Reevaluation of the Role of Bicarbonate and Formate in the Regulation of Photosynthetic Electron Flow in Broken Chloroplasts

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ABSTRACT

The stimulation of the Hill reaction in CO₂-depleted broken chloroplasts (Pisum sativum L. cv Rondo) by the total amount of dissolved CO₂ and HCO₃⁻ (bicarbonate*) was measured at several formate concentrations. Formate appears to be a competitive inhibitor of the bicarbonate* stimulation of electron flow. From these experiments we have obtained a reactivation constant (Kᵢ) of 78 ± 31 micromolar NaHCO₃ and an inhibition constant (Kᵢ) of 2.0 ± 0.7 millimolar HCOONa at pH 6.5. In the absence of formate, significant electron flow was measured at a bicarbonate* concentration well below Kᵢ, suggesting that electron flow from Q, the primary electron acceptor of photosystem II, to plastoquinone can proceed when no bicarbonate* is bound to the regulatory site at the Qₒ-protein. If so, bicarbonate* stimulation of electron flow is mainly a diminution of the inhibition of electron flow by formate. In view of the results, it is proposed that regulation of linear electron flow by bicarbonate* and formate is a mechanism that could link cell metabolism to photosynthetic electron flow.

The Hill reaction in chloroplasts that are depleted of CO₂ in the presence of formate, requires the presence of bicarbonate*. In the absence of formate electron flow is inhibited between Q and PQ (6, 7, 24): electron flow between Q and B⁺ is slightly inhibited and the reoxidation of B⁺ by PQ is almost completely blocked (2, 11, 22). Addition of bicarbonate* to these chloroplasts results in a large stimulation of electron flow (6, 7, 23, 24). There appears to be a Michaelis Menten type of relationship between the uncoupled Hill reaction rate and the bicarbonate* concentration in the reaction medium (12, 20), characterized by a maximal Hill reaction rate (Vₘₚ) and an apparent reactivation constant (Kᵢ' = [NaHCO₃] at half-maximal reactivation). At present it is not known whether CO₂, H₂CO₃, or HCO₃⁻ is the species involved in the regulation of electron flow (3, 21).

Although it has been recognized for a long time that formate is somehow involved in the regulation of electron flow by bicarbonate*, the mode of action of formate is still unknown. Good (1) first demonstrated that formate and acetate increase the dependency of the Hill reaction on bicarbonate*. It has been suggested (7, 17, 21) that formate and acetate compete with bicarbonate* for binding sites at the thylakoid membrane. Assuming that bicarbonate* is required for electron flow between Q and PQ, it was argued that displacement of bicarbonate* by formate would result in a lowering of the apparent affinity of the binding sites for bicarbonate*. This hypothesis was supported by Stemler (14), who described two pools of bicarbonate* binding sites and showed that formate can remove bicarbonate* from these binding sites. The pool of high-affinity binding sites was shown to be involved in the regulation of electron flow: removal of bicarbonate* from these high-affinity binding sites was correlated with an inhibition of electron flow. As bicarbonate* was actually removed from the thylakoid membrane by formate in these experiments, it is conceivable that the inhibition of electron flow is not a consequence of the removal of bound bicarbonate*, but may be due to an inhibitory action of formate. Stimulation of electron flow by bicarbonate* then can be explained by assuming that formate is displaced by bicarbonate*. In the former explanation (6, 17, 21), electron flow should be strongly inhibited in the absence of bicarbonate*, whether or not formate is present. As experimental data on this matter are conflicting (5, 7, 17, 23) we have designed experiments to elucidate the mode of action of formate.

Another reason for examining the role of formate in the regulation of linear electron flow in more details comes from the fact that formate is a metabolite which is involved in e.g. photorepiration (4, 10). If linear electron flow in vivo is regulated by bicarbonate* and formate, then metabolic pathways affecting bicarbonate* and formate levels in the chloroplast would also influence photosynthetic electron flow.

MATERIALS AND METHODS

Isolated broken chloroplasts (Pisum sativum L. cv Rondo) were obtained as described before (12). Thylakoids were depleted of CO₂ using a new procedure (Snell, Vermaas, and Van Rensen, unpublished data). Broken chloroplasts (0.5 ml stock suspension containing 2 mg Chl/ml) were washed once in 4.5 ml medium consisting of 0.3 M sorbitol, 50 mM sodium phosphate (pH 5.8), 10 mM NaCl, and 5 mM MgCl₂. The thylakoids were collected by centrifugation (10 min, 1000g) and resuspended in 4.0 ml depletion medium. This depletion medium contained 0.3 M sorbitol, 10 mM sodium phosphate (pH 5.8), 10 mM NaCl, 5 mM MgCl₂, and 10 mM sodium formate. The final Chl concentration was 250 μg Chl/ml. Depletion was accomplished by incubating the chloroplasts at 25°C in depletion medium for at least 60 min in the dark under a N₂ atmosphere. The Hill reaction was measured as O₂ evolution with FeCy as an electron acceptor at 25°C as described before (19). For the determination of the Hill reaction rate, we used the first 20 s of the electrode response. All media were made CO₂-free by bubbling with pure N₂ gas for at
least 1 h.

The kinetic constants $K'$ and $V_{max}$ were determined graphically from Lineweaver Burk plots that consisted of measurements (in duplo) of the Hill reaction rate at six different bicarbonate* concentrations.

**RESULTS**

**CO$_2$ Depletion of Chloroplasts.** The new procedure for depleting chloroplasts of CO$_2$ enables us to obtain large amounts of CO$_2$-depleted chloroplasts. Figure 1 shows the results of a typical CO$_2$-depletion procedure. At the indicated times, a sample of 100 l of the chloroplast suspension was injected into 1150 l of CO$_2$-free reaction medium and the Hill reaction was measured (Fig. 2). After 1 min dark equilibration, the Hill reaction was recorded during 30 s. Subsequently, the light was switched off and NaHCO$_3$ was added to a final concentration of 5 mM. The chloroplasts were incubated for 2 min in the dark and then the Hill reaction in the presence of NaHCO$_3$ was measured. Addition of 5 mM NH$_4$Cl causes a 4-fold stimulation of electron flow and therefore NH$_4$Cl was included in all reaction media. Figure 1 shows further that it takes about 90 min, in this particular experiment, to reach a steady-state situation in which the Hill reaction rate in the absence of added bicarbonate* is less than

![Figure 1](image1.png)

**FIG. 1.** The Hill reaction in the absence and presence of bicarbonate* as a function of the incubation time in the depletion medium. Reaction mixture: 0.3 mM sorbitol, 10 mM NaCl, 5 mM MgCl$_2$, 20 mM sodium phosphate (pH 6.5), 20 mM HCOONa, 5 mM NH$_4$Cl, 0.5 mM FeCy, and thylakoids equivalent to 20 lmg Chl/ml. Hill reactions were determined as in Figure 2.

![Figure 2](image2.png)

**FIG. 2.** Effect of NH$_4$Cl upon the reactivation of the Hill reaction of CO$_2$-depleted chloroplasts by bicarbonate*. Reaction medium as in Figure 1 with the exception that NH$_4$Cl was added at the indicated time at a final concentration of 5 mM. Numbers along the trace indicate Hill reaction rates in lmol O$_2$/mg Chl·h.

![Figure 3](image3.png)

**FIG. 3.** Double reciprocal plot of the Hill reaction rate against the bicarbonate* concentration added to the reaction mixture in the presence of 5.8 and 50.8 mM HCOONa. Chloroplasts were previously depleted of CO$_2$ and incubated in the reaction medium for 2 min in the dark. The reaction medium consisted of: 0.3 mM sorbitol, 20 mM sodium phosphate (pH 6.5), x mM HCOONa, (50.8–x) mM NaCl, 5 mM MgCl$_2$, 0.5 mM FeCy, and 5 mM NH$_4$Cl. The Chl concentration was 20 lmg Chl/ml.

![Figure 4](image4.png)

**FIG. 4.** The apparent reactivation constant ($K'$) as a function of the formate concentration in the reaction medium. Chloroplasts were previously depleted of CO$_2$. Reaction medium as in Figure 1; the chloroplasts were incubated in reaction medium plus bicarbonate* for 2 min before the Hill reaction was measured.

20 lmol O$_2$/mg Chl·h. After addition of 5 mM bicarbonate*, the uncoupled Hill reaction rate is about 130 lmol O$_2$/mg Chl·h. These rates approach the uncoupled rates of nondepleted control chloroplasts suspended in the same reaction mixture.

In the experiments illustrated in Figures 3 to 6, the determination of the bicarbonate* stimulation of the Hill reaction was performed on separate samples at the beginning and at the end of a series of experiments. This was done to avoid effects of light on the binding of bicarbonate* (15) and possibly formate (8). In the presence of 10 mM HCOONa, the stimulation of the Hill reaction by saturating amounts of bicarbonate* was always at least 8-fold in the presence of an uncoupler.

**Interaction of Formate and Bicarbonate* on the Hill Reaction.** In order to establish the kind of interaction of formate and
bicarbonate* on electron flow, we have measured the reactivation of the Hill reaction by various bicarbonate* concentrations at 5.8 and at 50.8 mM HCOONa. Figure 3 shows a double reciprocal plot of the results. It appears that the maximal Hill reaction rate ($V_{\text{max}}$) is not affected by formate, whereas the apparent reactivation constant ($K_r'$) is increased from 0.29 mM NaHCO$_3$ in the presence of 5.8 mM HCOONa to 1.6 mM NaHCO$_3$ in the presence of 50.8 mM HCOONa. Formate apparently acts as a competitive inhibitor of the reactivation of the Hill reaction by bicarbonate*. This inhibition can be interpreted in two ways: (a) Electron flow is only possible when bicarbonate* is bound to the binding site at the thylakoid membrane. Formate merely acts by competing with bicarbonate* for the same binding site or by lowering the affinity of the binding site for bicarbonate*. This view is presented in e.g. References 6, 17, and 21. (b) Electron flow is possible when no bicarbonate* is bound to the binding site. Formate inhibits electron flow and bicarbonate* can stimulate electron flow by displacing formate from its binding site. At high formate and bicarbonate* concentrations, the two mechanisms yield the same result: at these high concentrations the binding sites will be occupied by either formate or bicarbonate* and in both models electron flow should only occur when bicarbonate* is bound. Only at bicarbonate* and formate concentrations below the respective dissociation constants of their complex with the binding site, a significant number of binding sites will be unoccupied and under these conditions we should be able to distinguish between the two mechanisms. We have made an estimation of the dissociation constants of the bicarbonate*- and the formate-binding site complexes by measuring the true reactivation constant $K_r$ and the inhibition constant $K_i$ according to (12). Figure 4 shows that $K_r'$ is linearly dependent on the formate concentration in the reaction medium, as can be expected for competitive inhibition: $K_r' = K_r(1 + [\text{Formate}]/K_i)$. Extrapolation to [HCOONa] = 0 mM yields the reactivation constant $K_r$ and $K_i$ can be calculated from the slope of the line. $K_r$ appears to be 78 ± 31 mM NaHCO$_3$ and $K_i$ = 2.0 ± 0.7 mM HCOONa at pH 6.5 ($n = 5$).

Figure 5 shows the effect of low formate concentrations on the Hill reaction of CO$_2$-depleted chloroplasts in CO$_2$-free reaction medium (no bicarbonate* was added). The relationship between the Hill reaction rate and the formate concentration appears to be hyperbolic (E); a plot of the reciprocal Hill reaction rate against the formate concentration yields a straight line (O). The intercept with the y axis gives the Hill reaction in the absence of formate and (added) bicarbonate*. This Hill reaction rate is about 88 µmol O$_2$/mg Chl·h and $K_i$ in this experiment was estimated to be 3.45 mM HCOONa. This value is not significantly different from the value for $K_i$ given above. Figure 6 shows that CO$_2$-depleted chloroplasts from the same batch exhibit a $V_{\text{max}}$ of about 1.0 µmol O$_2}$/mg Chl·h in the presence of saturating amounts of NaHCO$_3$. It is obvious from Figure 5 that electron flow can proceed at [NaHCO$_3$] $\ll K_r'$, although $V_{\text{max}}$ is not reached.

**DISCUSSION**

**CO$_2$ Depletion of Chloroplasts.** In contrast to earlier methods (e.g. 6, 12, 17, 23), our modified CO$_2$ depletion procedure yields thylakoids that can evolve O$_2$ in excess of 150 µmol O$_2$/mg Chl·h at pH 6.5 at saturating bicarbonate* concentrations. Figure 1 shows that these high rates can only be observed after prolonged incubation of the chloroplasts in deplten medium at pH 5.8. The rather slow development of the dependency of the Hill reaction on bicarbonate* probably reflects the binding of formate. As the action of formate is pH dependent, i.e. more pronounced at low pH (16; H. H. Robinson, personal communication), it could be the protonated base that is involved in the rate-limiting step of the inhibition.

In the CO$_2$-depleted chloroplasts obtained by the new procedure electron flow in the presence of saturating bicarbonate* concentrations can be stimulated about 4-fold by 5 mM NH$_4$Cl or 5 µM Gramicidin J1J2 when FeCy is the electron acceptor. This strongly suggests that in these chloroplasts FeCy accepts electrons after PQ.

**Interaction of Formate and Bicarbonate* on the Hill Reaction.** Stemler (14) has demonstrated the existence of two pools of binding sites for bicarbonate*. The high-affinity binding sites, with a pool size of about one binding site per 400 Chl molecules, were shown to be involved in the regulation of electron flow. The dissociation constant of this binding site/bicarbonate* complex is 80 µM NaHCO$_3$ at pH 6.5 (18), which is practically identical to the value of 78 µM NaHCO$_3$ we obtained for $K_i$ at pH 6.5. In our opinion, this close agreement indicates that the initial Hill reaction rate reflects the state of the regulatory high-affinity binding sites at the Q$_B$-protein with respect to the binding of formate and bicarbonate*. This means that in the experiments described in Figure 5, a large fraction of the binding sites must have been empty, i.e. not occupied by either formate or bicarbonate*. Therefore, we suggest that the high-affinity binding sites...
do not require that a complex is formed with bicarbonate* to allow electron flow from Q to PQ.

However, at the moment the question whether the binding of bicarbonate* to the regulatory site at the Q₈-protein is a requirement for electron flow is still a rather hypothetical one. Our results do not exclude a possible involvement of a small pool of bicarbonate*-binding sites with a very high affinity for bicarbonate*. So far however, there are no indications that such a pool exists.

Another possibility is that binding of bicarbonate* brings the regulatory site in an 'active' conformation, which only slowly relaxes to an 'inactive' state after dissociation of the regulatory site-bicarbonate* complex. In this way only a few bicarbonate* molecules could keep all regulatory sites in the active state when no formate is present.

We did not succeed in achieving a total restoration of electron flow in the absence of formate (Figs. 5 and 6). An explanation for this observation is not known; obviously, further experiments are needed to clarify the exact mechanism of formate-bicarbonate* regulation of electron flow.

In a previous paper (12) we have analyzed the kinetics of the reactivation of the Hill reaction by bicarbonate*. There the assumption was made that electron flow from Q to PQ can only proceed when bicarbonate* is bound to the binding site. According to the present data, this assumption might be not correct. All experiments were done, however, in the presence of 100 mM formate, so the number of 'empty' binding sites must have been negligible. Therefore, the only conformation that allowed electron flow in these experiments was the one in which bicarbonate* was bound to the binding site.

**Physiological Consequences of the Formate/Bicarbonate Interaction on Electron Flow.** The results presented in this paper clearly show that formate and bicarbonate* can regulate linear electron flow in CO₂-depleted thylakoids. These CO₂-depleted chloroplasts were obtained by incubation with formate at pH 5.8. This incubation may seem a rather unphysiological treatment, but Stemler (16) already showed that CO₂-depleted chloroplasts can also be obtained by illuminating broken chloroplasts at pH 8 in the presence of high formate concentrations and an artificial electron acceptor.

Formate is a metabolite that is involved in several metabolic reactions, for instance the breakdown of glyoxylate by H₂O₂ to formate and CO₂ in peroxisomes (4, 10) and chloroplasts (25). Glyoxylate is an intermediate in photorespiration and high rates of glyoxylate decarboxylation have been observed in isolated peroxisomes (4). Figure 7 schematically shows how photorespiration and photosynthetic electron flow could interact in vivo via the formate and bicarbonate* pools. Under highly photosynthetic conditions, e.g. high light intensity and low CO₂ concentration, there is a large carbon flux through the glycolate pathway. If the amount of 'free' H₂O₂ (i.e. H₂O₂ not bound to catalase) is high enough, high rates of glyoxylate decarboxylation can be expected in the peroxisomes (4). The produced formate would have to enter the chloroplast in order to be able to inhibit electron flow. Alternatively, glyoxylate could enter the chloroplast and react with H₂O₂, generated in a reaction of O₂ with reduced Fd (13), to give formate and bicarbonate*. The bicarbonate* formed can be subsequently remetabolized by RUBISCO and the remaining formate will inhibit linear electron flow. So, at high O₂/CO₂ ratios, there is a direct path from photosynthetically produced O₂ to formate, which inhibits linear electron flow. Such a pathway could be regarded as a negative feedback mechanism that is involved in the regulation of linear electron flow. As inhibition of linear electron flow can stimulate cyclic electron flow (9), regulation of linear electron flow by formate and bicarbonate* will result in an altered linear to cyclic electron flow ratio, which in turn will affect the NADPH/ATP ratio in the chloroplast stroma.

**CONCLUSIONS**

These experiments show that formate inhibits linear electron flow in isolated broken pea chloroplasts. Bicarbonate* can diminish this inhibition, probably by displacing formate from the thylakoid membrane. The physiological significance of this regulation of electron flow is still far from being understood; photorespiration might be involved as a source of formate.

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