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Binding affinity of bicarbonate and formate in herbicide-resistant D1 mutants of *Synechococcus* sp. PCC 7942*

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Abstract

We examined the effects of mutations at amino acid residues S264 and F255 in the D1 protein on the binding affinity of the stimulatory anion bicarbonate and inhibitory anion formate in Photosystem II (PS II) in *Synechococcus* sp. PCC 7942. Measurements on the rates of oxygen evolution in the wild type and mutant cells in the presence of different concentrations of formate with a fixed bicarbonate concentration and *vice versa*, analyzed in terms of an equilibrium activator-inhibitor model, led to the conclusion that the equilibrium dissociation constant for bicarbonate is increased in the mutants, while that of the formate remains unchanged (11 ± 0.5 mM). The hierarchy of the equilibrium dissociation constant for bicarbonate (highest to lowest, ± 2 μM) was: D1-F255L/S264A (46 μM) > D1-F255Y/S264A (31 μM) ~ D1-S264A (34 μM) ~ D1-F255Y (33 μM) > wild type (25 μM). The data suggest the importance of D1-S264 and D1-F255 in the bicarbonate binding niche. A possible involvement of bicarbonate and these two residues in the protonation of Q_B^-, the reduced secondary plastoquinone of PS II, in the D1 protein is discussed.

Abbreviations: Chl *a* – chlorophyll *a*; DBMIB – 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCMU – 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DMQ – 2,5-dimethyl-*p*-benzoquinone; HEPES – N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; MES – 2-[N-morpholino]ethanesulfonic acid; PS I – Photosystem I; PS II – Photosystem II; Q_A – bound plastoquinone, a one-electron acceptor in Photosystem II; Q_B – another bound plastoquinone, a two-electron acceptor in Photosystem II

Introduction

Herbicides that inhibit photosynthesis have been developed during the last century (Brian 1976). Several of these herbicides inhibit electron transfer between Q_A and Q_B, the primary and secondary plastoquinone electron acceptors of PS II, by displacing plastoquinone from the Q_B binding site (Velthuys 1981, Wraight 1981). Experiments with the water soluble enzyme trypsin indicated that the binding sites of these herbicides are of a proteinaceous nature. The sensitivity of the Hill reaction to herbicides could be removed by treatment of chloroplasts with trypsin (Regitz and Ohad 1975, Renger 1976, Van Rensen and Krammer 1979). The use of photoaffinity labeling technique allowed the identifica-

* This paper is dedicated to the memory of my dear friend Robin Hill – Govindjee.

tion of a 32 kDa polypeptide subunit of PS II as the herbicide or Q_B-binding protein (Pfister et al. 1981).

Hirshberg and McIntosh (1983) found a single mutation in the chloroplast gene *psb*A in an atrazine-resistant mutant of *Amaranthus hybridus*. This mutation leads to an amino acid substitution of residue 264 in the D1 protein (also called herbicide binding protein) from serine to glycine. The same mutation in *psb*A has also been found in other atrazine-resistant mutants of higher plants. Several different structural PS II herbicide-resistant mutants have now been identified, some of which are homologous to mutations identified in photosynthetic bacteria (Hirschberg et al. 1987a).

In several herbicide-resistant D1 mutants in higher plants, the damping of variations in flash number dependent oxygen evolution yields was increased (Holt et al. 1983), the deactivation of the S_2 state of the oxygen evolving complex was faster (Holt et al. 1983), and the thermoluminescence peak corresponding to back reaction of S_2 with Q_B^- was replaced by a peak at lower temperatures due to the fact that a shift in the equilibrium for Q_A/Q_B electron transfer increases the concentration of Q_A^- (Demeter et al. 1985). In several cyanobacterial herbicide-resistant mutants, the initial phase of the electron transfer from Q_A^- to Q_B is unaltered but the electron transfer equilibrium between these two acceptors is displaced towards Q_A^- (Etienne et al. 1990, Gleiter et al. 1990, Prasil et al. 1991).

Indications for the involvement of the D1 protein in the bicarbonate effect have come from the studies on the interaction of herbicides and bicarbonate, and on herbicide-resistance mutants. Urea, triazine and phenol-type herbicides (known to interact with the Q_B site) increase the apparent dissociation constant K_d for bicarbonate by at least 2-fold (Van Rensen and Vermaas 1981); in other words, these inhibitors decrease the apparent affinity of the thylakoid membrane for bicarbonate, indicating the closeness of the sites of action of bicarbonate and PS II herbicides (Van Rensen and Vermaas 1981, Van Rensen 1982, Vermaas and Govindjee 1982, Snel and Van Rensen 1983). On the other hand, studies using ^{14}C-labelled atrazine revealed that bicarbonate depletion reduced the binding affinity of the herbicide to thylakoid membranes more than two fold; furthermore, trypsin treatment, known to modify the D1 protein and reduce Q_B binding, significantly decreased the stimulatory effect of bicarbonate on the electron transfer at the PS II acceptor complex (Khanna et al. 1981). Although interaction of bicarbonate with herbicides is established, bicarbonate does not function by displacing Q_B (see e.g. a model by Xu et al. 1991). A triazine-resistant mutant of *Amaranthus hybridus* (in which Ser-264 of D1 was changed to Gly) showed a 2-fold increase in the dissociation constant of bicarbonate (Khanna et al. 1981). Recent studies on several herbicide-resistant D1 mutants of the cyanobacterium *Synechocystis* sp. PCC 6714 (Govindjee et al. 1990) and of *Chlamydomonas reinhardtii* (Govindjee et al. 1991a) demonstrate differential formate sensitivities. We have assumed that formate replaces bicarbonate under our experimental conditions since formate is known to release CO_2 in thylakoid membranes (Govindjee et al. 1991b). Measurements on Chl *a* fluorescence transients in herbicide-resistant mutants of *Chlamydomonas reinhardtii* revealed differential sensitivity to 25 mM formate treatment: the most sensitive mutant is S264A and the most resistant mutant is L275F. The order of sensitivity is S264A $\gg$ V219I = F255Y $>$ wild type $\gg$ A251V $>$ L275F (Govindjee et al. 1991a). Based on the data of oxygen evolution, Chl *a* fluorescence decay and Chl *a* fluorescence transient kinetics, the order of sensitivity of *Synechocystis* sp. PCC 6714 mutants to formate is: S264A $>$ wild type $>$ F211S $\gg$ F211S/A251V (Govindjee et al. 1990).

In this paper, we have examined the effects of mutations in specific amino acids in the D1 protein on the binding affinity of the inhibitory and stimulatory anions formate and bicarbonate, respectively. The mutants used were D1-F255Y (Tyr5), D1-S264A (Dil), D1-F255Y/S264A (D5) and D1-F255L/S264A (Di22) from *Synechococcus* sp. PCC 7942. Measurements on oxygen evolution with different concentrations of formate in the presence of a fixed bicarbonate concentration and *vice versa*, analysed in terms of an equilibrium activator-inhibitor model (Segel 1975), led to the conclusion that it is the equilibrium dissociation constant (K_A) of bicar-

bonate binding that is increased in S264A and F255Y, and that this increase is greater when F255 is co-mutated to L, than when F255 is co-mutated to Y.

Materials and methods

1. *Growth of cyanobacteria*

Synechococcus PCC 7942 and mutants of this strain (Hirschberg et al. 1987b, Horovitz et al. 1989) were grown in BG-11 liquid medium at 30 °C as described by Cao et al. (1991).

2. *Steady-state oxygen evolution*

The steady-state oxygen evolution in cyanobacterial cells was determined polarographically under saturating light (3600 μmoles of photons/m^2s) filtered with a Corning CS3–68 yellow filter, using a Yellow Springs Instrument Clark-type electrode. A combination of two electron acceptors, 2,5-dimethyl-*p*-benzoquinone (DMQ) (0.5 mM) and $K_3Fe(CN)_6$ (1 mM), was used. DMQ acts as the electron acceptor and the non-penetrating ferricyanide keeps the DMQ in the oxidized state. (For use of this combination in cyanobacterial cells, see e.g., Vermaas et al. 1990.) Twenty μM 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB) was added in the reaction medium to block electron flow between the plastoquinone pool and PS I (Trebst 1980). Thus, the effect of CO_2 due to CO_2-fixing steps could be avoided (Cao and Govindjee 1990). The Chl *a* concentration used for oxygen evolution measurements was 20 μg/ml. The suspension medium was the reaction medium containing 0.1 M sucrose, 10 mM NaCl, 5 mM $MgCl_2$, 0.1 μM gramicidin D and 20 mM HEPES (pH 6.8) (see Cao and Govindjee 1988).

3. *Chlorophyll* a *fluorescence decay*

The kinetics of decay of variable Chl *a* fluorescence, after a single-turnover saturating flash, were measured at 685 nm by a weak measuring light. The measuring light was fired at variable times after each actinic flash. The actinic (FX-124, EG and G) and the measuring flashes (Stroboslave 1593A, General Radio) were filtered with two Corning blue (CS 4–96) glass filters; both had a 2.5 μs duration at half-maximum peak (see details in Eaton-Rye and Govindjee 1988a). Thylakoids were prepared by a procedure modified after Burnap et al. (1989). Thylakoid suspensions, at a Chl *a* concentration of 5 μg/ml, were dark adapted for 15 min. In order to calculate the rate constants of Q_A^- decay, the relative Q_A^- concentration was estimated from the variable Chl *a* fluorescence yield according to Joliot and Joliot (1964) using the formula given by Mathis and Paillotin (1981). The connection parameter, p, of 0.45 obtained from thylakoid membranes of a cyanobacterium *Phormidium laminosum* (Bowes and Bendall 1983) was used.

4. *Formate treatment*

Bicarbonate-depletion/formate treatment was done as follows: samples were incubated for 1 h in a medium containing 0.1 M sucrose, 10 mM NaCl, 5 mM $MgCl_2$, 10 mM NaH_2PO_4 (pH 5.8) and the indicated amount of sodium formate, over which CO_2-free N_2 gas was passed. These CO_2-depleted/formate-treated samples were then pelletted and resuspended in CO_2-free buffer at pH 6.8, as described by Eaton-Rye and Govindjee (1988a). To restore the Hill reaction, 10 mM bicarbonate was added to these samples at pH 6.8.

5. *Calculation of* K_A *and* K_i

K_A, the equilibrium dissociation constant for the activator (bicarbonate), and K_i, the equilibrium dissociation constant for the inhibitor (formate), were obtained by fitting all the data for each sample with Eq. (6) (see 'Results') using a multivariate non-linear regression, which was performed by a computer program *Mathematica* (Wolfram Research, Inc., 100 Trade Center Drive, Champaign, IL). In this program, all the values of (rate of reaction)$^{-1}$, [inhibitor] and [activator] were used as input, and the program was used to calculate K_A and K_i having the least square fit for the data. In this study, the [activator] of the control in which no additional bicarbonate was added to the cells was assumed

to be 0.1 mM according to Blubaugh and Govindjee (1988a).

Results

1. Sensitivity to herbicide DCMU: Chl a fluorescence yield measurements

Figure 1 shows the increase in variable Chl *a* fluorescence yield as a function of DCMU (diuron) concentration. The fluorescence yield was measured at 1 ms after a saturating flash. The ordinate of the graph is normalized by the measured fluorescence yields at fully bound states at saturating DCMU concentrations. The dissociation constants for DCMU calculated from the $[Q_A^-]$ vs. [DCMU] plots are 17.8 nM, 31.6 nM, 3.2 μM, 4.0 μM and 17.8 μM for wild type, D1-F255Y, D1-S264A, D1-F255Y/S264A and D1-F255L/S264A mutants, respectively. These data are in agreement with the I_{50} values of the mutants as measured from the reduction of DCPIP (Hischberg et al. 1987b, Ohad et al. 1987, Ohad and Hirschberg 1990, Ohad et al. 1990, Horovitz et al. 1989).

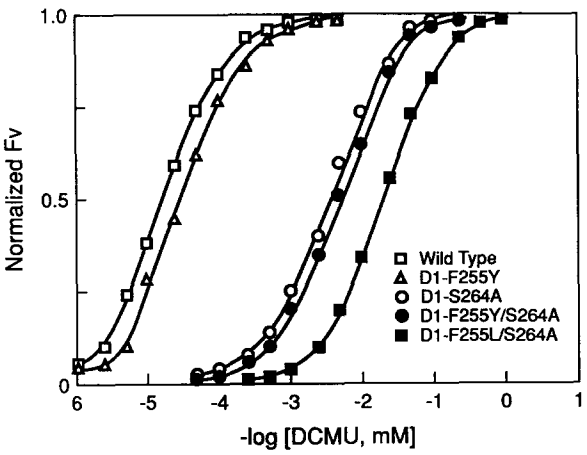


Fig. 1. Variable Chl *a* fluorescence yield (F_v) as a function of DCMU concentration in D1 mutants of *Synechococcus* sp. PCC 7942. The fluorescence yield was measured at 1 ms after a saturating actinic flash.

2. Sensitivity to formate: Hill reaction measurements

It has been demonstrated that bicarbonate stimulates the Hill reaction in bicarbonate-depleted *Synechocystis* 6803 cells (Cao and Govindjee 1988, 1990). Figure 2 shows the Hill reaction activity (measured as oxygen evolution) of bicarbonate-depleted wild type and mutant cells in the presence of various formate concentrations. 100% in wild type was equivalent to 185 μmoles O_2/mgChl · h, whereas 100% in mutants D1-F255Y, D1-S264A, D1-F255Y/S264A and D1-F255L/S264A were 146, 144, 131 and 120

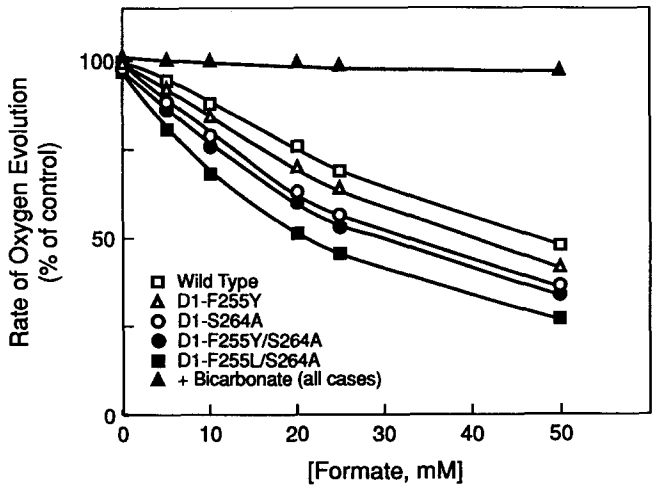


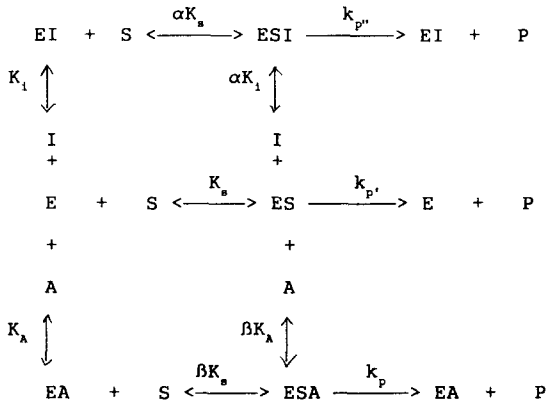
Fig. 2. The Hill reaction activity (measured as oxygen evolution) of bicarbonate-depleted/formate-treated wild type and mutant cells of *Synechococcus* sp. PCC 7942 in the presence of different concentrations of formate. Data were normalized to 100% in the absence of added formate. The inhibited reaction was fully recovered by the addition of 10 mM bicarbonate in all cases.

μmoles/mgChl · h, respectively. The I_{50} values, concentrations of formate at which half-maximal inhibition of oxygen evolution rate occurs in the absence of bicarbonate, are shown as averages of 5 experiments in Table 1. The inhibited reaction was fully recovered to that of their own controls by the addition of 10 mM bicarbonate in all cases (Fig. 2). The order of sensitivity (lowest to highest) to formate inhibition is: wild type < D1-F255Y ≳ D1-S264A ≳ D1-F255Y/S264A < D1-F255L/S264A.

3. *Analysis of Hill reaction measurements: Activator-inhibitor model*

In the previous studies of the reactivation by bicarbonate of Hill reaction in bicarbonate-depleted samples, the apparent dissociation constant of bicarbonate in the presence of formate was estimated by taking bicarbonate as substrate and formate as the inhibitor that competes with the substrate and blocks the enzymatic activity (Stemler and Murphy 1983, Snel and Van Rensen 1983, 1984, Blubaugh and Govindjee 1988a). However, the catalytic activity of PS II is to reduce plastoquinone to plastoquinol and to oxidize water to oxygen. It is not known if bicarbonate directly participates as a catalytic substrate in PS II electron transfer activity. It is more appropriate to consider its function as an activator that stimulates the PS II activity, and accordingly formate acts to replace the activator inhibiting the enzymatic function of PS II.

Here, we use a rapid equilibrium enzymatic model to describe the catalytic activity of PS II, the water-plastoquinone oxido-reductase. In this model, the binding of activator (bicarbonate) stimulates the enzymatic activity of PS II. We do not consider here the conditions, such as low pH, where the activator may not be necessary due to abundance of protons, as a major function of bicarbonate is suggested to be as a proton shuttle for the stabilization of Q_B^- (Blubaugh and Govindjee 1988b). The inhibitor (e.g. formate) (I) competes with the binding of the activator at its binding site that is distinct from that of the substrate (e.g. plastoquinone or water) (S); however, the inhibitor itself does not interfere with the binding of the substrate. As a result, the ESI complex can form but it is assumed to have a very low activity. The activator (A) competes with the inhibitor for the inhibitor site. When the activator occupies the inhibitor site, the ESA complex forms and produces EA and product (e.g. plastoquinol or oxygen) (P). I is a non-competitive inhibitor with respect to S; I and A are competitive. If the inhibitor acts non-competitively with respect to the substrate but the activator and inhibitor are mutually exclusive, then the equilibria of the system can be depicted as:



(1)

where E is the enzyme, S substrate, P product, A activator, and I inhibitor. K_s, K_A and K_I are the equilibrium constants. α and β are the factors by which the equilibrium constants change when I or A occupies the site of the enzyme other than S. k_p, $k_{p'}$ and $k_{p''}$ are rate constants for the breakdown of the enzyme complexes to P.

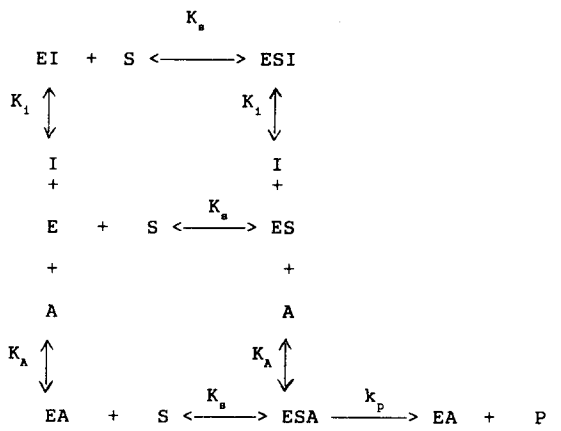
Our focus in this study is to examine the effects of point mutations in the D1 protein on

Table 1. I_{50} values for formate and equilibrium dissociation constants for formate (K_I) and bicarbonate (K_A) of the wild type and D1 mutants

	I_{50}	K_I (±0.5 mM)*	K_A (±2 μM)*
Wild type	50 mM	12 mM	25 μM
D1-F255Y	40 mM	12 mM	34 μM
D1-S264A	35 mM	11 mM	33 μM
D1-F255Y/S264A	32 mM	10 mM	31 μM
D1-F255L/S264A	20 mM	10 mM	46 μM

*These are estimated errors based on errors in O_2 evolution measurements.

the equilibrium dissociation constants K_A and K_i. To make the calculation of the constants feasible, we make the following simplifications and assumptions. We assume that the PS II centers occupied by formate have such a low activity that the rate constant $k_{p''}$ is negligibly small as compared to k_p. Data of El-Shintinawy et al. (1990), among others, show that $k_{p''}$ is small. Our existing hypothesis assumes that the PS II centers in vivo without bound bicarbonate have very small activity compared to those with bound bicarbonate (Blubaugh and Govindjee 1988a,b, Govindjee 1992). Thus, $k_{p'}$ is very small and k_p is the only valid pathway considered. We do not take into account the effects of A or I binding on the equilibrium constants and assume that α and β have the value of one. As a result, the equilibria of a simplified system are obtained as the following:



(2)

The various equilibrium constants for dissociation reactions are:

$$K_s = \frac{[E][S]}{[ES]} = \frac{[EI][S]}{[ESI]} = \frac{[EA][S]}{[ESA]} \quad (3)$$

$$K_i = \frac{[E][I]}{[EI]} \quad (4)$$

$$K_A = \frac{[E][A]}{[EA]} \quad (5)$$

Hereafter, we will call K_i and K_A as equilibrium dissociation constants for the inhibitor and activator, respectively.

The velocity equation for the system is (see Appendix):

$$\frac{1}{v} = \frac{\left(1 + \frac{K_s}{[S]}\right)}{K_i V_{max} \frac{[A]}{K_A}} [I] + \frac{1}{V_{max}} \left(1 + \frac{K_s}{[S]}\right)\left(1 + \frac{K_A}{[A]}\right) \quad (6)$$

where v is the initial velocity and V_{max} the maximum velocity.

In the above system, the K_A and K_i values can be determined by measuring the effect of different concentrations of A on the inhibition by I. This is most conveniently accomplished by Dixon plots of 1/v versus [I] at a constant [S] and different fixed concentrations of A (Segel 1975). Figure 3 shows several Dixon plots, the reciprocal of oxygen evolution rate versus formate concentration at different concentrations of bicarbonate. K_A and K_i values were obtained as described in section 5 of 'Materials and methods' from data on steady-state oxygen evolution rates measured after incubating samples in the dark for 10 min with various concentrations of bicarbonate and formate. Since HCO_3^- is considered the active species for the stimulation of electron transfer in PS II complex (Blubaugh and Govindjee 1986), the equilibrium concentration of HCO_3^- at the experimental pH value (pH 6.8) was used in the calculation; the equilibrium between bicarbonate and carbon dioxide is known to be completed within 1–3 min (Cooper et al. 1968, Blubaugh and Govindjee 1986).

Table 1 lists equilibrium dissociation constants for bicarbonate and formate (K_A and K_i) in the wild type as well as in the mutants. It indicates that all the mutants have increased dissociation constants for bicarbonate while the dissociation constants for formate are nearly unaltered (11 ± 0.5 mM) in all the cases. What is of interest to us is that the double mutant D1-F255L/S264A has a much higher k_A (46 μM) than not only the wild type (25 μM), but also the double mutant D1-F255Y/S264A (31 μM). This shows the importance of not only S264, but its binding environment (F255).

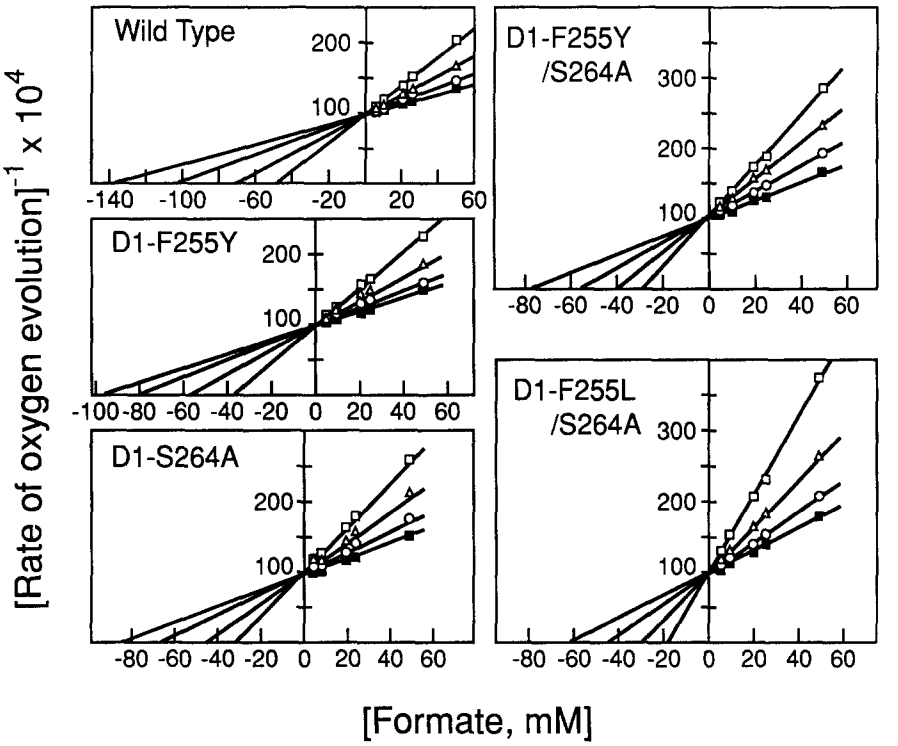


Fig. 3. Dixon plots for the wild type and the D1 mutants of *Synechococcus* 7942. The reciprocal of normalized oxygen evolution rates versus formate concentrations at different concentrations of bicarbonate were plotted. □: control; △: 0.5 mM bicarbonate; ○: 0.75 mM bicarbonate; ■: 1.0 mM bicarbonate.

Discussion

A single amino acid mutation in the D1 protein can lead to herbicide resistance in chloroplasts (Hirshberg and McIntosh 1983). Among several single amino acids in helix IV and V and in the interhelical loop, changes in Phe-255 and Ser-264 are frequently encountered (Brusslan and Haselkorn 1987, 1988), and these have terbutryn-resistant homologues in the L subunit of *Rhodobacter sphaeroides* and/or *Rhodopseudomonas viridis* at positions L-F215 and L-S223 (Paddock et al. 1987, Sinning and Michel 1987).

D1-S264 interacts with herbicides diuron and atrazine as well as the triazinones, while D1-S264→A is more resistant to diuron than to atrazine (Brusslan and Haselkorn 1988). D1-F255 interacts with atrazine (Trebst 1987). D1-F255Y has nearly wild type character regarding diuron resistance, D1-F255Y/S264A and D1-S264A are highly resistant to diuron. Substitution of Phe-255 with Leu instead of the aromatic amino acid Tyr in mutant D1-S264A leads to a double mutant D1-F255L/S264A with an extremely high tolerance to diuron. It is in this mutant that we have observed the highest sensitivity to formate ($I_{50} = 20$ mM; $K_A = 46$ μM; Table 1).

Among other amino acids, S264 and F255 are suggested to be in contact with Q_B binding domain (Trebst 1987). X-ray crystallographic studies on the reaction centers of *R. viridis* suggest that in contrast to the environment of Q_A, Q_B is surrounded by polar and charged amino acid residues. In PS II, hydrogen bonding between bicarbonate and amino acid residues in the Q_B binding site may play an important role in the binding and release of plastoquinones and inhibitors in PS II (Brusslan and Haselkorn 1988).

Several of the mutations in the Q_B domain not only affect herbicide affinity, but also Q_B properties. For example, in Ser-264-Gly mutants, the equilibrium between $Q_A^- \cdot Q_B$ and $Q_A \cdot Q_B^-$ shifts

considerably to the left as compared to the wild type (Ort et al. 1983, Vermaas and Arntzen 1983, Vermaas et al. 1984, Govindjee et al. 1991c). This causes a decrease in the quantum yield of charge separation because of an increase in the steady-state Q_A^- concentration, which leads to a lower maximum rate of photosynthesis. It should be emphasized that the primary physiological effect of triazine resistance on Q_B function is not a slowing down of electron transport between Q_A and Q_B, as often implied in the literature (e.g. Pfister and Arntzen 1979), but a decrease of the midpoint potential of the Q_B/Q_B^- redox couple, causing a shift in the semiquinone equilibrium between Q_A and Q_B (Etienne et al. 1990, Gleiter et al. 1990, Ohad et al. 1990, Prasil et al. 1991).

Measurements in several laboratories of a dissociation constant (K_d) for the bicarbonate in PS II complex of 80 μM (Stemler and Murphy 1983, Snel and Van Rensen 1984, Jursinic and Stemler 1986) must have been overestimated since nearly half of the added HCO_3^- was converted into CO_2. Considering the equilibrium concentration of HCO_3^- in the reaction medium, the K_d is about 40 μM (Blubaugh and Govindjee 1988b) in chloroplasts of higher plants. Results of the present study show that K_d for HCO_3^- in cyanobacterial cells is 25 μM at pH 6.8 (see Table 1).

The dissociation constant for formate is calculated to be about 11 mM, about 400 times higher than that of bicarbonate. In the kinetics of formate binding and HCO_3^--recovery studies, through measurements on F_v decay, the half time of F_v rise by formate binding is in the minute range, whereas that of F_v decline by HCO_3^--recovery is in second range (Diner and Petrouleas 1990). This is consistent with our results of K_A and K_i. Although Xu et al. (1991) suggest, as alternative, that formic acid, but not formate, may be the effective inhibitory species, this conclusion needs further consideration since the role of the binding protein remains unknown. However, nitric oxide (NO), another PS II inhibitor liganding to non-heme iron, has a K_d of about 30 μM (Diner and Petrouleas 1990). Therefore, HCO_3^- has K_d similar to that of NO. On the other hand, HCO_2^- has a similar structure and degree of charge delocalization as HCO_3^-. Both HCO_2^- and HCO_3^- have a carboxyl group, with the same degree of charge delocalization, but only HCO_3^- stimulates the Hill reaction. The main structural difference between the two is the presence of a hydroxyl group in HCO_3^- that is absent in HCO_2^-. It appears likely that the hydroxyl group is the functional moiety, whereas the carboxyl group is involved in binding (Blubaugh and Govindjee 1988b).

Upon reduction of Q_B by Q_A^-, Q_B^- is formed, and it stays at the Q_B site in D1 until Q_A is reduced again by a second turnover of the reaction center. Subsequent oxidation of Q_A^- and protonation of Q_B^{2-} will lead to the formation of Q_BH_2. This fully reduced Q_B can leave the Q_B site. A plastoquinone molecule from the pool in the thylakoid can then bind to the Q_B site and accept the next pair of electrons (see a review by Crofts and Wraight 1983). A possibility of a site of the bicarbonate effect dealing with protonation was based on the observations that the maximum inhibition of electron transfer occurs only after two or more actinic flashes (Govindjee et al. 1976, Robinson et al. 1984, Eaton-Rye and Govindjee 1988a,b, Xu et al. 1991) and that electron transport from Q_A^- to Q_B^- in bicarbonate depleted thylakoid membranes is pH dependent (Eaton-Rye and Govindjee 1988a,b).

Cao et al. (1991) have provided evidence for a role of D2-R-233 and D2-R-251 in stabilization of bicarbonate in Photosystem II. An arginine residue in the D1 protein has been proposed to be a plausible ligand to bicarbonate anion (Shipman 1981, Crofts et al. 1987, Blubaugh and Govindjee 1988b). In photosynthetic bacteria, Glu^{L212} and Asp^{L213} are involved in protonation of Q_B, and replacement of these ionizable residues with non-ionizable amino acids results in lesions at Q_B protonation steps (Takahashi and Wraight 1990, 1991); this phenomenon resembles the bicarbonate-depletion/formate-treatment in PS II. It is likely that the carboxylate group of bicarbonate forms ligands to Fe^{2+}, and the cationic side chains of Arg and/or Lys residues in the vicinity form hydrogen bonds to bicarbonate as in human lactoferrin (Anderson et al. 1989). The hydroxyl group of bicarbonate may be hydrogen bonded to other ionizable residues to participate in a proton translocation pathway to Q_B. The double mutation (S264→A;

F255→L) may have the strongest effect on carboxylate group linkage with Fe^{2+} and/or neighboring cationic side chains, and result in the largest change in K_A for HCO_3^- among the mutants.

The above discussion implies that bicarbonate is an essential activator for the PS II electron flow. This concept was challenged by Jurinsic and Stemler (1992) who proposed that bicarbonate may not be an essential requirement for the normal electron flow but it may function only to displace anion inhibitors present at PS II catalytic sites. Based on this hypothesis, it is expected that there would be a normal PS II activity without bicarbonate, and that the $k_{p'}$ in Eq. (1) should be equal to k_p. The velocity equation would then be:

$$\frac{1}{v} = \frac{\left(1 + \frac{K_s}{[S]}\right)}{K_i V_{max}\left(1 + \frac{[A]}{K_A}\right)} [I] + \frac{1}{v_{max}}\left(1 + \frac{K_s}{[S]}\right) \quad (7)$$

When this model (Eq. (7)) is applied, the y-axis intercept in the Dixon plots is expected to be independent of [A], whereas from Eq. (6) the y-axis intercept should be dependent on [A], and differ by a factor of $(1 + K_A/[A])$. Apparently, all the available data (Fig. 3) would fit the non-essential model. However, since the calculated K_A is in the μM range (Table 1) and [A] in the mM range, the ratio of $K_A/[A]$ is negligibly small. Thus, the two models cannot yet be distinguished in terms of the y-axis intercept in the Dixon plots. Until proven otherwise, we favor the essential activator model for explaining the mechanism of bicarbonate effects in PS II as bicarbonate clearly plays an important role in PS II under in vivo conditions (see Blubaugh and Govindjee 1988b).

To answer the question of essentiality of bicarbonate, new experiments are needed with thylakoid membranes thoroughly depleted of bicarbonate to which low concentrations of bicarbonate are added to stimulate the electron flow. Analysis of such data should help us in discriminating between the two competing models for the bicarbonate function. We suggest that when bicarbonate is bound, it functions not only to keep inhibitory anions away, but to protonate PS II. However, there remains the possibility that when all anions are absent, bound water may serve to replace the function of bicarbonate.

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Appendix

Equations (1) and (2) and the assumptions made for the kinetic model used in this paper are described in the text. The total enzyme concentration $[E_T]$ is given by

$$[E_T] = [E] + [ES] + [EI] + [EA] + [ESI] + [ESA] \qquad (A1)$$

v is the rate of breakdown of ESA.

$$v = k_p[ESA] \tag{A2}$$

By using Eq. (A2) for v and Eq. A1 for $[E_T]$, we get

$$\frac{v}{[E_T]} = \frac{k_p[ESA]}{[E] + [ES] + [EI] + [EA] + [ESI] + [ESA]} \tag{A3}$$

Solving and substituting Eqs. (3), (4) and (5) (see text) for [ES], [EI], [EA], [ESI] and [ESA]:

$$\frac{v}{[E_T]} = \frac{\dfrac{k_p[EA][S]}{K_s}}{[E] + \dfrac{[E][S]}{K_s} + \dfrac{[E][I]}{K_i} + \dfrac{[E][A]}{K_A} + \dfrac{[EI][S]}{K_s} + \dfrac{[EA][S]}{K_s}} \tag{A4}$$

Dividing both sides of Eq. (A4) by k_p gives

$$\frac{v}{k_p[E_T]} = \frac{\dfrac{k_p}{k_p}\dfrac{[E][A][S]}{K_sK_A}}{[E] + \dfrac{[E][S]}{K_s} + \dfrac{[E][I]}{K_i} + \dfrac{[E][A]}{K_A} + \dfrac{[E][I][S]}{K_sK_i} + \dfrac{[E][A][S]}{K_sK_A}} \tag{A5}$$

The maximal rate V_{max} is equal to the product of $[E_T]$ and k_p.

$$V_{max} = k_p[E_T] \tag{A6}$$

Thus,

$$\frac{v}{V_{max}} = \frac{\dfrac{[A][S]}{K_sK_A}}{1 + \dfrac{[S]}{K_s} + \dfrac{[I]}{K_i} + \dfrac{[A]}{K_A} + \dfrac{[I][S]}{K_sK_i} + \dfrac{[A][S]}{K_sK_A}} \tag{A7}$$

By rearranging Eq. (A7),

$$\frac{v}{V_{max}} = \frac{\dfrac{[S]}{K_s}\dfrac{[A]}{K_A}}{\left(1 + \dfrac{[A]}{K_A}\right)\left(1 + \dfrac{[S]}{K_s}\right) + \dfrac{[I]}{K_i}\left(1 + \dfrac{[S]}{K_s}\right)} \tag{A8}$$

Multiplying both sides of Eq. (A8) with V_{max} we obtain:

$$v = \frac{\dfrac{[S]}{K_s}\dfrac{[A]}{K_A}}{\left(1 + \dfrac{[A]}{K_A}\right)\left(1 + \dfrac{[S]}{K_s}\right) + \dfrac{[I]}{K_i}\left(1 + \dfrac{[S]}{K_s}\right)} V_{max} \tag{A9}$$

By taking the reciprocal of both sides of Eq. (A9), we get

$$\frac{1}{v} = \frac{\left(1 + \dfrac{[A]}{K_A}\right)\left(1 + \dfrac{[S]}{K_s}\right) + \dfrac{[I]}{K_i}\left(1 + \dfrac{[S]}{K_s}\right)}{\dfrac{[S]}{K_s}\dfrac{[A]}{K_A}} \frac{1}{V_{max}} \tag{A10}$$

Rearranging Eq. (A10), the final velocity equation for the system (Eq. (6) in the text) is:

$$\frac{1}{v} = \frac{\left(1 + \dfrac{K_s}{[S]}\right)}{K_iV_{max}\dfrac{[A]}{K_A}}[I] + \frac{1}{V_{max}}\left(1 + \frac{K_s}{[S]}\right)\left(1 + \frac{K_A}{[A]}\right)$$