Biochimica et Biophysica Acta 848 (1986) 147–151
Elsevier

BBA 40065

BBA Report

Bicarbonate, not CO_2, is the species required for the stimulation of Photosystem II electron transport

Danny J. Blubaugh and Govindjee *

Departments of Plant Biology, and Physiology and Biophysics, University of Illinois, 289 Morrill Hall, 505 South Goodwin, Urbana, IL 61801 (U.S.A.)

(Received August 12th, 1985)

Key words: Photosystem II: Bicarbonate effect; Photosynthesis; Electron transport; Quinone; (Spinach chloroplast)

Evidence is presented that the bicarbonate ion (HCO_3^-), not CO_2, H_2CO_3 or CO_3^{2-}, is the species that stimulates electron transport in Photosystem II from spinach (*Spinacia oleracea*). Advantage was taken of the pH dependence of the ratio of HCO_3^- to CO_2 at equilibrium in order to vary effectively the concentration of one species while holding the other constant. The Hill reaction was stimulated in direct proportion with the equilibrium HCO_3^- concentration, but it was independent of the equilibrium CO_2 concentration. The other two carbonic species, H_2CO_3 and CO_3^{2-}, are also shown to have no direct involvement. It is suggested that HCO_3^- is the species which binds to the effector site.

Bicarbonate appears to be an allosteric activator of the photosynthetic reduction of plastoquinone (PQ) in plant thylakoids. Warburg and Krippahl [1] demonstrated that the Hill reaction is impaired when CO_2 is removed from thylakoid membranes. It was later shown that this impairment is on the reducing side of Photosystem II (PS II; for a review, see Ref. 2). A number of anions, particularly formate and acetate, have been shown to interact with the binding of bicarbonate (HCO_3^-) suggesting a more general anion binding site (see e.g., Refs. 3 and 4). However, only HCO_3^- has been shown to exert a stimulatory effect on PS II. Although a partial inhibition of the quinone reactions has been observed in CO_2-depleted thylakoids in the absence of other anions [5], the full inhibitory effect seems to require their presence.

In the presence of formate, electron transfer from the secondary quinone Q_B to the PQ pool is blocked by HCO_3^- depletion [6–8], and electron transfer from the primary quinone Q_A to Q_B is slowed down [7,9]. Herbicides acting at the Q_B site bind less tightly when HCO_3^- is removed, supporting the conclusion that the site of HCO_3^- action is at the quinone level [10–12]. It is now generally accepted that Q_B is a bound PQ which, when fully reduced, exchanges for an oxidized PQ from the PQ pool [13,14]. Competitive binding between herbicides and quinones supports this view [15–18]. Our current hypothesis for the HCO_3^- requirement, as noted earlier, is that HCO_3^- acts as an allosteric activator for the reduction of bound PQ, and induces a conformational change which permits the efficient exchange between the bound PQH_2 and an oxidized PQ. Bicarbonate may also be involved in the protonation of PQH_2 [19,20]. The exact mechanism of action, however, is unknown. Before a reasonable mechanistic model

* To whom correspondence and reprint requests should be addressed.

Abbreviations: Chl, chlorophyll; [CO_2], the CO_2 concentration; DCIP, 2,6-dichlorophenolindophenol; [HCO_3^-], the HCO_3^- concentration; PQ, plastoquinone; PS II, Photosystem II; Q_A and Q_B, first and second quinone acceptors of PS II, respectively.

can be formulated, it must be known whether CO_2 or HCO_3^- is the species required.

The anion (HCO_3^-) had been implicated as the active species on the basis of competition by similar anions, such as formate and acetate [3,21]. However, the pH optimum of the requirement falls closely at the pK_a of HCO_3^-/CO_2 [21,22], and it has been suggested that both CO_2 and HCO_3^- are required [22]. From the effect of pH on the rate of HCO_3^- binding, it was suggested that CO_2 is involved [23]. Similarly, measurements of the lag time between CO_2 vs. HCO_3^- addition and restimulation of the Hill reaction suggest that CO_2 is required, at least for diffusion to the active site [24,25]. On the other hand, from the concentration dependence at pH 6.8 vs. that at pH 5.8, it was proposed that HCO_3^- is the active species [26]. We have sought to resolve this ambiguity by taking advantage of the pH dependence of the equilibrium concentrations of CO_2 and HCO_3^-, in order to hold effectively the concentration of one species constant while varying the concentration of the other. Here we present evidence that it is the anion HCO_3^- that is required for restimulation of the Hill activity in CO_2-depleted thylakoids. All of the other carbonic species (CO_2, H_2CO_3 and CO_3^{2-}) are shown to have no direct involvement in the stimulatory effect. This work was presented by the authors, in a preliminary fashion, at an international symposium [27].

Broken chloroplasts (thylakoids) were obtained by grinding fresh leaves of spinach (*Spinacia oleracea* L.) in a cold (7°C) grinding medium (0.5% (w/v) bovine serum albumin/1 mM EDTA/50 mM sodium phosphate/50 mM NaCl, pH 7.5) for 10 s in a Waring blender. The filtered homogenate was pelleted at $3500 \times g$ for 7 min. The thylakoids in the pellet were washed once and resuspended in 7°C isolation buffer (50 mM phosphate/50 mM NaCl, pH 7.5). The chlorophyll concentration ([Chl]) was then determined as in Ref. 28.

N_2 gas, used to purge containers and solutions of CO_2, was passed through a column of $CaCl_2$ and ascarite to remove any residual CO_2, and then bubbled through distilled H_2O to prevent solution evaporation. The thylakoids were depleted of CO_2 following the procedure in Ref. 22. Under N_2, aliquots of the CO_2-depleted thylakoids were diluted to [Chl] of 12 μg/ml using reaction medium (50 mM phosphate/100 mM NaCl/5 mM $NaHCO_2$/5 mM $MgCl_2$) of appropriate pH. CO_2-depleted thylakoids at four separate pH values (6.3, 6.5, 6.7 and 6.9) were thus prepared, all having identical [Chl], and having gone through the same isolation and CO_2-depletion procedures. The samples were kept on ice, under N_2, throughout the experiment.

The Hill reaction was measured as 2,6-dichlorophenolindophenol (DCIP) reduction at 600 nm [29], using a Cary-14 spectrophotometer. A cuvette with 60 μM of the anionic form of DCIP was flushed with N_2, then 4.0 ml of the CO_2-depleted thylakoid suspension was added, and the cuvette tightly stoppered. The gas space left in the cuvette was approx. 150 μl. This volume is small enough that we could ignore the escape of CO_2 into the gas space after HCO_3^- addition. The samples were illuminated with $1.5 \cdot 10^3$ W $\cdot$ m^{-2} red light (Corning CS2-59 filter). The photomultiplier was shielded with a 595 nm interference filter. The HCO_3^-/CO_2 was added after 1 min of dark adaptation, and the mixture was allowed to equilibrate for exactly 3 min before measuring the Hill reaction. At room temperature, the equilibration between carbonic species in aqueous solution is complete within 1 min [30], and the diffusion into the membrane and re-equilibration at the binding site was estimated to be over within 2.5 min by following the time-course of restoration by a half-saturating [HCO_3^-].

At equilibrium, the ratio of CO_2 to HCO_3^- in solution is dependent upon the pH, according to the reaction:

$$CO_2 + H_2O \overset{K_1}{\leftrightarrow} H_2CO_3 \overset{K_2}{\leftrightarrow} H^+ + HCO_3^- \overset{K_3}{\leftrightarrow} 2H^+ + CO_3^{2-} \quad (1)$$

where $K_1 (= (1.4 \pm 0.2) \cdot 10^{-3}$ M), $K_2 (= (3.2 \pm 0.4) \cdot 10^{-4}$ M) and $K_3 (= 4.70 \cdot 10^{-11}$ M) are equilibrium constants [31]. CO_2, in Eqn. 1 refers to dissolved, not gaseous, CO_2. Thus,

$$K_1 = \frac{[H_2CO_3]}{[CO_2]}; \; K_2 = \frac{[H^+][HCO_3^-]}{[H_2CO_3]}; \; K_3 = \frac{[H^+][CO_3^{2-}]}{[HCO_3^-]} \quad (2)$$

From these equations, the equilibrium (eq) con-

centrations of CO_2 and HCO_3^- can be calculated if the pH and the initial (i) total concentration of carbonic species are known:

$$[HCO_3^-]_{eq} = \frac{[HCO_3^-]_i}{\frac{[H^+]}{K_1K_2} + \frac{[H^+]}{K_2} + 1 + \frac{K_3}{[H^+]}} \quad (3)$$

$$[CO_2]_{eq} = \frac{[H^+]}{K_1K_2}[HCO_3^-]_{eq} \quad (4)$$

Fig. 1 shows the rate of DCIP reduction, expressed as a percentage of the fully restored rate, by CO_2-depleted thylakoids as a function of $[CO_2]_{eq}$. It is apparent that $[CO_2]$, at which the restored rate is a half maximal, is dependent on the pH. On the other hand, when the same data are plotted against $[HCO_3^-]_{eq}$ (Fig. 2), there is no apparent pH dependence; although the ratio of CO_2 to HCO_3^- at equilibrium varies nearly 4-fold over the pH range of the experiment, each curve falls on top of the others. From Eqn. 4 it is obvious that the ratio of CO_2 to HCO_3^- is constant at any given pH, but changes proportionately with any change in $[H^+]$. The lack of pH dependence in Fig. 2 means that $[CO_2]$ has no apparent effect on the degree of restoration, whereas the pH dependence in Fig. 1 indicates that $[HCO_3^-]$ is important. The inset in Fig. 1 shows that the Hill activity increases with increasing $[HCO_3^-]_{eq}$ with $[CO_2]_{eq}$ constant at 0.1 mM. From the inset in Fig. 2, which shows the effect of $[CO_2]_{eq}$ on the Hill activity, with $[HCO_3^-]$ constant at 0.2 mM, it is clear that the stimulatory effect of HCO_3^- is independent of the CO_2 level.

The other two carbonic species, H_2CO_3 and CO_3^{2-}, present at extremely low concentrations, can be ruled out as having any involvement. The H_2CO_3-to-CO_2 ratio at equilibrium is equal to K_1 of Eqn. 1 and is independent of pH. Since $[H_2CO_3]$ is directly proportional to $[CO_2]$ at all pH values, identical arguments apply for H_2CO_3 as made above for CO_2. Thus, H_2CO_3 cannot be the required species. The ratio of CO_3^{2-} to HCO_3^- at equilibrium, on the other hand, is equal to $K_3/[H^+]$. Since this ratio is inversely proportional to $[H^+]$, the data in Fig. 2 would be expected to show a pH dependence if CO_3^{2-} were involved in stimulating the Hill reaction. As was the case with CO_2, the lack of such pH dependence suggests that CO_3^{2-} is not involved.

The conclusion that CO_2 (or to a lesser extent, H_2CO_3) may be required for diffusion to the

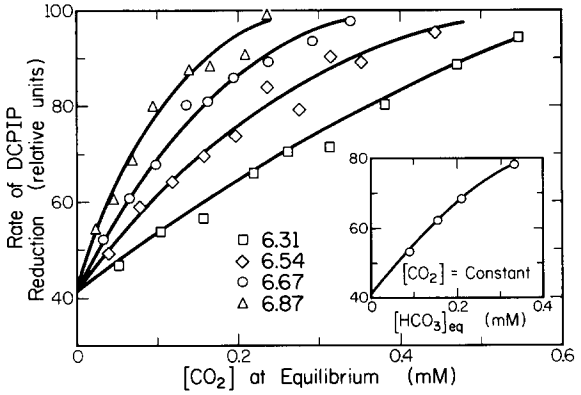

Fig. 1. The rate of DCIP reduction in CO_2-depleted thylakoids, expressed as a percentage of the control rate, as a function of the equilibrium CO_2 concentration. The reduction of DCIP was calculated from the decrease in absorbance at 600 nm. Illumination began 3 min after the addition of $NaHCO_3$. The control rate was determined separately for each curve by adding a saturating amount of HCO_3^- (2.5 mM) to the CO_2-depleted samples. The control rates, in μmol DCIP reduced per mg Chl per h, for each pH, were: pH 6.31, 209 (□); pH 6.54, 212 (◇); pH 6.67, 191 (○); and pH 6.87, 192 (△). Inset: the effect of the equilibrium HCO_3^- concentration on the Hill activity, with the CO_2 concentration held constant at 0.1 mM.

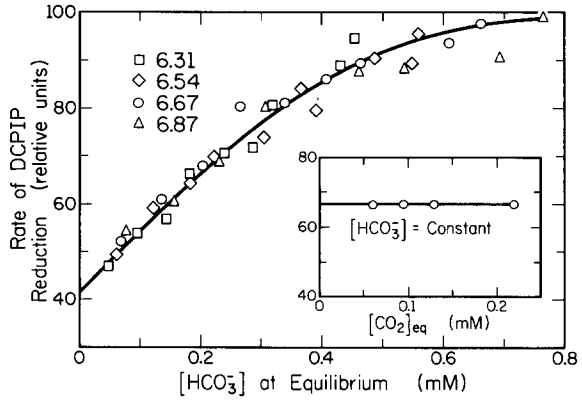

Fig. 2. The rate of DCIP reduction in CO_2-depleted thylakoids, expressed as a percentage of the control rate, as a function of the equilibrium HCO_3^- concentration. The symbols and the protocol are the same as in Fig. 1. Inset: the effect of the equilibrium CO_2 concentration on the Hill activity, with the HCO_3^- concentration held constant at 0.2 mM.

active site [24,25] is not disputed by the data presented here, since our measurements were made under equilibrium conditions and do not reflect the kinetics of HCO_3^- or CO_2 binding. While $[HCO_3^-]_{eq}$ is shown here to be a critical factor, $[CO_2]$ could be an important kinetic consideration if the binding site is buried beneath a hydrophobic domain.

The binding constant for HCO_3^- has been determined to be 80 μM [32,33]. Since $[CO_2]$ in photosynthesizing chloroplasts is estimated to be only 5 μM [34], it was suggested that under normal conditions all of the binding sites are empty, and there may be no real role for HCO_3^- in vivo [32]. However, since HCO_3^-, not CO_2, is the activating species, there is no good reason to assume that the binding sites are empty. For example, if, in the vicinity of the binding site, pH = 8 (as it is in the stroma), then $[HCO_3^-]$ in equilibrium with 5 μM CO_2 is 220 μM, well above the binding constant.

It is apparent from the data presented here, that HCO_3^-, not CO_2, H_2CO_3 or CO_3^{2-}, is the species required for PS II electron transport. This conclusion is consistent with the observed competition by anions, such as formate (HCO_2^-) and acetate ($CH_3CO_2^-$), which closely resemble HCO_3^- [3]. In fact, HCO_2^-, by itself, has been shown to inhibit electron transport in a manner similar to CO_2-depletion, presumably by outcompeting HCO_3^- for its binding site [35]. This effect of HCO_2^- was most pronounced at lower pH values, where the $[HCO_3^-]$ is diminished. Recently, nitrite (NO_2^-), but not nitrate (NO_3^-), has been shown to be as effective as formate at inhibiting PS II, apparently by competition with HCO_3^- [4,27]. It appears that the charge density on the oxygens may be an important parameter affecting the affinity of an anion to the HCO_3^- binding site. NO_3^- and CO_3^{2-}, which resemble each other in this respect, are both ineffective in stimulating electron transport. The effective NO_2^-, on the other hand, has the same degree of charge delocalization as does HCO_3^- and HCO_2^-.

Both HCO_2^- and HCO_3^- have a carboxyl group. However, only HCO_3^- has an hydroxyl group, which may be the functional moiety, while the carboxyl group could be involved in binding. The hydroxyl group, we speculate, could be involved in H^+ mediation during electron transfer from Q_A to Q_B and subsequent release of PQH_2, or it may be important as a source of H bonding to effect a conformational configuration necessary for efficient electron transfer.

Shipman [36] has suggested, as one possibility, that CO_2 may be complexed with a lysine residue to form a carbamate. However, the Q_B apoprotein, whose primary sequence has recently been elucidated (for a review see, e.g., Ref. 37) and where HCO_3^-/HCO_2^- seem to act [2,6,7,8,10,35], contains no lysine. The absence of lysine in the Q_B apoprotein is thus consistent with the conclusion that CO_2 is not the activating species. In view of our earlier results [2,6,7,8,10,35] the Q_B apoprotein seems to be a likely binding site for HCO_3^-. From the secondary structure of the Q_B apoprotein, based on hydropathy plots, arginine-257 appears to be within the hydrophobic matrix [37], and its positive charge is uncompensated by any nearby counter charge. Shipman had suggested [36] that HCO_3^- complexes with an arginine residue and provides a suitable binding environment for some herbicides that interfere with electron flow from Q_A^- to Q_B. Thus, we speculate that arginine-257 may be the binding site for HCO_3^-.

We thank the National Science Foundation (PCM 83-06061) for funds. D.B. was also supported by the National Institute of Health (PHS 5-T32GM7283).

References

1 Warburg, O. and Krippahl, G. (1960) Z. Naturforsch. 15B, 367–369
2 Vermaas, W.F.J. and Govindjee (1982) in Photosynthesis. Development, Carbon Metabolism and Plant Productivity, Vol. 2 (Govindjee, ed.), pp. 541-558, Academic Press, New York
3 Good, N.E. (1963) Plant Physiol. 38, 298–304
4 Stemler, A. and Murphy, J.B. (1985) Plant Physiol. 77, 974–977
5 Eaton-Rye, J.J. and Govindjee (1984) Photobiochem. Photobiophys. 8, 279–288
6 Govindjee, Pulles, M.P.J., Govindjee, R., Van Gorkom, H.J. and Duysens, L.N.M. (1976) Biochim. Biophys. Acta 449, 602–605
7 Siggel, U., Khanna, R., Renger, G. and Govindjee (1977) Biochim. Biophys. Acta 462, 196–207
8 Vermaas, W.F.J. and Govindjee (1982) Biochim. Biophys. Acta 630, 202–209
9 Jursinic, P., Warden, J. and Govindjee (1976) Biochim. Biophys. Acta 440, 332–330

10 Khanna, R., Pfister, K., Keresztes, A., Van Rensen, J.J.S. and Govindjee (1981) Biochim. Biophys. Acta 634, 105–116
11 Van Rensen, J.J.S. and Vermaas, W.F.J. (1981) Physiol. Plant. 51, 106–110
12 Vermaas, W.F.J., Van Rensen, J.J.S. and Govindjee (1982) Biochim. Biophys. Acta 681, 242–247
13 Wraight, C.A. (1981) Isr. J. Chem. 21, 348–354
14 Velthuys, B.R. (1981) FEBS Lett. 126, 277–281
15 Vermaas, W.F.J., Arntzen, C.J., Gu, L.Q. and Yu, C.A. (1983) Biochim. Biophys. Acta 723, 266–275
16 Jursinic, P. and Stemler, A. (1983) Plant Physiol. 73, 703–708
17 Oettmeier, W. and Soll, H.-J. (1983) Biochim. Biophys. Acta 724, 287–290
18 Laasch, H., Schreiber, U. and Urbach, W. (1984) in Advances in Photosynthesis Research (Sybesma, C., ed.), Vol. IV, pp. 25–28, Martinus Nijhoff Dr.W. Junk Publishers, Dordrecht, The Netherlands
19 Khanna, R., Wagner, R., Junge, W. and Govindjee (1980) FEBS Lett. 121, 222–224
20 Stemler, A. (1984) in Inorganic Carbon Transport in Aquatic Photosynthetic Organisms (Berry, J. and Lucas, W., eds.) pp. 376–387, American Society for Plant Physiology, Rockville, MD
21 Khanna, R., Govindjee and Wydrzynski, T. (1977) Biochim. Biophys. Acta 462, 208–214
22 Vermaas, W.F.J. and Van Rensen J.J.S. (1981) Biochim. Biophys. Acta 636, 168–174
23 Stemler, A. (1980) Plant Physiol. 65, 1160–1165
24 Sarojini, G and Govindjee (1981) in Photosynthesis, Vol 2, Photosynthetic Electron Transport and Photophosphorylation (Akoyunoglou, G., ed.), pp. 143–150, Balaban International Science Services, Philadelphia, PA
25 Sarojini, G. and Govindjee (1981) Biochim. Biophys. Acta 634, 340–343
26 Stemler, A. and Govindjee (1973) Plant Physiol. 52, 119–123
27 Eaton-Rye, J.J., Blubaugh, D.J. and Govindjee (1985) in Ion Interactions in Energy-Transducing Biomembranes (Papa, S., Barber, J. and Papageorgiou, G., Eds.), Plenum Press, New York, in the press
28 MacKinney, G. (1941) J. Biol. Chem. 140, 315–322
29 Armstrong, J.M. (1964) Biochim. Biophys. Acta 86, 194–197
30 Cooper, T.G., Tchen, T.T., Wood, H.G. and Benedict, C.R. (1968) J. Biol. Chem. 243, 3857–3763
31 Knoche, W. (1980) in Biophysics and Physiology of Carbon Dioxide (Bauer, C., Gros, G. and Bartels, H., eds.), pp. 3–11, Springer-Verlag, New York
32 Stemler, A. and Murphy, J.B. (1983) Photochem. Photobiol. 38, 701–707
33 Snel, J.F.H. and Van Rensen, J.J.S. (1984) Plant Physiol. 75, 146–150
34 Hesketh, J.E., Wooley, J.T. and Peters, D.B. (1983) in Photosynthesis. Development, Carbon Metabolism and Plant Productivity, Vol. 2 (Govindjee, ed.), pp. 387–418, Academic Press, New York
35 Robinson, H.H., Eaton-Rye, J.J., Van Rensen, J.J.S. and Govindjee (1984) Z. Naturforsch. 39c, 382–385
36 Shipman, L.L. (1981) J. Theor. Biol. 90, 123–148
37 Kyle, D.J. (1985) Photochem. Photobiol. 41, 107–116