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Formate Releases Carbon Dioxide/Bicarbonate from Thylakoid Membranes

Measurements by Mass Spectroscopy and Infrared Gas Analyzer

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Photosystem (PS) II acts as a water-plastoquinone oxidoreductase; it transfers four electrons from two molecules of water to plastoquinone producing molecular O_2 and two molecules of doubly reduced plastoquinone. During this process, water protons are released into the lumen and additional protons are taken up into the thylakoid membrane from its stromal side. These protons are utilized to produce plastoquinol from the doubly reduced plastoquinone [1, 2].

Bicarbonate has been suggested to regulate PS II electron flow under a variety of conditions [3]. This bicarbonate effect is assumed to be through the binding of HCO_3^- to the reaction center II complex, particularly the D1 and D2 proteins [3 – 5]. In this model, addition of formate removes HCO_3^-/CO_2 from their binding sites, thus causing inhibition of electron flow. Addition of bicarbonate to formate-treated samples restores the electron flow by displacing the bound formate ions. Another view is that the anion binding sites can be empty in the native membranes; addition of formate ions causes inhibition of electron flow as these ions bind to empty sites. Further addition of bicarbonate ions restores electron flow because the latter displace the inhibitory formate ions. In support of the latter view, Stemler [6] reported that formate addition, which caused drastic inhibition of electron flow in maize thylakoids at pH 6, did not result in CO_2 release. This challenged the hypothesis that formate inhibition of photosynthetic electron transport functions by displacing bicarbonate.

Using two independent methods, a sensitive membrane inlet mass spectrometer and a sensitive differential CO_2 gas analyzer, we show here that formate treatment releases micromolar quantities of CO_2 from spinach and pea membranes. This CO_2 release is pH-dependent and occurs within minutes of formate treatment. At pH 6.5, about 10 μM (1 CO_2/reaction center II) and at pH 6 about 4 μM CO_2 are released with a half-time in the range of 1 to 5 min. Our results are, thus, consistent with the hypothesis that native-bound bicarbonate is released from thylakoid membranes upon binding of formate.

Pea (*Pisum sativum*) thylakoid membranes were prepared as described in [7]. Frozen (−80 °C) pea thylakoids were thawed and suspended in a reaction medium that contained 0.3 *M* sorbitol, 50 m*M* sodium phosphate (pH 6 or 6.5), 10 m*M* NaCl and 5 m*M* $MgCl_2$. Spinach (*Spinacea oleracea*, brand Popi, USA, VegPak Produce Ltd, Toronto, Canada) thylakoids were prepared as described in [8]. Samples were either used fresh or frozen. Frozen (−80 °C) samples were thawed and suspended in a medium that contained 0.3 *M* sorbitol, 10 m*M* NaCl, 5 m*M* $MgCl_2$ and 25 m*M* sodium phosphate (pH 6 or 6.5).

Infrared gas analysis. CO_2 released from pea thylakoids was measured by a type 225-MK3 infrared gas analyzing system (IRGA; Analytical Development Co. Ltd, Hertsfordshire, England) as described in [9]. Two identical glass vessels were used; both vessels contained 4 ml reaction medium. Each vessel was connected separately to the IRGA via a closed circulating system. The total gas volume of the system was 260 ml. Thylakoids (4 mg Chl/ml in the reaction medium) were added to one of the vessels while the other served as a reference containing only the reaction medium. Additions to the two vessels were made simultaneously. Internal controls were run when both vessels contained reaction media or thylakoid suspensions. The instrument was calibrated by known amounts of bicarbonate.

Mass spectrometry. CO_2 was measured continuously as mass 44 ($^{12}CO_2$) by a VG Gas Analysis MM 14-80SC Mass Spectrometer (Middlewich, England) at 20 °C. Details of the experimental protocol are described elsewhere [10, 11] except that mass 44 was monitored continuously on a chart recorder. The sample cuvette was loaded with 6 ml of spinach thylakoids (4 – 6 mg Chl/ml) suspended in reaction buffer. Carbonic anhydrase was added to a concentration of 0.5 μg/ml to facilitate the equilibration between CO_2 and HCO_3^-. This allowed the calculation of the concentration of total dissolved inorganic carbon (DIC) by measurement of CO_2 alone. The instrument was calibrated by the addition of known amount of $NaHCO_3$. During formate addition experiments, the CO_2 signal was corrected by the subtraction of a formate artifact. The nature of this artifact is not known. Bicarbonate standard and formate additions were made on each experimental preparation to obtain internal controls.

Figure 1 shows CO_2 release data from pea thylakoids, as measured by IRGA, at pH 6 and 6.5. At pH 6, injection of 100 m*M* sodium formate led to the release of 1.4 ± 0.3 ppm ($n = 3$) CO_2 within 5 min (Fig. 1 A). Since the total gas volume was 260 ml, this corresponds to 15 ± 3 nmol CO_2. As this arose from a sample volume of 4 ml, the concentration of formate-displaceable HCO_3^-/CO_2 was 3.8 ± 0.8 μM. At pH 6.5, the injection of 100

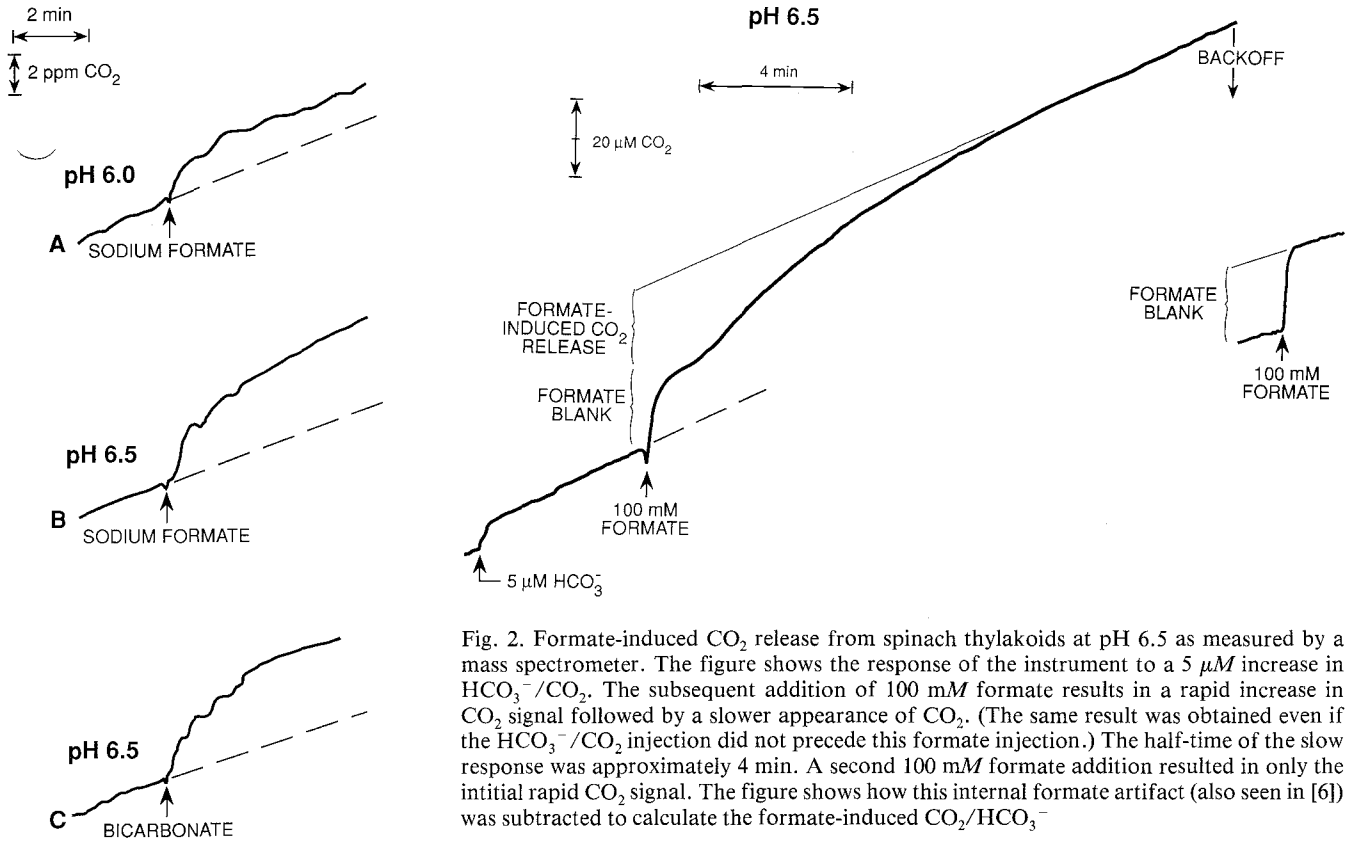

Fig. 2. Formate-induced CO_2 release from spinach thylakoids at pH 6.5 as measured by a mass spectrometer. The figure shows the response of the instrument to a 5 μM increase in HCO_3^-/CO_2. The subsequent addition of 100 mM formate results in a rapid increase in CO_2 signal followed by a slower appearance of CO_2. (The same result was obtained even if the HCO_3^-/CO_2 injection did not precede this formate injection.) The half-time of the slow response was approximately 4 min. A second 100 mM formate addition resulted in only the intitial rapid CO_2 signal. The figure shows how this internal formate artifact (also seen in [6]) was subtracted to calculate the formate-induced CO_2/HCO_3^-

Fig. 1. Formate-induced release of CO_2 from pea thylakoids as measured by IRGA. A) CO_2 release at pH 6 upon addition of 100 mM formate to a thylakoid suspension (4 mg Chl/ml), recorded as the difference with the simultaneous addition of the same amount of formate to the second vessel containing only the suspension medium (see text) without thylakoids. B) Same as (A), but at pH 6.5. C) Recording of the difference of CO_2 in the gas phase upon the addition of 10 μM bicarbonate to one of the vessels and water to the other, while both vessels contained 4 ml reaction medium without thylakoids at pH 6.5; temperature 23 °C

mM sodium formate led to the release of 4.2 ± 0.6 ppm ($n=3$) CO_2, also within 5 min (Fig. 1 B). This corresponds to 46 ± 7 nmol CO_2 per 4 ml sample or a concentration of 11.5 ± 2.3 μM CO_2.

Assuming 1 mol of HCO_3^-/CO_2 binding site per 400 mol of chlorophyll, we estimated that the 16 mg Chl in our 4-ml sample contains 40 nmol of bicarbonate binding sites. Thus, the concentration of expected binding sites is 10 μM. The observation of the release of 11.5 ± 2.3 μM CO_2 at pH 6.5 implies that formate caused the release of approximately 1.2 HCO_3^-/CO_2 per PSII reaction center. At pH 6 this declined to approximately 0.4 HCO_3^-/CO_2 per PSII. Internal controls showed that the injection of 10 μM $NaHCO_3$ to the reaction medium gave a deflection that corresponded to a 4-ppm change (Fig. 1 C). Thus, it appears that 100 mM sodium formate releases about 1 bound HCO_3^- per reaction center at pH 6.5.

Figure 2 shows the formate effect on CO_2 release at pH 6.5 in spinach thylakoids as measured by the mass spectrometer. Addition of 100 mM sodium formate caused a rapid increase in the mass 44 (CO_2) signal followed by a slower increase. This slow increase was complete within 12 min. Fifty percent completion occurred by 4 min. C. Xu, S. Taoka, A. R. Crofts, and Govindjee (unpublished, 1990) have observed a similar time course for formate binding, monitored by Chl *a* fluorescence yield measurements in spinach thylakoids at pH 6.5. A subsequent addition of 100 mM sodium formate showed that the initial rapid increase in CO_2 signal was a formate addition artifact (as also observed by Stemler [6]). Subtraction of the formate artifact from the formate-induced increase in HCO_3^-/CO_2 resulted in the detection of 3 ± 0.6 nmol dissolved inorganic carbon per mg Chl ($n=3$). As discussed earlier, we estimate that slightly more than one (1.3) HCO_3^-/CO_2 per reaction center was released upon formate treatment of spinach thylakoids. This is quite similar to the result (1.2) obtained with differential infrared gas analysis of pea thylakoids.

At pH 6, a formate-induced release of 1 ± 0.2 nmol CO_2 mg^{-1} Chl ($n=3$) was observed (data not shown). The formate-induced CO_2 release in spinach thylakoids was completed within 5 min. Fifty percent completion occurred within 2 min of formate addition. Using the same assumptions as for pH 6.5, approximately 0.4 HCO_3^-/CO_2 was released per reaction center II. Qualitatively, this is also in excellent agreement with the result obtained with differential infrared gas analysis of pea thylakoids.

Preliminary observations on dimethylquinone-ferricyanide Hill reaction in spinach thylakoids (for details, see leg-

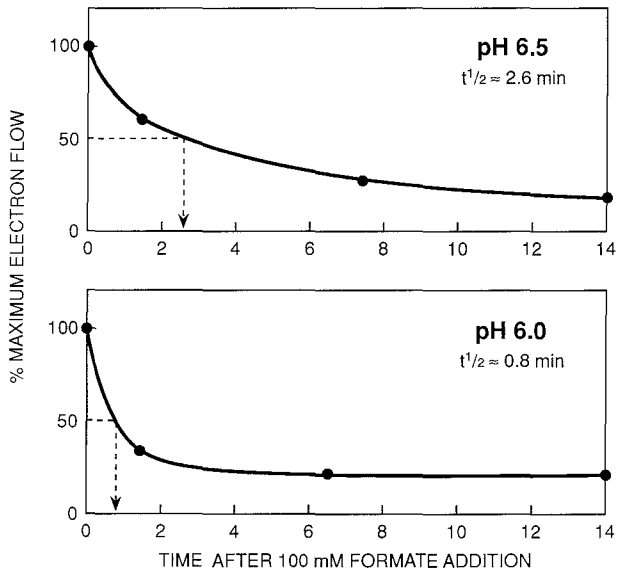

Fig. 3. The effect of formate incubation time on electron flow in spinach thylakoids at pH 6.5 and 6. Half-inhibition occurred after 2.6 min at pH 6.5, and after 0.8 min at pH 6. [A faster binding of formate at pH 6 than at pH 6.5 was also observed by C. Xu (unpublished, 1990) through chlorophyll *a* fluorescence measurements.] Electron acceptor mixture contained 200 μM dimethylquinone (DMQ), 1 mM ferricyanide and 5 mM NH_4Cl. 100 units of oxygen evolution at pH 6 were 150 μmol O_2 mg^{-1} Chl h^{-1} and 200 μmol O_2 mg^{-1} Chl h^{-1} at pH 6.5 when fresh thylakoids were used. [Chl] was 50 μg/ml. (Quantitative comparisons with mass spectrometry and infrared gas analyzer data are not possible due to the unavoidable use of 4 – 6 mg Chl/ml in the latter measurements and due to other experimental differences.)

end to Fig. 3) suggest that formate addition inhibits electron flow within a few minutes in a pH-dependent manner. Depending upon the time of bicarbonate (10 mM) addition, a large part (70 – 80 %) of this inhibition was relieved. These observations, along with the unpublished observations of C. Xu and coworkers, suggest that the release of CO_2 from thylakoids leads to inhibition of electron flow.

Considering the differences in techniques (IRGA versus mass spectroscopy) and samples (peas versus spinach), the agreement in conclusion is remarkable: CO_2 is indeed released when formate is added to thylakoid membranes. Data presented here clearly demonstrate that within minutes after the addition of 100 mM sodium formate, both pea and spinach thylakoids release CO_2, the release being much greater (about 1 CO_2 per PSII reaction center) at pH 6.5 than at pH 6. These results lend support to the hypothesis that CO_2/HCO_3^- plays an important role in the functioning of photosystem II. It is considered likely that under conditions when thylakoids have been depleted of CO_2, formate may not release CO_2 as seems to be the case in. Under physiological conditions, however, bicarbonate is expected to remain bound and function uniquely in electron flow in PSII [3].

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