PHOTOSYSTEM II ELECTRON FLOW REQUIRES BOUND BICARBONATE

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1. Introduction

Carbon dioxide is required for photosynthesis as it is fixed by ribulose 1,5-bisphosphate carboxylase to produce carbohydrates. In addition, CO_2 (or HCO_3^-) is also involved in Photosystem II (PSII) electron transport, both on its electron donor (see e.g. 1,2), as well as its electron acceptor (see e.g. 3-5) side. This phenomenon is known as the bicarbonate effect. Experiments in several laboratories (6-8) have provided new information on this important phenomenon. Incubation of plant thylakoids and cyanobacteria in media with anions (formate, acetate, nitrate, etc.) inhibits electron flow. This inhibition is reversed fully and uniquely by the addition of bicarbonate (see e.g., 9, 10). Bicarbonate has been shown to be liganded to the non-heme iron in the Q_A-Fe-Q_B complex of PSII (11, 12).

Jursinic and Stemler (13) discussed two hypotheses for the bicarbonate effect: (1) the "bound-bicarbonate" hypothesis: here, a high-affinity binding of bicarbonate is required for high rates of electron flow; and (2) the "inhibitory-anion" hypothesis, also called the "empty-site" hypothesis. Here, for high rates of electron flow, the binding site may be entirely free of any inhibitory anion, and bicarbonate functions only to displace the inhibitory anions. These two explanations differ in the residency state of the binding site for bicarbonate when normal (rapid) electron flow occurs. Jursinic and Stemler (13) failed to demonstrate the presence of CO_2 in CO_2-depleted thylakoid suspensions in low (10 mM) NaCl medium that had high rates of electron flow. Thus, they concluded that the inhibitory-anion or "empty-site" hypothesis is correct, implying that bicarbonate is not a requirement for PSII reactions at this site. On the other hand, Cao et al. (14) using a kinetic model, were unable to distinguish between what they had called the essential and non-essential activator models, respectively, equivalent to the "bound bicarbonate" and "empty-site" hypothesis.

In this paper, we demonstrate CO_2 release under the experimental conditions of Jursinic and Stemler (13) and, thus, support the "bound bicarbonate" and not the "empty-site" hypothesis.

2. Materials and Methods

Broken chloroplasts (thylakoids) were prepared from 10 to 15 day old pea plants (*Pisum sativum* L. var. Rondo) and 7 to 10 day old maize plants (*Zea mays* L.) grown in climate-controlled rooms. Thylakoids were isolated as described earlier (15), suspended in a medium containing 0.4 M sorbitol, 20 mM tricine-NaOH (pH 7.8), 10 mM NaCl, 5 mM $MgCl_2$, 2 mM Na-ascorbate and 0.2% (w/v) bovine serum albumin, and stored at -80°C. Thylakoids were thawed on ice before use.

Maximum rates of O_2 evolution in continuous saturating light was measured as described earlier (15). Thylakoids were pretreated exactly as described by Jursinic and Stemler (13). As was already indicated by Jursinic and Stemler, it is difficult to measure small changes in CO_2-concentration in the gas phase above a thylakoid

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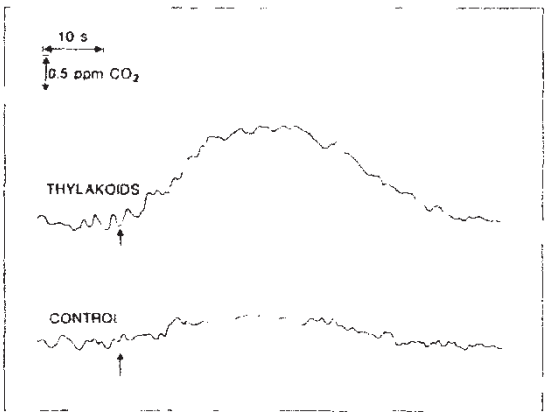
suspension using a closed LICOR infra-red gas analyzer system. They had to take special precautions (such as covering the entire instrument with plastic) to avoid, e.g., leakage. To avoid this problem, we have chosen to use an open infra-red gas analyzing system, and to inject samples taken from the gas phase above the thylakoid suspension. Carbon dioxide concentrations in the gas phase above the samples were measured with a type 225-MK2 infra-red gas analyzing system (IRGA; Analytical Development Co. Ltd., Hertsfordshire, England). This IRGA system is based on a differential measurement method. In both the sample and the reference cell the reference gas (pure N_2) was passed. The difference in the concentration of CO_2 between the sample and the reference cell caused a difference in absorption of IR radiation. This exerted a difference in pressure between the measuring units (which are separated by a membrane) of both cells. This pressure difference was measured. Calibration of the IRGA was carried out with a calibration gas which contained 400 ppm CO_2 in N_2 gas. There was a perfect linearity in the response of our system. The flow rate of reference N_2 gas through both the analyzing and the reference cells was 350 $ml.min^{-1}$. Because of the positive pressure in our system, we encountered no problem of leakage of CO_2 into the analyzer.

Thylakoids were incubated at 20°C in the dark for 30 min in a reaction medium containing 50 mM sodium phosphate and 10 mM NaCl at pH 6.4 at a concentration of 50 μg $Chl.ml^{-1}$, whereafter they were collected by centrifugation at 1000 x g for 8 min and resuspended in 3.8 ml reaction medium at a concentration of 4.2 mg $Chl.ml^{-1}$. The thylakoid suspension was transferred into a septum vial with a magnetic stir bar. To facilitate equilibration between CO_2 and HCO_3^-, 100 μl of carbonic anhydrase solution (20 $\mu g.ml^{-1}$, pH 6.4) was added to a final concentration of 0.5 $\mu g.ml^{-1}$. To examine whether the high affinity binding sites of the pretreated thylakoids were depleted of bicarbonate or not, 100 μl of CO_2-depleted 4 M sodium formate solution at pH 6.4 was injected to a final concentration of 100 mM. Both the carbonic anhydrase and the formate solution were prepared in CO_2-depleted reaction medium at pH 6.4 and stored in septum vials. The gas volume above the thylakoid suspension in the septum vial was 4.8 ml. The thylakoid suspension was incubated at 20°C for 15 min in room light and was continuously stirred during the incubation period. A sample of 0.3 ml was taken from the gas phase above the thylakoid suspension and its CO_2 content was analyzed by injecting the sample into the IRGA via a rubber septum into the gas stream which then entered the analyzing sample cell. We also measured controls in which, instead of thylakoid suspension, reaction medium was incubated.

3. Results and Discussion

Stemler (16) and Jursinic and Stemler (13) concluded that PSII electron flow can proceed normally when the bicarbonate binding site at PSII is empty of all monovalent anions, including bicarbonate. Govindjee et al. (17) had challenged Stemler's conclusion at pH 6 (16), based on the fact that there was much more CO_2 release at pH 6.5 than at pH 6. The conclusion of Jursinic and Stemler (13) was, however, based on the following result, done at pH 6.4.

In maize thylakoids, incubated in the dark for 30 min in a CO_2-free medium at pH 6.5 containing 10 mM NaCl, the rate of O_2 evolution without the addition of bicarbonate was found to be only 5% lower than when bicarbonate was added two min before the start of the measurement. This must have been an almost uninhibited condition, in which the bicarbonate binding site has bicarbonate bound according to the bound-bicarbonate hypothesis, and is empty, according to the inhibitory-anion hypothesis. A choice in favour of the empty-site hypothesis was made when Jursinic



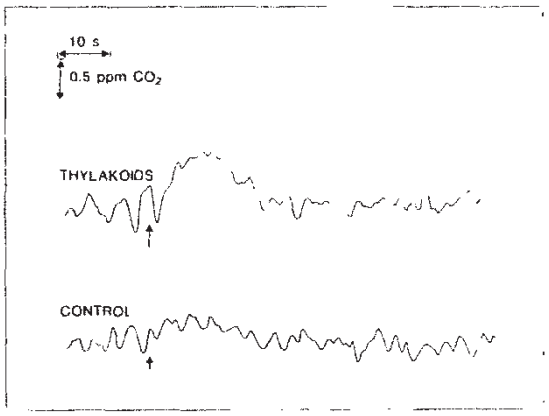


Figure 1. (left) CO_2 release by pea thylakoids after injection of 100 mM formate. At the time of the upward arrow, 0.3 ml gas sample was injected in the IRGA, taken from the gas phase above a thylakoid suspension which was incubated with 100 mM formate during 15 min. Control is from an experiment in which the same medium was used without thylakoids. One representative result out of four experiments.
Figure 2. (right) CO_2 release by maize thylakoids after injection of 100 mM formate. Same protocol as in Fig. 1. One representative result out of four experiments.

TABLE I. The rate of oxygen evolution by maize and pea thylakoids after incubation in the dark at 20 °C for 30 min in a CO_2-free medium containing 50 mM sodium phosphate and 10 mM NaCl at pH 6.4 (n=5). Then, after collection by centrifugation and resuspension of the thylakoids in fresh CO_2-depleted reaction medium to 50 μg Chl.ml^{-1}, 10 mM bicarbonate was added and, after a further 2 min dark incubation, the electron acceptor system (5 mM NH_4Cl, 40 μM DCBQ and 0.4 mM FeCy) was added and oxygen evolution was measured (A. "+ 10 mM $NaCO_3$") The B. "no addition" sample was treated in the same way, but no bicarbonate was added. A third sample (C. "+ 100 mM $NaHCO_2$") was again treated in the same way, but CO_2-free formate was added. Oxygen evolution in μmol O_2.mg Chl^{-1}.h^{-1}. The horizontal line D. "CO_2 release" indicates the number of CO_2 released per total PSII reaction centers upon the addition of 100 mM formate to sample B.

	maize thylakoids	pea thylakoids
A. + 10 mM $NaHCO_3$	198 ± 13	177 ± 11
B. no addition	178 ± 14	153 ± 14
Percent inhibition	10 ± 2	13 ± 3
C. + 100 mM $NaHCO_2$	61 ± 6	37 ± 7
Percent inhibition	69 ± 3	79 ± 3
D. CO_2 release	0.54 ± 0.04	1.04 ± 0.10

and Stemler (13) did not observe any CO_2 release after injection of 100 mM formate to thylakoids, as used above. Because there occurred an unidentified, what they called, respiratory process in their spinach and pea thylakoids, they did not use spinach and pea.

In this work we have repeated this critical experiment. First, we could not find significant differences between CO_2 and O_2 exchanges in peas and maize, used

in this research, in darkness as reported by Jursinic and Stemler (13). We observed a small inhibition (10 - 13%) after incubation of pea thylakoids in the dark for 30 min in a medium containing 10 mM Cl^- (Table I). This is a somewhat larger inhibition than the 5% found by Jursinic and Stemler (13). Since the inhibition is partly reversed by the addition of bicarbonate, this result indicates that this treatment removes bicarbonate to a certain extent from its binding site at PSII. The critical result, however, was that we found a substantial release of CO_2 by the addition of formate to NaCl-treated thylakoids of not only peas (Fig. 1), but also of maize (Fig. 2). This means that, although the thylakoids were incubated in CO_2-free medium for 30 min, they still have bicarbonate bound at their binding site which may be removed by the addition of formate. On the basis of the results presented above, we have calculated the number of CO_2 released by the addition of 100mM formate to be in the range of 0.6 to 1 (Table I).

In conclusion: in both maize and pea thylakoid suspensions depleted of CO_2 without any inhibitor, but with 10 mM NaCl in the medium, high rates of electron flow are associated with the presence of 0.6 to 1 CO_2 per photosystem II reaction center. These data support the essential activator model for bicarbonate, the details of which remain to be elucidated.

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