Interaction of Photosystem II Herbicides with Bicarbonate and Formate in Their Effects on Photosynthetic Electron Flow

J. J. S. van Rensen

Laboratory of Plant Physiological Research, Agricultural University Wageningen, Gen. Foulkesweg 72, 6703 BW Wageningen, The Netherlands

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The reactivation of the Hill reaction in CO_2-depleted broken chloroplasts by various concentrations of bicarbonate was measured in the absence and in the presence of photosystem II herbicides. It appears that these herbicides decrease the apparent affinity of the thylakoid membrane for bicarbonate. Different characteristics of bicarbonate binding were observed in chloroplasts of triazine-resistant *Amaranthus hybridus* compared to the triazine-sensitive biotype. It is concluded that photosystem II herbicides, bicarbonate and formate interact with each other in their binding to the Q_B-protein and their interference with photosynthetic electron transport.

Introduction

Isolated broken chloroplasts can be depleted from bicarbonate by suspending them in a formate-containing medium at a pH below 6, and flushing the suspension with nitrogen gas. These CO_2-depleted chloroplasts, transferred to a reaction medium at pH 6.5 and containing formate, do not show a substantial rate of Hill reaction upon illumination. By incubation of the CO_2-depleted chloroplasts with bicarbonate in the dark the Hill reaction rate can be largely restored. It is not known whether HCO_3^- or CO_2 is the active species in this effect. For convenience this effect has been named the "bicarbonate-effect", although by addition of HCO_3^- also CO_2 is added, the relative concentrations of both compounds being dependent upon pH [1, 2].

It was demonstrated by Govindjee and coworkers that this bicarbonate-effect is caused by an inhibition and reactivation of the photosynthetic electron transport between the primary quinone electron acceptor of photosystem II, Q_A, and the plastoquinone pool [1, 2]. DCMU-type herbicides are also inhibitors of electron transport between Q_A and plastoquinone [3]. Since a relationship between the bicarbonate-effect and the action of herbicides was anticipated, the interaction of herbicides with bicarbonate and formate was investigated. It was found that photosystem II herbicides decrease the affinity of the thylakoid membrane for bicarbonate.

Materials and Methods

Peas (*Pisum sativum* L. cv. Rondo), triazine-resistant and -susceptible *Amaranthus hybridus* plants were grown in a growth chamber at 20 °C and broken chloroplasts were isolated as described elsewhere [4]. Photosynthetic electron transport was measured as oxygen evolution with a Gilson oxygraph as described earlier [5]. The *Amaranthus* chloroplasts lost their activity in Hill reaction rate within a few hours. Their activity was stabilized by addition of 5% glycerol and 0.5% dimethyl sulfoxide to both the suspension and the reaction medium.

Broken chloroplasts were depleted from bicarbonate by suspending them in a medium at pH 5.0 which contained 50 mM Na-phosphate, 100 mM Na-formate, 100 mM NaCl and 5 mM $MgCl_2$, and flushing the suspension with nitrogen gas, as described in [6]. The Hill reaction activity of the chloroplasts was assayed at pH 6.5.

Results and Discussion

After incubation of CO_2-depleted chloroplasts with various concentrations of bicarbonate during 2 min in the dark different rates of reactivated Hill reactions were obtained. Assuming that bicarbonate-binding is equivalent to activation of electron

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Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; dinoseb, 2,4-dinitro-6-*sec*-butylphenol; *i*-dinoseb, 2,4-dinitro-6-isobutylphenol; DNOC, dinitro-*o*-cresol; ioxynil, 4-hydroxy-3,5-diiodobenzonitrile; Q_A, primary quinone electron acceptor of photosystem II; Q_B, secondary quinone electron acceptor of photosystem II; simeton, 2-methoxy-4,6-bis(ethylamino)-1,3,5-triazine.

Reprint requests to Dr. J. J. S. van Rensen.

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transport, it is possible to plot log $(v/V_{max} - v)$ *versus* log bicarbonate concentration (v is the actual Hill reaction rate; V_{max} is the maximal Hill reaction rate at saturing HCO_3^--concentration). Fig. 1 shows these so-called Hill plots for three independent experiments. They show linear relationships with values near 1. The slope defines the Hill coefficient, n_H. The straight line indicates that the system thylakoid membrane *versus* bicarbonate has a Michaelis-Menten behaviour and that there is only one type of activation site for bicarbonate at the thylakoid membrane. The n_H-values of unity mean that there is no cooperativity between the sites. The system can be treated like a system enzyme *versus* substrate.

The same type of experiment was done in the absence and in the presence of a fixed concentration of a herbicide. Fig. 2 shows a double reciprocal plot of the results of an experiment with dinoseb. Qualitatively the same results were obtained with other herbicides like DCMU, simeton, DNOC [6], *i*-dinoseb [7], and ioxynil [8]. From these Lineweaver-Burk plots the apparent dissociation constant (K_d) of the thylakoid-bicarbonate complex and the inhibitor constant (K_i) of the thylakoid-herbicide complex can be calculated. The inhibitor constant of the thylakoid-dinoseb complex was calculated from Fig. 2 to be 48 nM. K_i-values obtained for other herbicides were: 30 nM for DCMU, 250 nM for DNOC [6], 31 nM for *i*-dinoseb [7], and 51 nM for ioxynil. The control experiment of Fig. 2 yields an apparent dissociation constant of 1.13 mM bicarbonate. In the presence of 100 mM Na-formate the K_d-value for bicarbonate always appears to be around 1 mM. In the presence of 100 nM of the herbicide dinoseb the apparent K_d is increased to 3.47 mM HCO_3^-. It appeared that also in the presence of 100 nM of DCMU DNOC, *i*-dinoseb, or ioxynil, the apparent K_d for bicarbonate is increased to at least 2-fold. Also Stemler and Murphy [9] reported a 2.5 times higher apparent K_d for bicarbonate in the presence of 5 μM DCMU. This means that these herbicides decrease the apparent affinity of the thylakoid membrane for bicarbonate.

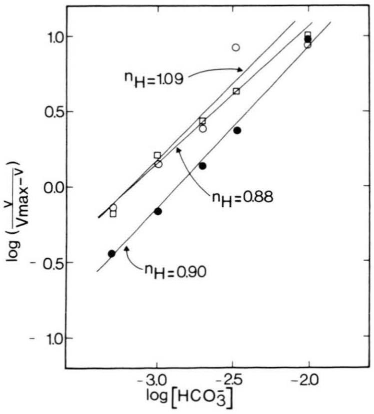

Fig. 1. Hill plots of Hill reaction rates in CO_2-depleted pea chloroplasts after addition of various concentrations of bicarbonate. Reaction medium: 50 mM Na-phosphate (pH 6.5), 100 mM Na-formate, 100 mM NaCl, 5 mM $MgCl_2$, 0.5 mM ferricyanide, and chloroplasts equivalent to 33 μg Chl ml^{-1}. v, Hill reaction rate at the indicated bicarbonate concentration; V_{max} was calculated from Lineweaver-Burk plots of these data.

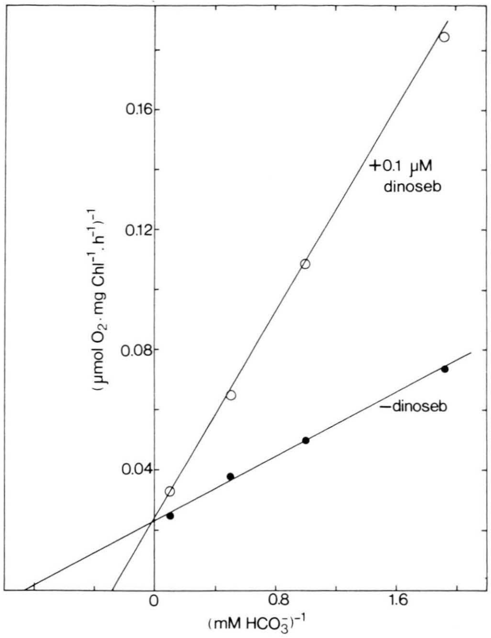

Fig. 2. Double-reciprocal plot of the Hill reaction rate as a function of bicarbonate concentration in CO_2-depleted pea chloroplasts in the absence and in the presence of 100 nM dinoseb. Reaction medium as in Fig. 1.

Herbicide binding studies using [^{14}C]atrazine [10] or [^{14}C]ioxynil [8] revealed that the number of binding sites of these herbicides is the same in control, CO_2-depleted, and HCO_3^--reactivated chloroplasts. However, the binding constant (K_b) for both herbicides was increased 3-fold in CO_2-depleted chloroplasts compared to control and HCO_3^--reactivated chloroplasts. This implies that in CO_2-depleted chloroplasts the affinity of the herbicides to their binding sites is decreased.

Thus there appeared a contradiction: in the presence of herbicides the apparent affinity of the thylakoid membrane for bicarbonate is decreased; in the absence of bicarbonate the affinity of the thylakoid membrane for herbicides is decreased. This apparent contradiction could be explained as follows [8]. Electron transport chains which have no bicarbonate bound to their binding site at the thylakoid membrane appear to be fully inactive [10, 11]. Thus, in these experiments electron transport in CO_2-depleted chloroplasts is only due to chains to which HCO_3^- is bound. The herbicide binding studies showed that in CO_2-depleted chloroplasts the affinity for herbicides is decreased. This means that the concentration of free herbicide is higher at low HCO_3^--concentration than at high bicarbonate concentration, resulting in a higher inhibition of the few active chains at low HCO_3^--concentration. This is actually observed [6, 8]. An alternative explanation may be that there are allosteric interactions [3, 12] between herbicide and bicarbonate binding. Both the binding of a herbicide and the absence of bicarbonate may cause a conformational alteration of the Q_B-protein, which changes the affinity for another herbicide or for bicarbonate.

It seems that phenol-type herbicides behave differently. It was found that DNOC [6], *i*-dinoseb [7] and dinoseb (Fig. 2) decrease the apparent affinity of the thylakoid membrane for bicarbonate. Moreover, these herbicides do not influence the maximal Hill reaction rate (V_{max}), indicating competitive inhibition of bicarbonate binding. Vermaas [13] showed that the affinity of *i*-dinoseb in the absence of bicarbonate was about 3-fold higher than in its presence. These results indicate a pure competitive interaction of the binding of bicarbonate and phenol-type herbicides. However, also in this case the interactions may be allosteric.

Snel and van Rensen [7] measured the kinetics of the reactivation of electron transport by varying the dark incubation time of CO_2-depleted chloroplasts with bicarbonate. The half-time of this reactivation appeared to be 25 s when 2 mM bicarbonate was added. In the presence of 100 nM *i*-dinoseb or 100 nM DCMU the half-time of the reactivation by 2 mM bicarbonate appeared to increase to about 58 s. These observations were explained by an analysis according to which any inhibitor which is competitive with respect to the bicarbonate-stimulation of the Hill reaction, should increase the half-time of the reactivation of the Hill reaction.

Additional evidence for an interaction of herbicides with bicarbonate was obtained by studies with triazine-resistant and -susceptible biotypes of *Amaranthus hybridus* [14, 15]. Measurements of the reactivation of the Hill reaction in CO_2-depleted chloroplasts by various concentrations of bicarbonate showed that the apparent K_d for bicarbonate is about 1 mM in triazine-susceptible chloroplasts. In the triazine-resistant chloroplasts the K_d showed about 2-fold increase, *i.e.* the affinity of the thylakoid membrane for bicarbonate is lower in the resistant chloroplasts [10]. It appears that the alteration in the Q_B-protein which causes the triazine-resistance also changes the binding of bicarbonate, indicating a close spatial relationship between the binding of bicarbonate and of the DCMU-type herbicides. It was suggested by Gressel [15] that the lowered rate of electron flow between Q_A and the plastoquinone pool in triazine-resistant plants could be caused by the lowered affinity of the resistant thylakoids for bicarbonate.

Stemler [16] and Snel *et al.* [17] have shown that by illumination of isolated chloroplasts in a medium containing formate the Hill reaction rate decreases with time. This is correlated with exchange of bicarbonate for formate. We studied this phenomenon in isolated chloroplasts of triazine-resistant and -susceptible biotypes of *Amaranthus hybridus*. Table I shows that both in coupled and uncoupled electron transport conditions it takes less time to obtain inhibition of the Hill reaction in the resistant chloroplasts, indicating a faster exchange of bicarbonate for formate in resistant chloroplasts. This again means that in resistant chloroplasts

Table I. Time required to deplete chloroplasts of *Amaranthus hybridus* biotypes from bicarbonate.

Treatment	Triazine sensitive	Triazine resistant
without NH_4Cl	67.4 ± 4.8	36.8 ± 2.0
+ 5 mM NH_4Cl	56.0 ± 3.9	36.1 ± 2.5

The chloroplasts were illuminated in a reaction medium containing 50 mM Na-phosphate (pH 6.5), 100 mM Na-formate, 100 mM NaCl, 5 mM $MgCl_2$, 0.5 mM ferricyanide, and chloroplasts equivalent to 33 µg Chl ml^{-1}. Due to exchange of bicarbonate for formate, which is an inhibitor of electron flow, the Hill reaction decreases with time. Numbers indicate the half-times in seconds needed to obtain complete inhibition of electron flow; $n = 16$.

bicarbonate is more loosely bound than in the sensitive chloroplasts.

The reported results were interpreted to indicate that a possible mode of action of photosystem II herbicides could be a decrease of the affinity of the thylakoid membrane for bicarbonate. Since bicarbonate-binding was thought to be indispensable for electron transport, herbicide-induced decrease of bicarbonate-binding should result in a lower rate of electron transport. However, all reported experiments were performed in media containing 100 mM formate. It was recently demonstrated that formate is a competitive inhibitor of bicarbonate-binding and it was questioned, whether bicarbonate is required for electron transport, or that bicarbonate displaces the inhibitor formate from the thylakoid membrane [17, 18]. At present it is obvious that photosystem II herbicides, bicarbonate and formate bind to the Q_B-protein and interfere with electron transport. However, the mutual relationships of the action of these compounds are not yet clear.

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