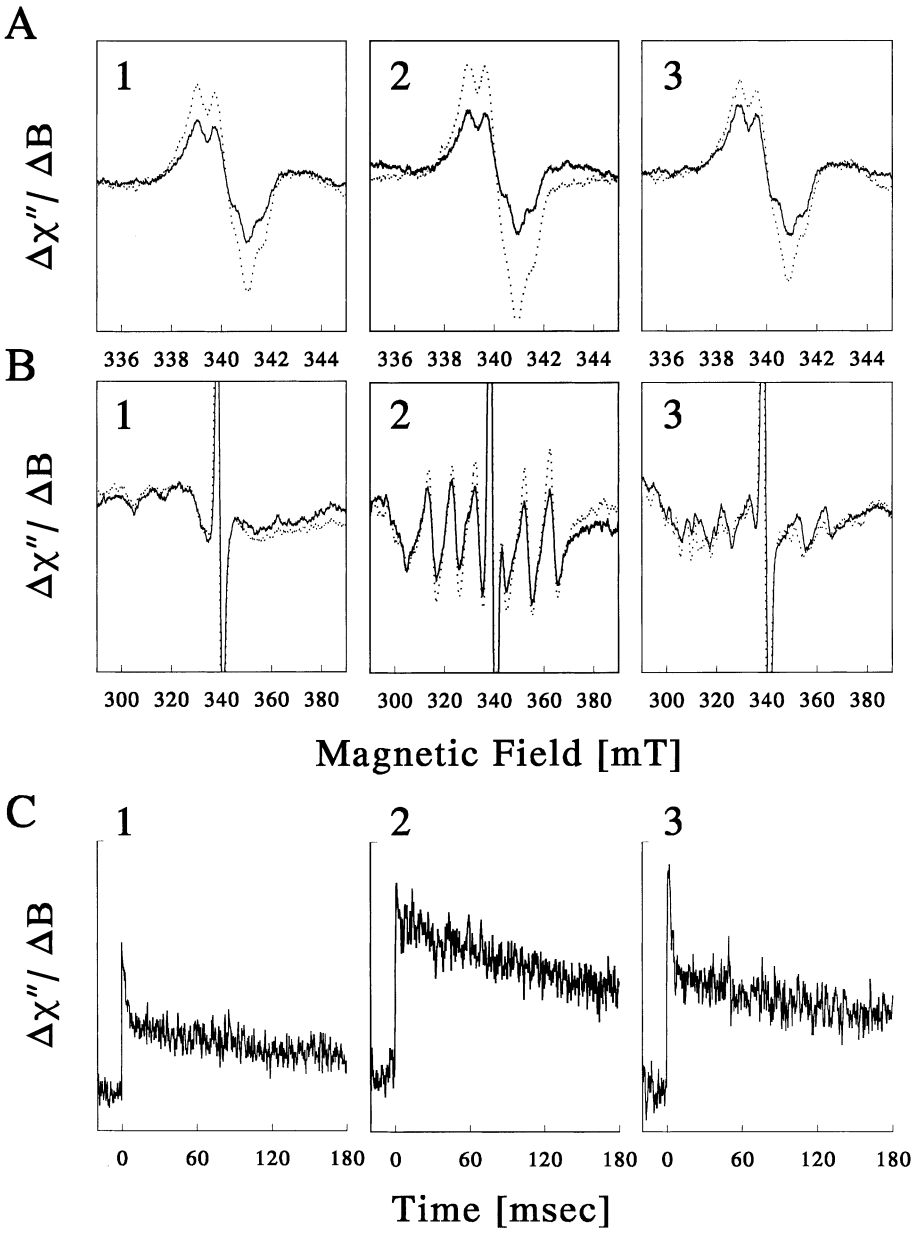

FIGURE 1: Effect of formate and bicarbonate on the EPR spectra of signal II (A) and Mn^{2+} (B) measured in the dark (solid lines) and under continuous illumination (dotted lines), and kinetics of the EPR signal II induced by microsecond light flashes (C), in untreated oxygen-evolving DT-20 membrane fragments. Trace 1, no additions; 2, in the presence of 100 μM sodium formate (after 15 min incubation in the dark at 0 °C); 3, in the presence of 100 μM sodium formate and 200 μM $NaHCO_3$ (after 15 min incubation in the dark at 0 °C). The kinetics were measured at the low-field peak of the spectrum, which does not overlap with the narrower $g = 2$ signal arising from Chl radicals (*18*). An average of 100 flashes was given for each trace. The Chl concentration was 2.8 mg/mL (corresponding to about 14 μM of PS II reaction centers).

0.1 M KCl, and 10% glycerol at a Chl concentration of 2 mg/mL.

For EPR measurements, samples were put into a flat EPR quartz cell 0.1 mm × 10 mm × 30 mm. The EPR experiments were done in the absence of artificial electron acceptors, except for the flash-induced kinetics of signal II, which were measured in the presence of 0.3 mM PPBQ. All measurements were carried out at room temperature. EPR measurements were performed with a Varian E-9 spectrometer as in (*14*), using 100 kHz field modulation at a microwave frequency of 9.52 GHz, microwave power 10 mW, modulation amplitude 0.4 mT for signal II, and 2.0 mT for the EPR signal of Mn^{2+}. Continuous illumination with red light provided by a 300 W xenon lamp (filtered by 5 cm water, a Balzers Calflex filter and a glass OG3 filter) or saturating xenon flashes were used as actinic illumination. The kinetics of the flash-induced EPR signal II were measured with 1 ms time resolution with the averaging of 100 flashes fired at 20 s intervals using a LeCroy 9410, 150 MHz, digital oscilloscope equipped with a signal-averaging facility. Flash-induced UV absorbance changes were measured as described previously (*8*).

RESULTS

The EPR spectra in Figure 1A show that in untreated, oxygen-evolving DT-20 membrane fragments the light-induced increase of signal II, attributed to photoaccumulation of oxidized tyrosine Y_Z^+, is about half the amplitude of the signal present in the dark, attributed to tyrosine Y_D^+. The kinetics of the flash-induced EPR signal II, measured with a 1 ms time resolution (Figure 1C) revealed, in addition to

a spike due to the partially resolved rapid decay phases of Y_Z^+ attributed to reduction by the oxygen-evolving complex (OEC), a substantial component of about 0.1 s lifetime which is stable enough to allow photoaccumulation of the signal in continuous light. This finding is consistent with the earlier conclusion from flash-induced UV absorbance changes that about half of the PS II centers in DT-20 membrane fragments are inactive in oxygen evolution (*8*). In the presence of 100 μM formate the rapid reduction of Y_Z^+ was blocked (Figure 1C) and the light-induced Y_Z^+ signal was about equal to the dark-stable Y_D^+ signal (Figure 1A). This is consistent with evidence that, in the presence of formate, oxidation of the Mn cluster is blocked (*6*−*8*) and shows in addition that oxidation of tyrosine Y_Z is not blocked. Apparently, formate blocks electron transfer from the Mn cluster to Y_Z^+. Addition of 200 μM bicarbonate largely prevented the inhibition (Figure 1C) as was observed also in UV absorbance changes (*8*), although the amount of slowly decaying Y_Z^+ still is larger than in the control, presumably at the expense of the (unresolved) sub-millisecond decay phases.

The broader magnetic field scans presented in Figure 1B show that the inhibition of electron transfer from the Mn cluster to Y_Z^+ was accompanied by the appearance of the six-line signal of free hexaaquo Mn^{2+}. Its amplitude corresponded to about 10 μM Mn^{2+} or 0.7 Mn^{2+} per PS II. Upon illumination, the amplitude increased to 0.9 Mn^{2+} per PS II. These numbers suggest, assuming that Mn was released only from centers that had a functional OEC before formate treatment, that nearly 2 Mn per PS II were released. Again, the effect of formate was largely suppressed by bicarbonate. These observations indicate that bicarbonate may function as a structurally essential ligand to the Mn normally oxidized by Y_Z^+.

The influence of bicarbonate was studied on rebinding of Mn to DT-20 membrane fragments from which all Mn (>95%) had been removed by treatment with 1 M Tris and 0.5 M $MgCl_2$ (*13*). When $MnCl_2$ was added to Mn-depleted PS II with a concentration of 2 Mn/PS II (Figure 2A) or 4 Mn/PS II (Figure 2C) the six-line EPR spectrum of Mn^{2+} (traces 1) was substantially decreased by illumination (traces 2) and largely recovered by a subsequent dark period of 5 min (traces 3). Most likely this is due to reversible photoxidation of Mn^{2+} to Mn^{3+}. In the presence of 1 mM bicarbonate (Figure 2B and 2D) the results were quite different. The Mn^{2+} signal was already nearly halved in darkness and disappeared irreversibly upon illumination. The small signal remaining in the case of 4 Mn^{2+}/PS II (Figure 2D, traces 2 and 3) suggests that nearly 4 Mn/PS II could be converted irreversibly to an EPR-silent form. The control measurements in Figure 2E show that neither bicarbonate (trace 2) nor PS II alone (trace 3) had any effect on the amplitude of the Mn^{2+} signal (trace 1). Only when both bicarbonate and PS II were present was the Mn^{2+} signal halved (trace 4). It is interesting that in all cases described above illumination by much stronger white light (inhibiting PS II as followed from the considerable decrease of both dark and light-induced EPR signals II) resulted in a reappearance rather than a disappearance of the Mn^{2+} signal (data not shown).

The results of Figure 2 suggest that bicarbonate is essential for rebinding of Mn to Mn-depleted PS II. Figure 3 shows that under the same conditions, rapid (≤5 ms) electron

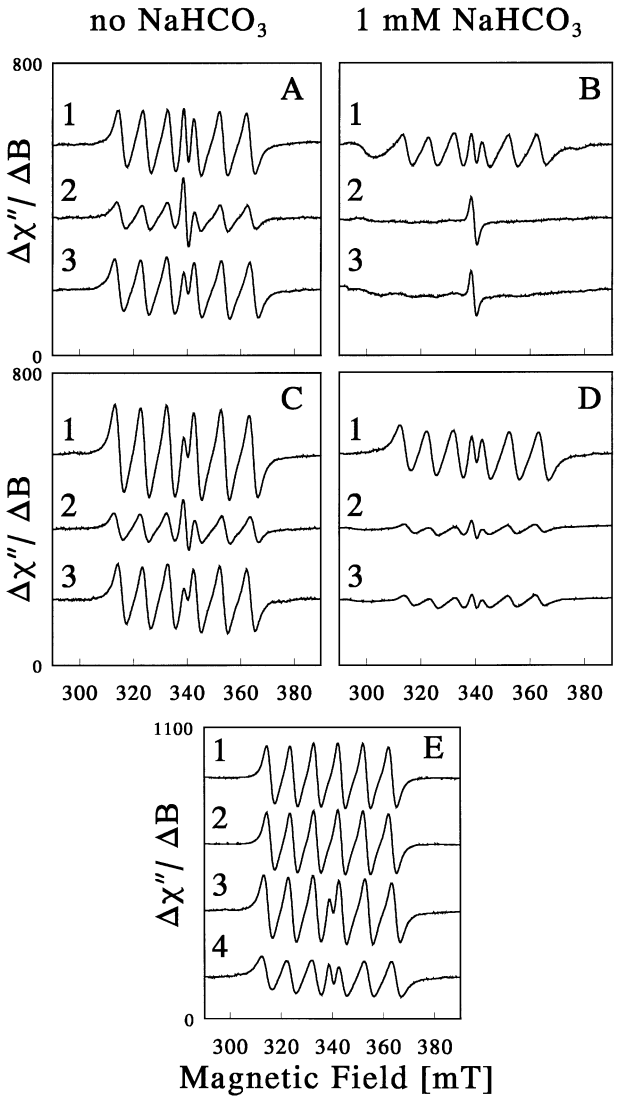

FIGURE 2: Effect of bicarbonate and illumination by continuous light on the EPR signal of Mn^{2+} added to Mn-depleted DT-20 membrane fragments at concentrations of (A, B) 28 μM $MnCl_2$ (2 Mn/PS II) and (C, D) 56 μM $MnCl_2$ (4 Mn/PS II), before (A, C) and after (B, D) the addition of 1 mM $NaHCO_3$. 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. (E) Dark EPR spectra of 56 μM $MnCl_2$ (corresponding to 4 Mn/PS II) measured in the absence (1, 2) and presence (3, 4) of PS II membrane fragments (2.8 mg Chl/mL) depleted of Mn without other additions (1, 3) and after the addition of 1 mM $NaHCO_3$ (2, 4). The receiver gain was four times lower than that for Figure 1B. For other conditions see Materials and Methods.

donation to Y_Z^+ is restored (traces B2 and B3) but not by bicarbonate only (trace B1) nor by Mn^{2+} without bicarbonate (traces A2 and A3). The restoration of electron donation to Y_Z^+ is in line with earlier observations, but the physiological relevance of the reactions involved is not immediately obvious, since oxygen evolution is not restored under these conditions in the absence of Ca^{2+}. However, the rebinding of Mn with concomitant recovery of the fast Y_Z^+ reduction may well be an essential first step in the photoactivation process, because that too was found to be strictly dependent on the presence of bicarbonate. Figure 4A shows the characteristic UV absorbance changes induced by a series of saturating flashes in oxygen evolving PS II, reflecting the four-flash redox cycle of the OEC. No such oscillation with flash number was observed after Mn depletion (Figure 4B),

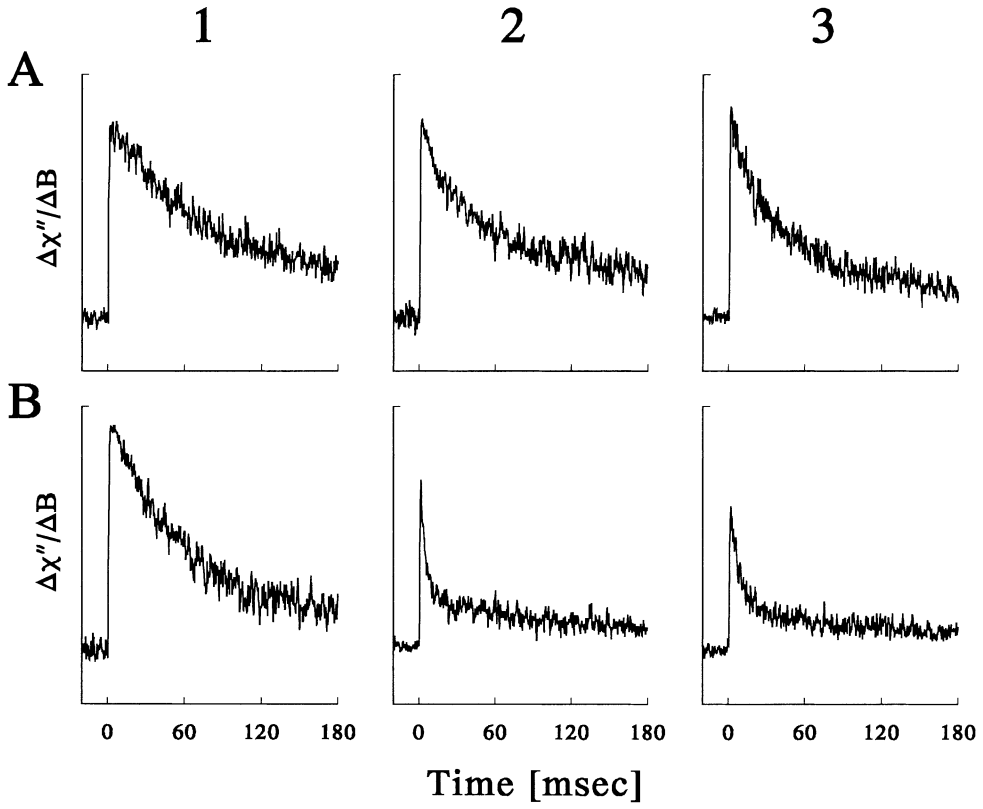

FIGURE 3: Kinetics of flash-induced EPR signal II in DT-20 membrane fragments deprived from Mn before (1) and after (2, 3) the addition of $MnCl_2$ at concentrations of 28 μM (2 Mn/PS II; trace 2) and 56 μM (4 Mn/PS II; traces 3), without other additions (A) and after addition of 1 mM $NaHCO_3$ (B).

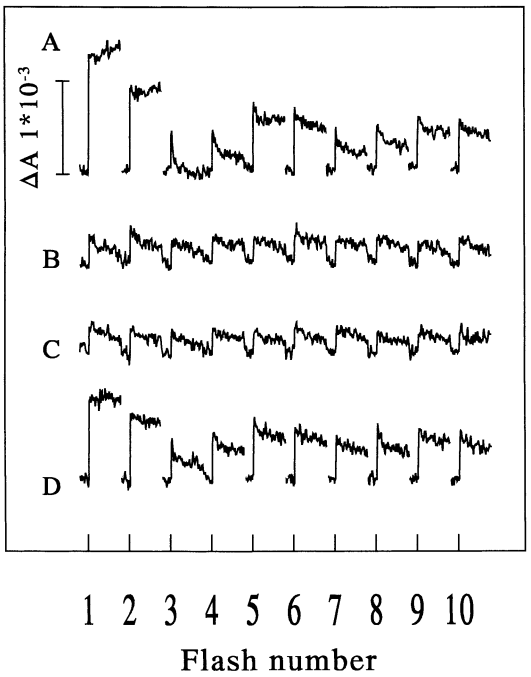

FIGURE 4: Absorbance changes at 295 nm on illumination by a series of 10 saturating flashes spaced at 1 s, in untreated (A) and Mn-depleted (B−D) DT-20 particles suspended in the medium containing 100 mM KCl, 100 mM MES-NaOH (pH 6.2), and 50 μM DCBQ. (B) Without other additions; (C, D) after the addition of 4 Mn/PS II and subsequent photoactivation in the absence (C) or presence (D) of 1 mM $NaHCO_3$. Each trace represents kinetics of absorbance changes during 100 ms. The Chl concentration was 200 μg/mL; the optical path length was 1.2 mm.

and it could only be recovered, to nearly 40% of the original amplitude, by the photoactivation treatment, described in Materials and Methods, in the presence of both Mn and bicarbonate (Figure 4C and 4D).

DISCUSSION

The finding that 0.1 mM formate severely disturbs the oxygen-evolving complex in DT-20 PS II membranes (Figure 1) is consistent with the observation that it blocks photoxidation of the Mn cluster (*8*). The present data allow us to specify that the inhibited reaction is electron transfer from Mn to Y_Z^+ and suggest that the cause of inhibition is a release of Mn from its functional binding site. If bicarbonate is present in excess over formate no such effects are observed. Merely, dilution of a DT-20 suspension in a medium deprived of bicarbonate by flushing with CO_2-free air as well as the addition of 20−50 μM formate have been reported to cause a bicarbonate-reversible inhibition of electron transfer at the donor side of PS II (*6*, *7*). Thus, the effects of formate are likely due to removal of bicarbonate. If that results in the release of Mn^{2+}, as Figure 1 suggests, a role of bicarbonate as a structurally essential ligand of the Mn cluster comes readily to mind. Recently, Stemler and Lavergne confirmed that formate has effects on the electron donor side of PS II (*15*). They showed that in the presence of DCBQ formate accelerates the S1−S0 state transition probably due to destabilization of the manganese tetrad by formate. On the other hand, Jursinic et al. did not see a significant effect of formate and bicarbonate on the EPR signal II in chloroplasts (*16*), which may be due to a lower accessibility of the donor side of PS II to the anions in these preparations.

Reactivation of electron transport in Mn-depleted DT-20 by addition of $MnCl_2$ was earlier shown to require the

presence of bicarbonate (*6*, *7*). Oxygen-evolving activity was not restored in those studies, but only catalytic amounts of $MnCl_2$ were required (*13*), perhaps mediating oxidation of water to H_2O_2. The data presented here (Figure 2) indicate that the requirement of bicarbonate in this reaction cannot be attributed only to the much lower midpoint potential of Mn^{3+}/Mn^{2+} in the presence of bicarbonate (*17*), because also in its absence a light-induced oxidation of added Mn^{2+} seems to occur. On the other hand, the data in Figure 2 do reveal a strictly bicarbonate-dependent, irreversible binding of 2−4 Mn per PS II. It is probably this bound Mn which restores electron donation in PS II, in agreement with the earlier observation that the restored electron transport activity persists after resuspension of the PS II particles in Mn-free buffer (*7*).

Figure 3 shows that also the rapid (≤5 ms) reduction of Y_Z^+ was restored by Mn addition to Mn-depleted DT-20 if, and only if, bicarbonate was present. The results on restoration of the rapid reduction of Y_Z^+ by added Mn^{2+} are very similar to those obtained earlier by Hoganson et al. (18) on "BBY" PS II membrane fragments without bicarbonate depletion and re-addition, and indicate that the protonatable ligand with pK between 6.0 and 7.5 postulated by Hoganson et al. is in fact bicarbonate (pK 6.4). With Hoganson et al. (*18*), we note that the kinetics suggest that Y_Z^+ is reduced by a Mn bound at or near the site it occupies in intact PS II and that the binding of this Mn might well be an essential first step in the photoactivation of the OEC [although we cannot specify presently which of the earlier shown steps for OEC photoactivation (*19*−*21*), requires bicarbonate]. Figure 4 confirms that strict requirement of bicarbonate was observed for photoactivation of oxygen evolution, as was indicated by the recovery of the characteristic UV absorbance changes. With DCBQ as an electron acceptor, this requirement cannot be attributed to the acceptor side (*8*).

The amount of Mn involved in bicarbonate-dependent binding (2−4 Mn/PS II) and recovery of the fast electron donation to Y_Z^+ (2 Mn/PS II) was larger than the amount released from untreated DT-20 upon formate addition (1 Mn/PS II), but only about half of the PS II in DT-20 is active in oxygen evolution. So, it is possible that actually 2 Mn/PS II are involved in both cases and that they are bound as a dimer. We conclude that a direct ligation of this Mn pair by bicarbonate explains our data well. Isotopic labeling may help to verify this explanation. If confirmed, the readily exchangeable bicarbonate ligand should be a useful tool to study the Mn cluster in DT-20, even if its presence in the native system remains uncertain. We cannot exclude that its function is normally performed by a carboxylate of the 33 kDa or other protein.

ACKNOWLEDGMENT

The authors are thankful to Dr. R. Picorel for the help in performing this work.

REFERENCES

1. Govindjee and Van Rensen, J. J. S. (1993) in *The Photosynthetic Reaction Center* (Deisenhofer, J., and Norris, J. R., Eds.) Vol. 1, pp 357−389, Academic Press, San Diego, CA.
2. Stemler, A., and Govindjee (1973) *Plant Physiol. 52*, 119−123.
3. Stemler, A. (1980) *Biochim. Biophys. Acta 593*, 103−112.
4. Wydrzynski, T., and Govindjee (1975) *Biochim. Biophys. Acta 387*, 403−408.
5. Diner, B. A., and Petrouleas, V. (1990) *Biochim. Biophys. Acta 1015*, 141−149.
6. Klimov, V. V., Allakhverdiev, S. I., Feyziev, Ya. M., and Baranov, S. V. (1995a) *FEBS Lett. 363*, 251−255.
7. Klimov, V. V., Allakhverdiev, S. I., Baranov, S. V., and Feyziev, Ya. M. (1995b) *Photosynth. Res. 46*, 219−225.
8. Wincencjusz, H., Allakhverdiev, S. I., Klimov, V. V., and Van Gorkom, H. J. (1996) *Biochim. Biophys. Acta 1273*, 1−3.
9. Allakhverdiev, S. I., Yruela, I., Picorel, R., and Klimov, V. V. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 5050−5054.
10. Barry, B. A., and Babcock, G. T. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 7099−7103.
11. Shutilova, N. I., Klimov, V. V., Shuvalov, V. A., and Kutyurin, V. M. (1975) *Biofizika 20*, 844−847 (in Russian).
12. Allakhverdiev, S. I., Klimov, V. V., and Proskuryakov, I. I. (1988) *Biofizika 33*, 600−604 (in Russian).
13. Klimov, V. V., Allakhverdiev, S. I., Shuvalov, V. A., and Krasnovsky, A. A. (1982) *FEBS Lett. 148*, 307−312.
14. Gast, P., and Hoff, A. J. (1979) *Biochim. Biophys. Acta 548*, 520−535.
15. Stemler, A. J., and Lavergne, J. (1997) *Photosynth. Res. 51*, 83−92.
16. Jursinic, P., Warden, J., and Govindjee (1976) *Biochim. Biophys. Acta 440*, 322−330.
17. Kozlov, Yu. N., Kazakova, A. A., and Klimov, V. V. (1997) *Biological Membranes 14*, 93−97 (in Russian).
18. Hoganson, C. W., Ghanotakis, D. F., Babcock, G. T., and Yocum, C. F. (1989) *Photosynth. Res. 22*, 285−293.
19. Tamura, N., and Cheniae, G. M. (1987) *Biochim. Biophys. Acta 890*, 179−194.
20. Miller, A.-F., and Brudvig, G. (1990) *Biochemistry 29*, 1385−1392.
21. Miyao-Tokutomi, M., and Inoue, Y. (1992) *Biochemistry 31*, 526−532.

BI9717688