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THE INTERACTION BETWEEN BICARBONATE AND THE HERBICIDE IOXYNIL IN THE THYLAKOID MEMBRANE AND THE EFFECTS OF AMINO ACID MODIFICATION ON BICARBONATE ACTION

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Bicarbonate depletion of chloroplast thylakoids reduces the affinity of the herbicide, ioxynil, to its binding site in Photosystem (PS) II. This herbicide is found to be a relatively more efficient inhibitor of the Hill reaction when HCO_3^- is added to CO_2-depleted thylakoids in subsaturating rather than in saturating concentrations. The reason for this dependence of the inhibitor efficiency on the HCO_3^- concentration is that the inactive HCO_3^--deficient PS II reaction chains bind less ioxynil than the active PS II electron-transport chains that have bound HCO_3^-, and, thus, after addition of a certain amount of ioxynil the concentration of the free herbicide increases when the HCO_3^- concentration decreases. Therefore, the inhibition of electron transport by ioxynil increases at decreasing HCO_3^- levels. Measurements on the effects of modification of lysine and arginine residues on the rate of electron transport are also presented: the rate of modification is faster in the presence than in the absence of HCO_3^-. Therefore, we suggest that surface-exposed lysine or arginine residues are not involved in binding of HCO_3^- (or CO_2 or CO_3^{2-}) to its binding protein, but that HCO_3^- influences the conformation of its binding environment such that the affinity for certain herbicides and the accessibility for amino acid modifiers are changed.

Introduction

The presence of HCO_3^- (or CO_2 or CO_3^{2-}) is indispensible for efficient photosynthetic electron transport in chloroplast thylakoids [1–3]. Since it is not yet certain whether HCO_3^-, CO_2 or CO_3^{2-} (or a combination of these) is (are) responsible for this 'bicarbonate effect', we use 'HCO_3^-*' to indicate the species that binds to the thylakoid membrane and that is necessary for electron transport. It has been shown by a number of different techniques (electron transport, Chl *a* fluorescence, and absorption change measurements) that the most important site of HCO_3^-* action is between the first PS II electron-accepting quinone Q and PQ (plastoquinone) [4–12]. The strong influence of HCO_3^-* on electron transport at the quinone level might imply that HCO_3^-* interacts with the 32–34 kDa B-apoprotein, which is known to bind herbicides like atrazine and diuron [13–15], or with a 41–47 kDa protein suggested to bind phenolic herbicides [16,17]. Competition studies between HCO_3^-* and herbicides, carried out by Van Rensen and Vermaas [6], showed that diuron, simeton (a triazine) and 4,6-dinitro-*o*-cresol (a phenolic

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Abbreviations: PS, photosystem; Chl, chlorophyll; DCIP, 2,6-dichlorophenolindophenol; diuron, 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU); atrazine, 2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine; dinoseb, 2,4-dinitro-6-(1-methylpropyl)phenol; ioxynil, 4-hydroxy-3,5-diiodobenzonitrile; simeton, 2-methoxy-4,6-bisethylamino-1,3,5-triazine.

herbicide) are relatively more potent inhibitors when a smaller number of the HCO_3^-*-binding sites are occupied by HCO_3^-*. A Lineweaver-Burk plot indicated full competition between HCO_3^-* and 4,6-dinitro-*o*-cresol, and a partial competition between HCO_3^-* and diuron or simeton. However, Khanna et al. [7] reported a decrease in the affinity of [^{14}C]atrazine for its binding site in the absence of HCO_3^-*; this result is apparently opposite to what may be expected in the case of a competition between HCO_3^-* and herbicides. In this paper, we present data on interactions between HCO_3^-* and the herbicide ioxynil, and provide an explanation for the above-mentioned apparent disagreement between competition and binding studies.

Renger and co-workers [18] reported the effects of glutaraldehyde on the acceptor side of PS II: a modification of (probably) lysine residues by glutaraldehyde treatment led to a decrease in the reduction of Q, but not to a significant decrease in the rate of electron transport from Q to PQ. We extended the protein modification experiments to investigate if lysine or arginine residues may be involved in the bicarbonate effect.

Materials and Method

Chloroplast isolation and Hill reaction measurements. Chloroplasts were isolated from 10–15-day-old pea (*Pisum sativum* L.) leaves and were broken as described earlier [19]. CO_2 depletion was performed by washing the thylakoids once in a formate-containing buffer (50 mM sodium phosphate, 100 mM NaCl, 100 mM HCO_2Na, 5 mM $MgCl_2$), pH 5.3, or by shaking the thylakoids suspended in this buffer for 10 min while CO_2-free N_2 was led over the suspension ([Chl] = 30–50 μg/ml). The CO_2-depleted thylakoids were resuspended in a buffer of the same composition at pH 6.5. The buffers and tubes were kept CO_2 free. For a more detailed description of the CO_2-depletion procedure, see Refs. 11 and 20.

The ferricyanide Hill reaction was measured as oxygen evolution using a Clark-type electrode. The DCIP Hill reaction was monitored at 595 nm as DCIP reduction using a Cary 14 spectrophotometer. The difference in the extinction coefficient between oxidized and reduced DCIP at 595 nm was taken to be 18 $mM^{-1} \cdot cm^{-1}$ at pH 7.0 [21]. The actinic light was filtered through a Corning 2-67 red filter, and the photomultiplier of the spectrophotometer was shielded by a 595 nm interference filter.

Chl a fluorescence measurements. The instrument used to measure fluorescence induction kinetics in dark-adapted chloroplasts has been described elsewhere [11]. Excitation light, obtained from a tungsten lamp, was passed through a narrow-band 660 nm interference filter before it impinged on the sample. Chl *a* fluorescence, after having passed a monochromator (set at 690 nm, with a half-bandwidth of 5 nm), was detected by an S-20 photomultiplier.

Ioxynil binding. [^{14}C]Ioxynil (spec. act. 33.5 μCi/mg) was added to 1 ml of a suspension (80 μg Chl) of CO_2-depleted thylakoids with or without the addition of 10 mM $NaHCO_3$. The suspension was incubated for 15 min at room temperature in the dark, and then the thylakoids were pelleted by centrifugation; 0.8 ml of the supernatant was transferred to a scintillation vial with 10 ml scintillation fluid. The amount of free [^{14}C]ioxynil was measured and the amount bound to the thylakoid membrane was then calculated.

Protein modification. Thylakoids were CO_2 depleted and resuspended into 50 mM sodium phosphate, 20 mM HCO_2Na, 10 mM NaCl, 5 mM $MgCl_2$ and 0.2 M mannitol (final pH = 7.0). Then, 10 mM $NaHCO_3$ and 2 mM HCl (to keep the pH at 7.0) were added to half of the samples. Subsequently, 50 mM phenylglyoxal (to modify arginine residues [22]) or 5 mM pyridoxal 5-phosphate (to modify lysine residues [23]) was added. After incubation with the modifier for the time indicated at room temperature in the dark, the suspension was centrifuged (5 min; 1000 × *g*) and the thylakoid pellet was resuspended into a buffer containing 50 mM sodium phosphate, 10 mM $NaHCO_3$, 10 mM NaCl, 5 mM $MgCl_2$, 0.2 M mannitol (final pH 7.0) ([Chl] = 33 μg/ml). Then, 50 μM DCIP was added and the Hill reaction was measured. The modification was also monitored by Chl *a* fluorescence measurements. Control experiments, in which no amino acid modifier was added, were also performed.

Results and Discussion

Ioxynil binding

For ioxynil, the I_{50} value (i.e., the herbicide concentration at which 50% inhibition of electron transport is observed) and the binding constant are comparable [17]; this indicates that there is little ioxynil binding to the groups in the thylakoid membrane that are not directly involved in photosynthesis at ioxynil concentrations lower than I_{50}. Thus, binding experiments using ioxynil concentrations lower than I_{50} can be reliably used to measure the ioxynil affinity for the site influencing electron transport. A double-reciprocal plot of the concentrations of ioxynil that is free and that is bound to the thylakoids with or without HCO_3^- addition is shown in Fig. 1. This plot indicates that the number of binding sites (1 per 480 Chl molecules) is not significantly changed by HCO_3^- addition to CO_2-depleted thylakoids. The binding constant, K_b, however, is approx. 10 nM in the presence and approx. 30 nM in the absence of HCO_3^-. This means that the presence of HCO_3^- leads to an increased affinity of [^{14}C]ioxynil to the binding site, and, thus, these data would not suggest any competition between HCO_3^-* and ioxynil. (Atrazine affinity has been shown to depend on CO_2 depletion in qualitatively the same way as that of ioxynil [7].). However, our measurements on the sensitivity of the ferricyanide Hill reaction to ioxynil in CO_2-depleted thylakoids after addition of various concentrations of HCO_3^- suggest a (partial) competition between HCO_3^-* and ioxynil (Fig. 2): the thylakoids are more sensitive to ioxynil at lower than at higher HCO_3^-* concentration. Similar results have been reported for HCO_3^-*-diuron and HCO_3^-*-simeton interactions [6]. At first glance, the results shown in Figs. 1 and 2 seem to be contradictory. However, other experiments [7,11] suggest that electron-transport chains, in which no HCO_3^-* is bound to a site in the vicinity of or at the 32–34 kDa herbicide-binding protein, are fully inactive; thus, electron transport in CO_2-depleted chloroplasts is only due to chains in which an HCO_3^-* molecule is bound near the quinones Q and B. Since the binding constant of ioxynil for its site is lower when no HCO_3^-* is bound near that site, the concentration of free ioxynil is higher at low concentrations of HCO_3^- than at higher HCO_3^- levels (less ioxynil is bound to the site), resulting in a greater inhibition of the still active electron-transport chains at low than at high HCO_3^- concentrations. Indeed, the observed HCO_3^- dependence of inhibition by 100 nM ioxynil (approx. 35% at saturating HCO_3^- levels, approx. 62% in the presence of 0.5 mM $NaHCO_3$; see Fig. 2) can be easily explained by a 3-fold change

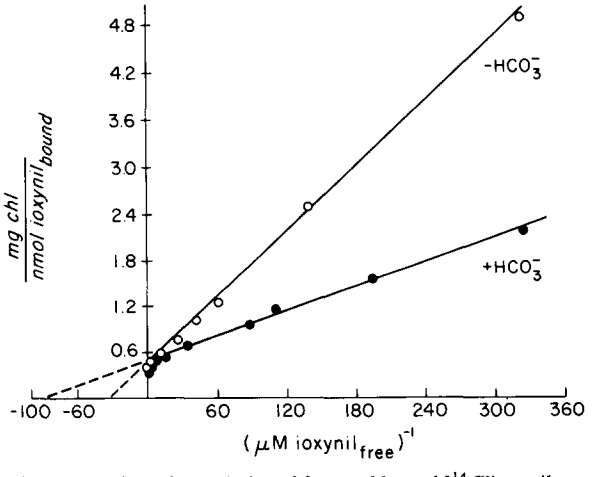

Fig. 1. Double-reciprocal plot of free and bound [^{14}C]ioxynil to CO_2-depleted thylakoids with (●) and without (○) addition of 10 mM $NaHCO_3$ prior to ioxynil addition (20–700 nM ioxynil).

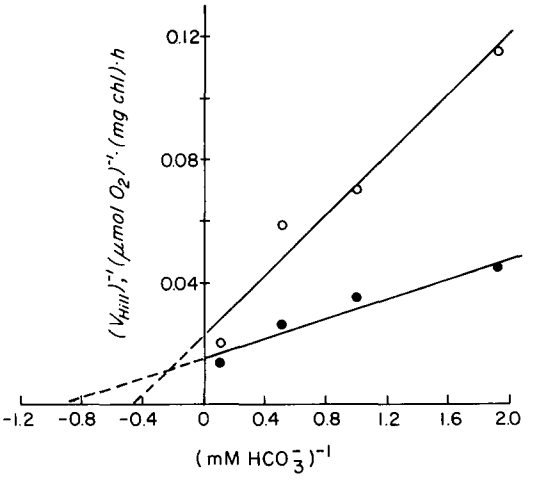

Fig. 2. Double-reciprocal plot of the ferricyanide Hill reaction rate (v_{Hill}) as a function of the HCO_3^- concentration in the absence (●) and presence (○) of 100 nM ioxynil. The thylakoids were allowed to incubate with HCO_3^- for 2 min. The time between ioxynil addition and the measurement of its effect was 3.5 min. The ferricyanide concentration was 0.5 mM.

in ioxynil affinity. Therefore, the observed increased sensitivity for ioxynil of the ferricyanide Hill reaction in CO_2-depleted thylakoids resupplied with subsaturating amounts of HCO_3^- is probably due to a higher ioxynil concentration in solution.

This explanation meets with problems in the case of 4,6-dinitro-*o*-cresol, which was suggested, based on studies of the inhibition of the Hill reaction by 4,6-dinitro-*o*-cresol at various HCO_3^- levels, to interact with HCO_3^-* competitively: the I_{50} value for 4,6-dinitro-*o*-cresol is relatively high (approx. 10^{-5} M), and, thus, in the presence of a concentration of 4,6-dinitro-*o*-cresol that inhibits electron transport by 30–50%, the amount of 4,6-dinitro-*o*-cresol released from binding sites (10–100 nM) due to a lower affinity when no HCO_3^-* is bound in the vicinity is negligible compared to the free 4,6-dinitro-*o*-cresol concentration already present. Therefore, when the only interaction between HCO_3^-* and 4,6-dinitro-*o*-cresol would be one comparable to that between HCO_3^-* and ioxynil, then the inhibition by 4,6-dinitro-*o*-cresol would be expected to be independent of the HCO_3^-* level. We suggest that the interaction between HCO_3^-* and herbicides containing a phenol group (such as ioxynil and 2,4-dinitro-*o*-cresol) may be strongly dependent on the structure of the phenolic herbicide: the ioxynil affinity is decreased in the absence of HCO_3^-*, whereas such behavior does not seem to explain the results obtained with 4,6-dinitro-*o*-cresol [6], or with the slightly more powerful phenolic herbicides i-dinoseb and dinoseb (Van Rensen, J.J.S., unpublished results). This might indicate that the side groups of the phenol-group containing herbicides are very important in determining the mode of interaction (cf. Ref. 24). This would point to very closely related, but not identical, binding sites for HCO_3^-* and herbicides.

Protein modification

As mentioned in the Introduction, the influence of modification of amino acids of thylakoids proteins on photosynthetic electron transport is still largely unknown. However, such modifications may provide information on the groups of a protein involved in an electron-transport process, and, furthermore, may provide insight into the mechanism of HCO_3^-* action. Therefore, we investigated the effect of lysine and arginine modification on the bicarbonate effect. The results of lysine modification experiments are shown in Table I. These data indicate that modification of certain lysine residues in proteins influencing electron transport occurs at a somewhat higher rate in the presence than in the absence of HCO_3^-*. If this was due to a modification of some amino acid residues at the HCO_3^-*-binding site, then the opposite result would have been expected: in the absence of HCO_3^-* (the binding site not occupied by HCO_3^-*) a faster modification of the HCO_3^-*-binding site would occur than in its presence. In control experiments (without protein modifier) the DCIP Hill reaction rate was decreased by only approx. 35% after a 2 h incubation. Fig. 3 shows that the variable Chl *a* fluorescence (F_v) almost disappears after lysine modification and that the long-term (greater than 10 s after illumination) fluorescence yield (F_{max}) is lowered. Modification in the absence of HCO_3^-* leads to qualitatively identical fluoresence induction curves. It is interesting to note that the initial fluoresence yield (F_0) after modification is higher than before modification. Incubation with 1 or 10 mM NH_2OH (which donates electrons, presumably indirectly, to P-680)

TABLE I

EFFECT OF LYSINE RESIDUE MODIFICATION IN THE PRESENCE AND THE ABSENCE OF HCO_3^-* ON THE DCIP HILL REACTION

All thylakoids used in this experiment were previously CO_2 depleted; to all samples, except those used for measuring the control activity, 5 mM pyridoxal 5-phosphate was added when the incubation was started; furthermore, 10 mM $NaHCO_3$ + 2 mM HCl was added to the '+HCO_3^-' samples at the beginning of the incubation. The DCIP Hill reaction was measured in the presence of 10 mM $NaHCO_3$ after washing out the modifier. 100% activity corresponds to 130 μmol DCIP reduced/mg Chl per h.

Incubation time (min)	Activity (%) ($n=4$)		Control activity (%) (+ or $-HCO_3^-$)
	$-HCO_3^-$	$+HCO_3^-$	
0	100	100	100
10	95±5	93±8	98
30	75±7	62±6	89
60	58±5	32±4	80
120	24±3	14±3	64

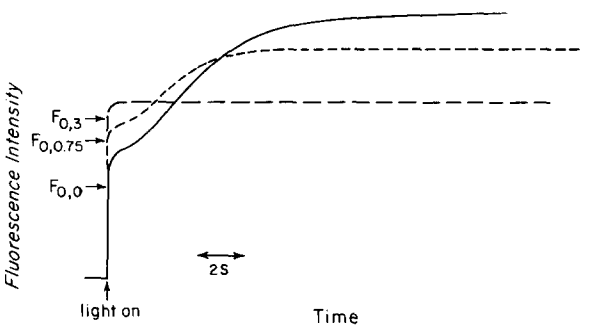

Fig. 3. Chl *a* fluorescence induction curves of CO_2-depleted thylakoids after 0 (———), 0.75 (- - - - -) and 3 (— — —) h of incubation with 5 mM pyridoxal 5-phosphate. The initial level (F_0) in all the three cases is indicated. At the beginning of the incubation, 10 mM $NaHCO_3$ and 2 mM HCl were added. After incubation for the indicated time, the thylakoids were pelleted and resuspended into pyridoxal 5-phosphate-free medium; [Chl] = 10 μg/ml.

for 10 min does not change the fluorescence induction curves significantly (data not shown), indicating that the observed modification effects are not located on the donor side of PS II. Using control chloroplasts (without modifier), neither the ratio of F_{max} to F_0 nor F_{max} changes very significantly during a 3 h incubation at room temperature: both the F_{max}/F_0 ratio and F_{max} decreased by only approx. 25%. These results might indicate that the lysine modification changes the conformation of the vicinity of the PS II reaction center and antenna (resulting in an increase in the F_0 yield), and influences stacking properties of the thylakoids, or charge separation between P-680 and Q, or both (resulting in a loss of F_v). We note that the lysine modification results reported here somewhat resemble the results of glutaraldehyde treatment of thylakoid membranes as presented by Renger and co-workers [18]. This is not surprising because glutaraldehyde is supposed to react with lysine residues [25].

In order to investigate if arginine might play a role in HCO_3^-* binding, we performed experiments with the arginine modifier phenylglyoxal. Modification by 50 mM phenylglyoxal gives results qualitatively similar to those with lysine modification: the modification rate (as measured by a decrease in the DCIP Hill reaction) is higher in the presence of HCO_3^-* than in its absence, and the variable fluorescence disappears after 2 h of modification. Hydroxylamine treatment does not appear to change the fluorescence characteristics, again suggesting an effect on the acceptor rather than on the donor side of PS II.

The absence of a positive correlation between the absence of HCO_3^-* and lysine or arginine modification indicates that the HCO_3^-*-binding site (1) is inaccessible to the modifier (thus, not surface exposed, which is in agreement with the idea that the HCO_3^-*-binding site is hidden under the membrane surface [20]); or (2) does not contain a lysine or arginine that is uncharged at the pH used. The latter possibility would imply that the mechanism of HCO_3^-* action on photosynthetic electron transport is not the formation of a carbamate ($R\text{-}NH\text{-}CO_2^-$) from CO_2 and a free amino group. Consistent with this possibility is the preliminary observation that diazomethane (that stabilizes carbamates) does not change the $H^{14}CO_3^-$ binding characteristics to CO_2-depleted chloroplasts (Govindjee and co-workers, unpublished results). However, carbamate formation has been shown to be involved in the stimulation of ribulose-1,5-bisphosphate carboxylase/oxygenase by CO_2 [26].

We consider it very likely that HCO_3^-* binding changes the conformation of a protein, presumably located on the outer side of the thylakoid membrane, in such a way that lysine and arginine residues become somewhat better accessible to amino acid modifiers. Our data, both on herbicide binding (Figs. 1 and 2) and protein modification (Table 1; Fig. 3) point to a direct influence of HCO_3^-* on the conformation of the herbicide-binding protein(s) and, possibly, of other proteins in the vicinity. This effect of HCO_3^-* on protein conformation is presumably responsible for the large modification of electron transport at the quinone level by HCO_3^-*.

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