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Chloroacetates as inhibitors of Photosystem II: Effects on electron acceptor side

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Abstract

The two-electron gate of Photosystem II (PSII) is known to function by transferring electrons from the reduced one electron acceptor Q_A^- (a bound plastosemiquinone) to the oxidized two-electron acceptor Q_B (a bound plastoquinone), and then again from Q_A^- to the singly reduced Q_B^-, producing plastoquinol Q_BH_2. In this article, we have used three chloroacetates (monochloroacetate, MCA; dichloroacetate, DCA; and trichloroacetate, TCA), having different geometry and hydrophobicity, to probe the binding environment of the two-electron gate in spinach thylakoids. We first established that these chloroacetates up to 100 mM act specifically in the Q_AQ_B region by monitoring partial reactions of PSII as well as PSI, measuring thermoluminescence, specific for recombination reactions between the donor and acceptor sides of PSII, and studying chlorophyll (Chl) *a* fluorescence decay in the micro to millisecond region, specific for electron flow from Q_A^- to Q_B (Q_B^-). Further, the site of action was located on the D1–D2 protein through observations on the differential sensitivity of chloroacetates on specific D1–D2 mutants of the cyanobacterium *Synechocystis* sp. PCC 6803.

Detailed measurements were then done to characterize the effect of chloroacetates on Q_AQ_B reactions. Data on the [Q_A^-] decay kinetics led to the following observations: (1) chloroacetates (and acetate) not only increase the time constant of electron flow from Q_A^- to Q_B(Q_B^-), but increase the equilibrium [Q_A^-] both after flash 1 and 2, and the degree of these effects (lowest to highest) is correlated with the geometry (increased number of chlorine moiety) and increased hydrophobicity of these inhibitors; the hierarchy is: acetate < TCA. (2) In comparison with flash 1, data after flash 2 (at pH 6) show relatively larger increases in [Q_A^-] equilibrium with DCA and TCA. At pH 7.5, however, flash 1 effects were larger than flash 2 effects with all chloroacetates. (3) Bicarbonate reverses the inhibitory effect on Q_A^- to Q_B(Q_B^-) reactions also in a differential manner; the hierarchy for the most reversible (or least irreversible) is: acetate $\gtrsim$ MCA > DCA > > TCA. (4) The pH dependence of the inhibitory effects on Q_A^- to Q_B(Q_B^-) are: the MCA and DCA effects are larger at pH 6 than at pH 7.5, but the TCA effects are higher at pH 7.5 than at pH 6. The above results, taken together with those in the literature, are in agreement with a picture of the Q_A – Fe–Q_B niche in the D1–D2 protein of PS II where quinones, herbicides, chloroacetates, formate as well as bicarbonate bind, but differently with different overlapping sites. Chloroacetates show effects on the two-electron gate that place them 'in-between' the herbicides and the bicarbonate-reversible formate.

Keywords: (Chloro)acetate; Bicarbonate effect; Thermoluminescence; Chlorophyll *a* fluorescence; D1–D2 mutants; Photosystem II; Q_A^- reoxidation; (Spinach chloroplast)

1. Introduction

The photosystem II (PS II) reaction center (RC) of plants and cyanobacteria consists of, at least, five polypeptides (D1, D2, 4 and 9 kD subunits of Cyt b559 and a 4.5 kD product of the psb I gene) as well as several electron donors and acceptors [1]. A two-electron gate mechanism involving primary (Q_A) and secondary (Q_B) plastoquinone electron

Abbreviations: Chl: chlorophyll; DBMIB: 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone; DCA: dichloroacetate; DCMU: 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCPIP: 2,6-dichlorophenolindophenol; DPC: diphenylcarbazide; FeCy: potassium ferrihexacyanate; HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MCA: monochloroacetate; MES: 2-(N-morpholino)-ethanesulfonic acid; MV: methyl viologen; pBQ: 1,4-benzoquinone; PD: p-phenylenediamine; PQ: plastoquinone; PS II: photosystem II; Q_A: bound plastoquinone, one-electron acceptor of photosystem II; Q_B: loosely bound plastoquinone, two-electron acceptor of photosystem II; [Q_A^-] equilibrium: refers to equilibrium concentration of the reduced form with respect to the oxidized form, Q_A; SiMo: silicomolybdate; TCA: trichloroacetate; Tris: tris(hydroxymethyl)aminomethane

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acceptors explains the electron transfer from the PSII RC to the plastoquinone pool [2]. When the dark adapted PSII RC is activated by a single-turnover saturating flash of light, an electron is transferred from P680, the primary electron donor of PS II, to Q_A via pheophytin and then to Q_B [3]. The negative charge on the quinone induces a pK shift of nearby amino acid residues, resulting in the protonation of the RC and the stabilization of the charge on Q_B^- [4]. After the second actinic flash, a second electron is transferred to Q_B^-, and then two protons are delivered to the doubly reduced Q_B to form a plastoquinol. This plastoquinol is replaced by a plastoquinone from the membrane pool. Some important PS II-directed herbicides (e.g., atrazine and diuron) act by displacing Q_B from its binding site [5,6] in the D1 protein. The target area in the D1 protein varies with different PS II-directed herbicides [7].

In contrast to the herbicides [7], and, in addition, to effects on the electron donor side of PSII [8–11], anions (such as formate, acetate and nitrite) and nitric oxide show bicarbonate-reversible inhibition of PS II electron flow at the plastoquinone reductase site [12–15]. Major bicarbonate/formate/nitric oxide binding sites are suggested to be on the electron acceptor side of PS II in the $Q_A - Fe - Q_B$ complex, where Fe is the non-heme iron that sits between Q_A and Q_B (see e.g., discussions in [16,17]). The binding of bicarbonate at these sites is suggested not only to interact with herbicide binding [18–20], but also to promote protonation associated with PQ reduction at the Q_B site [21–24]. Thus, at the $Q_A - Fe - Q_B$ binding niche in the D1/D2 protein, interactions between the plastoquinone, herbicide, bicarbonate and bicarbonate-reversible inhibitors exist. In contrast to diuron-type herbicides, halogenated 4–hydroxypyridines have been shown to have unimpaired inhibitor potency in Tris-treated chloroplasts [25]. However, the nature of most of these interactions has not yet been elucidated. In this article, we seek to probe the $Q_A - Fe - Q_B$ binding niche by examining the effects of specific inhibitory acetates of different pK_a, geometry and hydrophobicity and their reversibility by bicarbonate. Each chloroacetate has a carboxyl group on one side similar to that of formate, bicarbonate and acetate, but a different number of chlorine groups on the opposite side, each having a different hydrophobicity. For our purpose, we chose three chlorinated acetates (MCA, monochloroacetate; DCA, dichloroacetate; TCA, trichloroacetate). On the basis of partial electron transfer reactions, Chl *a* fluorescence, and thermoluminescence, these acetates are shown here to specifically, but differentially, inhibit Q_A^- to Q_B (or Q_B^-) reactions. The specific location of the site of action on the D1 protein, that harbors Q_A/Q_B, was supported by observation of differential inhibitory effects on PSII reactions of some D1/D2 mutants. Comparison of results on Chl *a* fluorescence decay after flash 1 (Q_A^- to Q_B) and after flash 2 (Q_A^- to Q_B^- and involvement of protonation) revealed that trichloroacetate (pK_a, 0.7) does not function as formate (pK_a, 3.7) since it inhibits almost as strongly after flash 1 as after flash 2, i.e., it leads to inhibition of electron flow. Furthermore, the degree of inhibition and bicarbonate irreversibility increased with increases in the hydrophobicity. Such a correlation is reminiscent of that observed with herbicides [7].

2. Materials and methods

2.1. Sample preparation

Spinach (*Spinacia oleracea*) thylakoids were isolated as previously described [23]. Thylakoids, suspended in 0.3–0.4 M sorbitol, 15 mM NaCl, 5 mM $MgCl_2$ and 20 mM HEPES (pH 7.8), were frozen rapidly after isolation, and stored in liquid nitrogen (77 K) or at −80°C until the experiment was carried out. Chlorophyll concentration was spectrophotometrically determined in 80% acetone (v/v) extracts of thylakoids [26]. Frozen thylakoids were thawed immediately before use. These thylakoids were suspended in 0.3 (or 0.4) M sorbitol, 50 mM NaCl and 2 (or 5) mM $MgCl_2$, without NaCl, to a final Chl concentration of 10 or 25 μM, as specified [23]; pH of the suspension was adjusted by using 20–50 mM MES (from pH 5.5–6.5) or 20–50 mM HEPES (from pH 7.0–7.5); whenever mentioned, 50 mM Tricine was used for pH 7.6 measurements. When chloroacetates were added, the pH of the stock solutions was adjusted before they were mixed with the thylakoid suspensions. MES, HEPES, monochloroacetic acid, dichloroacetic acid and trichloroacetic acid were purchased from SIGMA, and sodium acetate from J.R. Baker, and used directly without further purification.

2.2. Tris treatment and electron donors

Thylakoids were suspended to a final Chl *a* concentration of 50 μg ml^{-1} in a 0.8 M Tris buffer, pH 8.0, and then incubated for 10 min at 4°C in dim light or total darkness [27]. The samples were centrifuged for 7 min at 0°C in dark at 5 000 x g.

For the fluorescence measurements the pellet was rinsed and resuspended in a pH 6 buffer (20 mM MES, 5 mM $MgCl_2$, 15 mM NaCl and 0.4 M sorbitol) to which 80 μM quinhydrone was added (when specified) to ensure that most [Q_B^-] was converted to [Q_B]. The electron donor was benzidine (60–200 μM) as reduced by ascorbate (1 mM), added in dark. Alternatively, diphenyl carbazide (0.2–2 mM range), reduced by ascorbate (2 or 3 mM), was used as electron donor. The order of addition was: suspension buffer followed by chloroacetates, quinhydrone, electron donor and then the thylakoids. The incubation time was 5 min before measurements began.

For the electron transfer measurements the centrifuged Tris-treated samples were suspended in a reaction medium containing 0.3 M sorbitol, 5 mM $MgCl_2$ and 1 μM gramicidin; 50 mM Tris was used for pH 7.8; for pH 6.5, 50 mM MES was used. Electron transport rates were measured using

3 mM DPC as an electron donor and 0.2 mM DCPIP as acceptor.

2.3. Electron transport assays

Whole chain electron transport (both PSI and PSII) with water as the electron donor and MV (100 μM) as the electron acceptor was assayed as O_2 uptake, which results from aerobic oxidation of reduced MV (for details, see [28]). A membrane-covered Clark-type oxygen electrode was used; illumination was provided with yellow light from a Kodak Carousel 4200 slide projector after its white light was filtered with a Corning C.S. 3–68 glass and 2 in. of 1% $CuSO_4$ solution. 200 units ml^{-1} of superoxide dismutase was used to accelerate the dismutation of superoxide radical anion to H_2O_2 and to prevent the anion from reacting with electron donors. One mM NaN_3 was added to inhibit the possible catalase activity of the thylakoid membranes. For PSI assays, electron flow from PS II was blocked by the addition of 10 μM DCMU, and the electron donor was a mixture of 0.5 mM DCPIP and 2 mM sodium ascorbate (see e.g., [29]). The PSII reaction from water to plastoquinone (PQ) was assayed in two ways: (a) using phenylenediamine (100 μM) plus ferricyanide (500 μM) while electron flow to PSI was blocked by 1 μM DBMIB, as described earlier [30], and (b) using 100 μM pBQ as electron acceptor. The PSII reaction from water to Q_A was assayed using silicomolybdate (100 μM) as an electron acceptor, and 1 μM DBMIB to block the electron flow to PSI [31,32]. The PSII reaction that includes the electron flow from the donor D (or Z) to Q_A only (i.e., the initial charge separation) was measured through DCMU (10–100 μM) insensitive electron transfer from DPC (1 mM) to DCPIP (0.2 mM) in Tris-treated thylakoids, as described in section 2.2.

2.4. Chlorophyll a fluorescence yield decay: Q_A^- to Q_B or (Q_B^-) reaction

To monitor the redox state of Q_A, the Chl *a* fluorescence yield after a single-turnover saturating flash (EG and G FX-124 flash lamp, 2.5 μs duration) was measured by an instrument described elsewhere [23]. Using weak measuring flashes (exciting only 1% of the RCs/flash), the 'O' (or F_o) level of Chl *a* fluorescence yield of 5 min dark-adapted thylakoids, as well as the decay of the variable (F_v) Chl *a* fluorescence yield, after actinic flashes (dark interval, 1 s), were measured at 685 nm. This was done by using a 10 nm bandwidth 685 nm interference filter and a S-20 response photomultiplier (EMI 9558). The F_o is a measure of the fluorescence yield when all the Q_A is in the oxidized state, and the decay of F_v reflects the reoxidation of Q_A^- to Q_A. 80 μM quinhydrone was included in the suspension medium to ensure that all centers started with Q_B and not Q_B^-. Assuming that the probability of the intersystem energy transfer is 0.5 [33], the concentration of Q_A was calculated from the variable Chl *a* fluorescence yield, as outlined earlier [34], without consideration for the complexities involved [35]. The $[Q_A^-]$ decay involves multiple processes (see e.g., [36–38]). Results were quantitated by describing the decay in terms of three major (fast, intermediate and slow) exponential components. Their amplitudes (A_f, A_i, A_s) and lifetimes (t_f, t_i, t_s) were derived from $[Q_A^-]$ decays (up to 1 s) by the GLOBALS UNLIMITED™ global analysis software [39]. $[Q_A^-]$ is given in relative units with 1 being $[Q_A^-]_{max}$ obtained in the presence of 6 μM DCMU. Thus, the sum of the analyzed amplitudes (A's) need not add to 1 in our analysis. The fast (sub ms) component reflects the kinetics of direct reoxidation of Q_A^- by Q_B and/or Q_B^-, (see e.g. [40]). The interpretation of the intermediate component (ms) is still unclear. Here, we assume that it reflects the $[Q_A^-]$ equilibrium, partially controlled by the movement of plastoquinone to PS II without bound Q_B [36–38]. The slow component (s) reflects the back-reaction between Q_A^- and the S_2 state of the oxygen evolving complex, mostly in the non Q_B centers [36–38]. It is assumed that the sum of the amplitudes of the intermediate and the slow components, $A_i + A_s$, represents a quasi-steady-state $[Q_A^-]$ due to $[Q_A^-]$ equilibria. $(A_i + A_s)/A_f$ is the ratio between the quasi-steady-state $[Q_A^-]$ and the $[Q_A^-]$ that is oxidized by Q_B (or Q_B^-). To study the concentration dependence of the inhibition of Q_A^- reoxidation and equilibration by chemicals, the increase in the concentration of Q_A^- in the PSII reaction centers is plotted as $[Q_A^-]_{treated} - [Q_A^-]_{control}$. The increase was estimated after a 1 min incubation of thylakoids with the chemical and at 5 ms after the actinic flashes.

2.5. Thermoluminescence

Thermoluminescence (TL) is an excellent probe of PSII reactions (see a minireview, [41]). Recombination of charges in $S_2Q_A^-$ and $S_2(S_3)Q_B^-$ give the Q (-10–$+10$°C range) and B ($+15$–$+35$ °C range) bands, respectively. TL measurements were first made in collaboration with T. Ono and Y. Inoue, then, with D. Kramer, and finally confirmed in the Wageningen laboratory, using a TL-apparatus constructed by S. Demeter. All measurements gave identical results. We present here data obtained with an instrument described by Kramer et al. [42]. Thylakoid samples were suspended in 50 mM phosphate buffer at pH 6.5; 10 μl aliquots (total 10 μg Chl) were absorbed onto Miracloth strips and TL measurements were made by heating (0.7°C s^{-1}) illuminated and cooled samples.

2.6. Measurements on Synechocystis sp. PCC 6803 mutants

Wild type (WT) and mutants (D1-S264A; D1-ΔAA; and D2-SE) of *Synechocystis* sp. PCC 6803 were grown as described by Kless et al. [43]. The D1-S264A is a well known D1 mutant in which serine 264 in the Q_B-binding niche has been changed to alanine; it is resistant to DCMU-type herbicides, but more sensitive to formate (see, e.g., Vernotte et

al. [44]). The D1-ΔAA mutant lacks two alanines (250 and 251) but has an additional tyrosine between residues 246 and 247 in the D1 protein [45]. The D2-SE is a D2 mutant in which an extra loop of 31 amino acids has been added in the D-de loop of D2 [43]. These three mutants were chosen for their diversity to test the involvement of the D1-D2 proteins in chloroacetate binding. We have measured the effect of trichloroacetates on the DCPIP Hill reaction of thylakoids from the WT and mutant cells, just as was done for the effects of herbicides [45].

3. Results and Discussion

3.1. Site and specificity of chloroacetates

In order to use chloroacetates as a probe for the $Q_A - Fe - Q_B$ binding niche, we first had to establish its site of action. The effect of up to 100 mM chloroacetates was located mainly between Q_A and Q_B; it was not on PSI, or on the donor side of PSII. Only much higher concentrations lead to the release of donor side extrinsic 17, 23, and 33 kD polypeptides in thylakoids and in isolated PSII particles [46]. Thus, like other inhibitors, specificity is a function of concentration and other experimental conditions. Data in Table 1, with $DCPIPH_2$ as the electron donor and methylviologen as the electron acceptor, show that 100 mM chloroacetates do not inhibit PSI electron flow at pH 6.0; similar results were obtained at pH 7.6 (data not shown). However, the whole chain electron flow (H_2O to MV) was inhibited differentially: TCA > DCA ≈ MCA at the three pH values examined (6.0, 6.5 and 7.6). Thus, the inhibitory site must be somewhere in PS II. Table 2 shows the effects of chloroacetates on three partial reactions of PSII. (A) H_2O to PQ electron flow (with PD/FeCy as electron acceptor and DBMIB to block electron flow to PSI). This reaction includes both donor and acceptor sides of PSII; it is inhibited by TCA > DCA > MCA at pH 6.5. At pH 7.6 the effects are smaller, with only TCA showing an inhibition. (B) DCMU-insensitive electron flow in Tris-treated thylakoids from DPC to DCPIP. This reaction is unaffected by chloroacetates suggesting that they do not affect the primary charge separation steps. (C) Water to SiMo reaction (in the presence of 1 mM DBMIB). Silicomolybdate binds to the acceptor side of PSII close to the non-heme iron [32]. This reaction measures water to Q_A electron flow and is not affected by acetates. The reduction of SiMo is sensitive to donor side inhibition and the binding of chloroacetates to the acceptor side might interfere with the binding of SiMo. Lineweaver-Burk plots of SiMo titration curves in the absence of chloroacetates or in the presence of 100 mM MCA or TCA, respectively, show no interaction between SiMo and MCA or TCA (result not shown). This indicates that SiMo reduction is not sensitive to the binding of chloroacetates on the acceptor side of PSII and allows the use of SiMo as a probe for donor side effects of chloroacetates. In Fig. 1 the time dependence of the inhibition of electron flow to SiMo (H_2O to Q_A) and to pBQ (H_2O to PQ or beyond) by three concentrations of TCA is shown. The electron transport to pBQ was inhibited almost instantaneously and the inhibition by 100 mM TCA was almost complete. However, the electron flow to SiMo was inhibited more gradually and the extent of the inhibition was

Table 1
Effects of 100 mM chloroacetates on PS II + PS I electron transport (whole chain electron transport) and on PS I electron transport. Experimental conditions are described under Materials and Methods. Average of 3–5 experiments

	Whole chain electron flow		Photosystem I electron flow	
pH	6.0	6.5	7.6	6.0
Control	$100^a \pm 150$	$100^b \pm 10$	$100^c \pm 10$	$100^d \pm 11$
MCA	44 ± 5	62 ± 5	70 ± 5	104 ± 9
DCA	31 ± 4	67 ± 5	90 ± 5	113 ± 10
TCA	10 ± 5	28 ± 5	56 ± 6	130 ± 15

Percent of control values. Control values in μmol O_2.mg $Chl^{-1}.h^{-1}$: [a], about 280; [b], about 580; [c], about 530; [d], about 200.

Table 2
Effects of 100 mM chloroacetates on photosystem II reactions. Experimental conditions are described under Materials and Methods. (A) Electron flow from water to plastoquinone (PD/FeCy as electron acceptor, DBMIB as inhibitor of electron flow to PSI) (n = 3); (B) Electron flow in Tris-treated thylakoids from DPC to Q_A in a DCMU-insensitive DCPIP reduction (n = 4); (C) Electron flow from water to SiMo in the presence of DBMIB (n = 3)

	(A)	(A)	(B)	(B)	(C)
pH	6.5	7.6	6.5	7.8	6.5
Control	$100^a \pm 5$	$100^b \pm 15$	$100^c \pm 10$	$100^d \pm 10$	$100^e \pm 5$
MCA	77 ± 4	96 ± 10	102 ± 10	100 ± 10	100 ± 1
DCA	61 ± 2	102 ± 5	119 ± 20	135 ± 20	86 ± 2
TCA	18 ± 5	48 ± 11	113 ± 5	95 ± 15	105 ± 7

Percent of control values. Control values in μequivalents.mg $Chl^{-1}.h^{-1}$: [a], 1400; [b], 1100; [c], 52; [d], 84; [e], 600

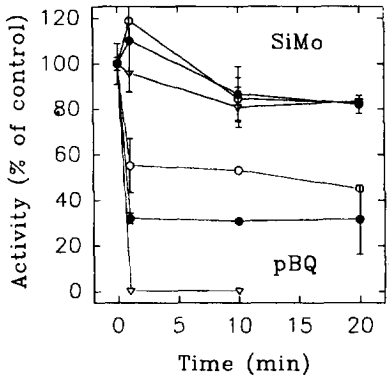

Fig. 1. Effects of trichloroacetate (open circle, 10 mM; closed circle, 30 mM; upside down triangle, 100 mM TCA) on electron flow reactions from water to silicomolybdate (SiMo) and to para-benzoquinone (pBQ) at pH 6.5. Control activity of the SiMo-reaction was 210, that of the pBQ-reaction was 432 μmol O_2.mg $Chl^{-1}.h^{-1}$. Average of 3 experiments. See Materials and Methods for further details.

less than 20%, whereas it was more than 90% for pBQ. These data indicate that TCA inhibits the acceptor side of PSII completely and almost instantaneously whereas the donor side of PSII is much less sensitive to TCA, since longer incubation times are needed, and even in that case the effects are much smaller than on the acceptor side.

In conclusion, although there are effects of high concentrations of chloroacetates on the donor side of PSII [46], most of the effects are on the acceptor side of PSII even at 100 mM TCA. This becomes obvious in the Chl *a* fluorescence decay measurements (see sections 3.2–3.4).

3.2. Chlorophyll a fluorescence decay in tris-treated thylakoids

In Tris-washed thylakoids that are known to be blocked on the water oxidation side, reduced benzidine (or DPC) can act as an electron donor. Chl *a* fluorescence yield decay in Tris-washed thylakoids with reduced benzidine, after flash 2, showed an inhibition of electron flow from Q_A^- to Q_B^- and an increase in equilibrium $[Q_A^-]$ upon the addition of chloroacetates (Fig. 2). In samples without chloroacetates, the Chl *a* fluorescence yield at 70 μs (the first measured point) was much lower than in the samples treated with chloroacetates. Since Chl *a* fluorescence yield is related to $[Q_A^-]$ this result is suggested to be due to a higher $[Q_A^-]$ in treated samples due to a blockage of electron transfer beyond Q_A^- by the chloroacetates. If these chemicals had inhibited only the electron donor side, we should have seen a lowering, not an increase, of variable Chl *a* fluorescence. The true F_{max} was identical in all cases, when measured with 6 μM DCMU (data not shown). However, the hierarchy of this inhibition (TCA > DCA > MCA) qualitatively followed that in the whole chain electron flow (see Table 1).

Electron flow from Q_A^- to Q_B (flash 1) was also inhibited by chloroacetates, but, in contrast to the Q_A^- to Q_B^- reaction, the hierarchy of inhibition was altered with MCA giving a larger inhibition than DCA (data not shown). Since Tris-washing may alter the native system, and since almost identical and reliable results on Q_AQ_B reactions were obtained with untreated thylakoids, we have used the latter allowing us to compare these data with those published earlier with other inhibitors [16–21].

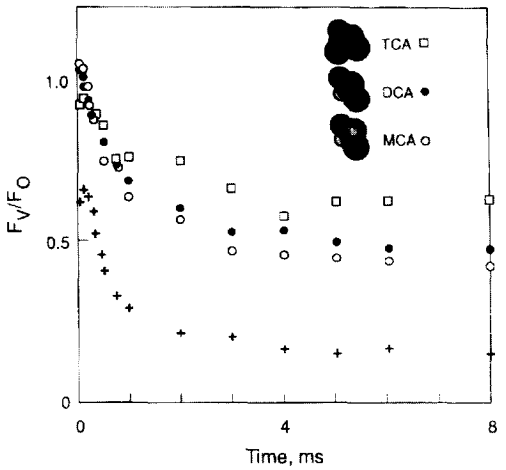

Fig. 2. Chl *a* fluorescence decay after flash 2 in Tris-washed spinach thylakoids in the presence of reduced benzidine as electron donor without further addition (crosses) or with 100 mM monochloroacetate (MCA) or dichloroacetate (DCA) or trichloroacetate (TCA). F_v = variable Chl *a* fluorescence yield; F_o = Chl *a* fluorescence yield at very low light intensities. See Materials and Methods for experimental details.

3.3. Concentration dependence of chloroacetates on $[Q_A^-]$

To quantitatively analyze the effect caused by chloroacetates on Q_A^- reoxidation and equilibration, the increase of $[Q_A^-]$ upon chloroacetate addition was measured after each of four flashes. The values of $[Q_A^-]_{treated} - [Q_A^-]_{control}$, calculated at 5 ms after flashes 1–4, are plotted against concentration of acetate, mono-, di- or tri-chloroacetate for two pH values (A = 7.5; B = 6.0) (Fig. 3, the numbers after A and B indicate the flash number). The increase caused by TCA is the largest and the one by acetate is the weakest of all concentrations used. The initial slope of increase in $[Q_A^-]_{treated} - [Q_A^-]_{control}$, as a function of inhibitor concentration, shows the same hierarchy as that at 100 mM in most cases: TCA > DCA > MCA > acetate. At pH 6, more than at pH 7.5,

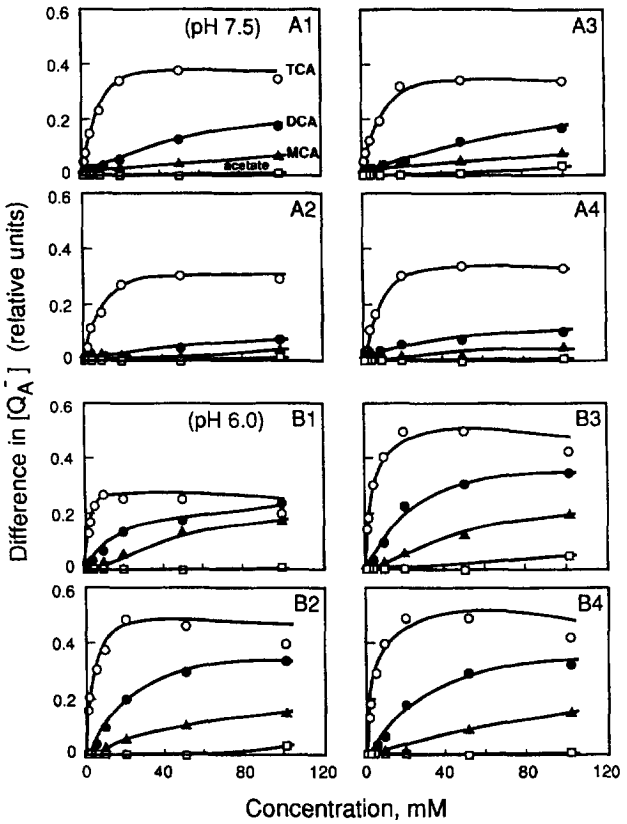

Fig. 3. Differences in $[Q_A^-]$ at 5 ms, after flashes 1–4, between treated and control spinach thylakoids as a function of concentration of acetate, monochloroacetate (MCA), dichloroacetate (DCA) or trichloroacetate (TCA) at two pHs (panels A for pH 7.5 and panels B for pH 6; numbers after A and B indicate the flash number). See Materials and Methods for experimental details.

DCA and TCA cause larger increases in $[Q_A^-]_{treated} - [Q_A^-]_{control}$ after the second and subsequent, than after the first flash (panels B, Fig. 3). This is consistent with a larger inhibition at pH 6.5 than at pH 7.6 under steady state (Table 1). However, when compared with formate [21,23], chloroacetates lead to a larger inhibition after the first flash relative to that after the second. Further, at pH 7.5, the effect is even larger after the first than after the second flash (Fig. 3, panels A). Thus, in contrast to formate, chloroacetates inhibit strongly Q_A^- to Q_B electron flow. These novel observations on flash number dependence and pH dependence are of obvious importance and are more fully explored and discussed in the following sections. Our results show that we can choose 20–100 mM of chloroacetates, that give large effects, to analyze in detail Q_AQ_B reactions.

3.4. Kinetics of Chl a fluorescence yield decay at pH 6.0 after flash 1: the bicarbonate effect

The decay of Chl *a* fluorescence yield after flash 1 monitors mostly electron transfer from Q_A^- to Q_B, and can be used to investigate the effects of chloroacetates, as has been done for formate [21,23]. Fig. 4 shows the results on Chl *a* fluorescence, and thus $[Q_A^-]$, decay up to 8 ms after the actinic flash. In the series: acetate /MCA /DCA /TCA, the $[Q_A^-]$ decay appears progressively slowed with an increasing number of chlorine moiety (hatched areas within the figures) in these inhibitors. However, the reversibility by 10 mM bicarbonate progressively decreases in the series; it was fully reversible in acetate to almost fully irreversible in TCA. Analysis of these data into three exponential components (*f*ast, *i*ntermediate and *s*low) led to the following results for 100 mM acetate, MCA, DCA, and TCA: amplitude A_f decreased from 0.63 (control) to 0.53, 0.47, 0.41 and 0.16, respectively, whereas $A_i + A_s$ increased leading to an increased $A_i + A_s/A_f$ ratio from 0.46 (control) to 0.7, 1.0, 1.3 and 3.3, respectively. In parallel, lifetimes t_f (and t_i) increased from 420 μs (and 3.3 ms) (control) to 610 μs (9 ms), 710 μs (70 ms), 970 μs (65 ms) and 1500 μs (99 ms), respectively. Thus, the hierarchy was observed not only in the lifetime of electron transfer from Q_A^- to Q_B, but also in the equilibration reaction between $Q_A^-Q_B$ and $Q_AQ_B^-$. Here, we have assumed that the fluorescence decay curve can be fitted by three components. This is not necessarily true and the change in lifetimes may also be caused by the induction of components with longer lifetimes by the chloroacetates. In contrast to chloroacetates, bicarbonate-reversible formate treatment [21,23] provides a much smaller effect on Q_A^- to Q_B reaction, as already mentioned earlier. In summary, chloroacetates affect the Q_AQ_B reactions differently from that by formate; they inhibit the electron flow instead of protonation, and are only partially displaced by bicarbonate depending upon the specific chloroacetate used.

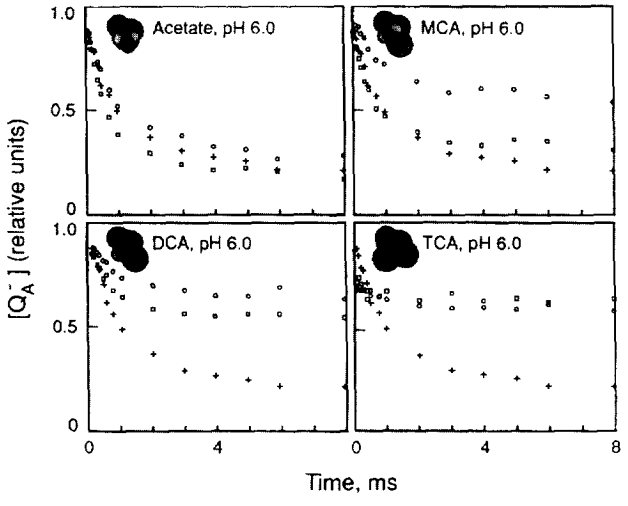

Fig. 4. $[Q_A^-]$ as a function of time after flash 1 at pH 6 in controls (crosses), 100 mM acetate, monochloroacetate (MCA) or dichloroacetate (DCA) or trichloroacetate (TCA) treated spinach thylakoids (open circles), and those to which 10 mM bicarbonate was added after the addition of acetates (squares). See Materials and Methods for experimental details.

3.5. Flash 1 versus flash 2 effects on Chl a fluorescence decay

As mentioned above, Eaton-Rye and Govindjee [23] and Xu et al. [21] showed that bicarbonate-reversible formate slowed $[Q_A^-]$ decay more after the second, rather than after the first flash, leading them to suggest that bicarbonate functions by providing protons to nearby amino acids [17] to stabilize Q_B^-. A detailed analysis of the chloroacetate effect was done here. Fig. 5 shows a comparison of $[Q_A^-]$ decay, at pH 6.0, up to 30 ms after flash 1 and 2 in the chloroacetate series. At pH 6, DCA and TCA (both 100 mM) show results qualitatively similar to that of formate, but 100 mM MCA shows the opposite result; and acetate shows a very small difference between flash 1 and 2. Analysis of these data into three amplitude and lifetime components showed that what changed most is the sum of the amplitudes of the intermediate

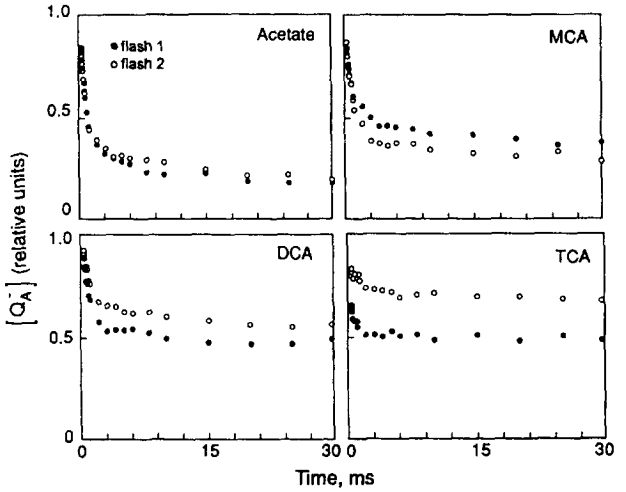

Fig. 5. $[Q_A^-]$ as a function of time up to 30 ms after flash 1 (closed circles) and flash 2 (open circles) in the presence of 100 mM acetate or monochloroacetate (MCA) or dichloroacetate (DCA) or trichloroacetate (TCA) in spinach thylakoids. See Materials and Methods for experimental details.

and slow components ($A_i + A_s$). For example, $A_i + A_s$ decreased from a value of 0.48 (flash 1) to 0.38 (flash 2) with MCA present, and it increased from 0.52 to 0.65 with DCA, and from 0.52 to 0.73 with TCA. These changes were accompanied by opposite changes in the amplitude of the fast component (A_f). There seemed to be no significant change in the lifetime of the fast component in all cases except perhaps TCA. It is *important* to note that although DCA and TCA show qualitatively similar results to that of formate, they differ remarkably from formate because even the decay after flash 1 is drastically slowed from that in the control (Fig. 4). These chloroacetates inhibit, therefore, both the electron flow as well as protonation events. The situation with MCA is unique; here $[Q_A^-]$ decay after flash 1 is slowed more than after flash 2. Similar results were obtained in Tris-washed thylakoids (data not shown). Several interpretations are possible: (1) ratio of Q_B^-/Q_B in dark is increased by MCA; (2) MCA slows Q_A^- to Q_B, because it can exchange with Q_B, but not with Q_B^- (for further discussion, see Xu et al. [47]). Thus, the mechanism by which chloroacetates act on the Q_AQ_B complex is different from that with formate, although formate causes also inhibition of Q_A^- to Q_B, but only after long-term treatment [48].

3.6. Thermoluminescence studies: a 'block' between Q_A and Q_B by chloroacetate

Thermoluminescence is an excellent probe for identifying a 'block' in electron flow between Q_A and Q_B since a 'B' band appears at around 20–40°C as a result of $S_2(S_3)Q_B^-$ recombination, and a 'Q' band appears at around 0–10°C as a result of $S_2Q_A^-$ recombination [41]. Thus, a block, as with several herbicides, e.g., diuron, leads to the appearance of the Q band at the expense of the B band. Fig. 6 shows that both after the first (1) and the second (2) flash, 100 mM TCA leads to the disappearance of the B and appearance of the Q band, respectively.

3.7. Decay of $[Q_A^-]$ at two pH values; effect of different pK_a

In view of the conclusion of Blubaugh and Govindjee [49] that HCO_3^- is the active species for stimulating PS II reactions, HCO_2^- (formate $pK_a = 3.75$), has been accepted as the inhibitory species [17]. However, Xu et al. [21] have provided data and arguments for an alternative: formic acid, not formate. This is based mainly on the fact that the inhibitory effect is higher at the lower than at the higher pH. In view of these arguments, we measured the effect of MCA ($pK_a = 2.81$), DCA ($pK_a = 1.30$), and TCA ($pK_a = 0.70$). Fig. 7 shows the results for DCA and TCA at two pH's for flash 2. In agreement with the data for formate, 100 mM acetate ($pK_a = 4.73$), MCA (data not shown) and DCA show large inhibitory effects on $[Q_A^-]$ decay at pH 6, the effect at pH 7.5 being much smaller. However, 100 mM TCA shows almost equal effects at both pH 6 and 7.5.

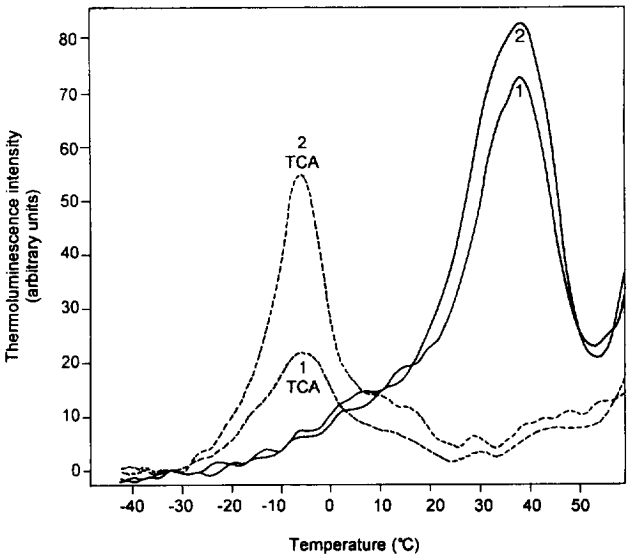

Fig. 6. Thermoluminescence bands after flash 1 and 2 in untreated and trichloroacetate (TCA) treated thylakoid samples. Note that TCA treatment produces the Q band (due to $S_2Q_A^-$ recombination) at the expense of the B band ($S_2Q_B^-$ recombination) showing that TCA blocks electron flow from Q_A^- to Q_B. Data obtained in collaboration with T. Ono and D. Kramer.

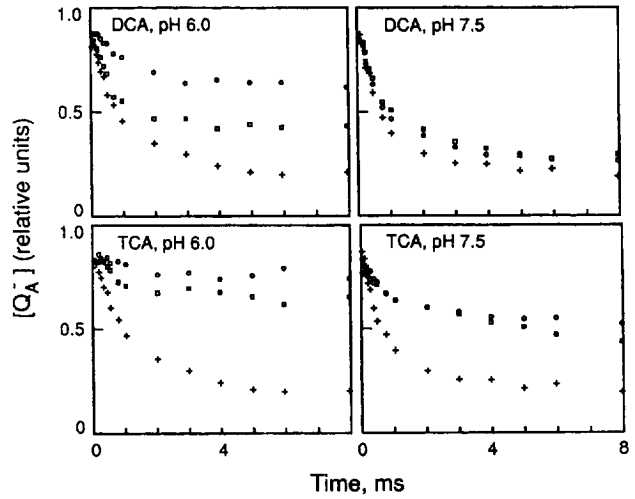

Fig. 7. $[Q_A^-]$ as a function of time after flash 2 at two different pHs in spinach thylakoids (crosses), with 100 mM dichloroacetate (DCA), or trichloroacetate (TCA) (open circles) and those to which 10 mM bicarbonate was added after the addition of chloroacetates (squares). See Materials and Methods for experimental details.

To confirm precisely the pH dependence, we made further measurements at lower concentrations (0–10 mM) of TCA at different pH's. $[Q_A^-]_{treated} - [Q_A^-]_{control}$, estimated at 5 ms after the flashes, is plotted against the concentration of TCA (Fig. 8). Lowering the pH of the suspension medium from 7.5 to 5.0 leads to an increase in the initial slope of changes in $[Q_A^-]_{treated} - [Q_A^-]_{control}$ plotted as a function of TCA concentration. The flash number dependence of the inhibition is clear: a larger increase in $[Q_A^-]_{treated} - [Q_A^-]_{control}$ can be seen after flashes 2 – 4 than after the first flash. The $[Q_A^-]$ decays in acetate, MCA- and DCA- treated samples also show stronger inhibition of the Q_A^- reoxidation at pH 5.5 than at pH 6 (data not shown).

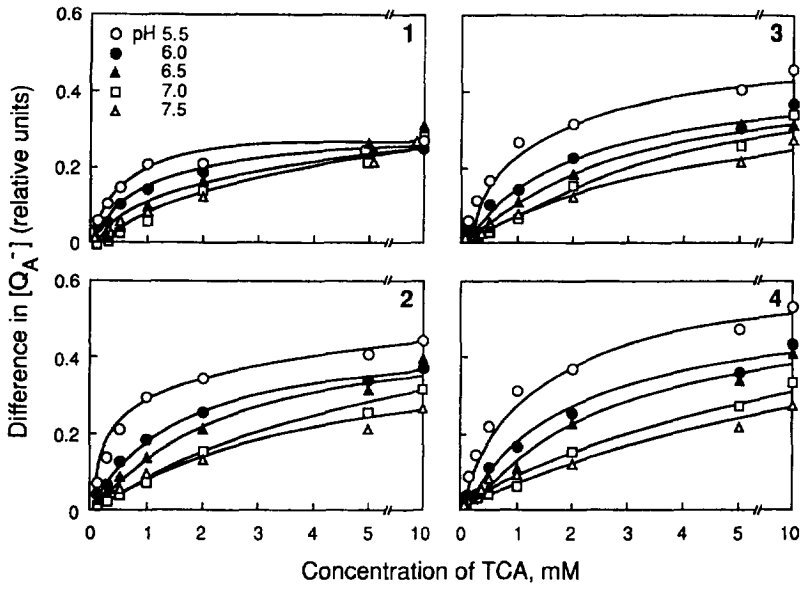

Fig. 8. Differences in [Q_A^-] at 5 ms, after flashes 1, 2, 3, and 4, between treated and control spinach thylakoids as a function of [trichloroacetate] at several different pHs. See Materials and Methods for experimental details.

There are two interpretations of the above results on the pH dependence of the effects of chloroacetates on Q_A^- to Q_B (or Q_B^-) reactions: (1) Acetic acid, monochloroacetic acid, dichloroacetic acid and trichloroacetic acid are active species of the chloroacetate effects. (2) The pH dependence of the inhibitory species is convoluted with the pH effects on the PS II RC protein. Thus, RC protein may play a significant role in the observed pH dependence and that chloroacetates may function as anions. The anion hypothesis is a much more satisfactory explanation: since positively charged arginines have been suggested to stabilize bicarbonate when it is bound [17,50]. Good [51] had examined the effect of various anions on the Hill reaction rate in higher plant chloroplasts and had concluded that: (1) smaller monofunctional anions (formate, acetate) cause a bicarbonate-dependent inhibition of the Hill reaction rates; (2) anions with hydroxyl close to the carboxyl (glycolate, salicylate, lactate) produce a bicarbonate-independent inhibition of the Hill reaction rate; and (3) anions consisting mainly of large or polyfunctional ions (citrate, oxalate, malonate, maleate, arsenate, phosphate and pyrophosphate) do not affect the Hill reaction rate.

The pH dependence of the inhibitory effect of chloroacetates is dependent on several factors: (1) As the pH is decreased, the surface charge changes from negative to neutral to positive; this leads to an absence of binding of anions at the higher pH and an increased binding at the lower pH; (2) in contrast to the above, the concentration of anions in solution is low at the lower pH and high at the higher pH. The exact shape of the convoluted curve of (1) and (2) would depend on the pK_a of the chemical being considered and its local binding characteristics. However, in general, the effect of chemicals with low and high pK_as would have a rising and a declining portion of curves in different pH ranges. Our data on MCA ($pK_a = 2.8$) show a small effect. These data are consistent with this halogenated acetate being effective in its anion form. However, data on TCA ($pK_a = 0.7$) show equal effectiveness at pH 6 and 7.5 suggesting that not only pK_a but local binding effects on the protein play a role here. Data on DCA ($pK_a = 1.3$) are intermediate between MCA and TCA. The above discussion implies that pH dependence of the inhibitory effects of chloroacetates can be explained by invoking anion binding on RC proteins. Thus, the alternative suggested by Xu et al. [21] that formic acid, not formate, is the active inhibitory species remains only an alternative. Further research and analysis are needed to decipher the nature of the binding species of chloroacetates.

3.8. Studies with D1/D2 mutants of cyanobacteria

The role of D1 and D2 proteins in the binding of bicarbonate-reversible formate has been established through observations of differential sensitivity of formate inhibition of Q_AQ_B reactions in various D1/D2 mutants [44,50,52–54]. In this article, we have observed differential sensitivity of trichloroacetate on the Hill reaction rate (measured as H_2O to DCPIP electron flow) of a few chosen D1/D2 mutants of *Synechocystis* sp. PCC 6803 (Fig. 9). It is shown here (obtained in collaboration with H. Kless.) that two D1 mutants, S264A and D1-ΔAA, are much more sensitive than the wild type, but the D2-mutant D2SE is rather resistant to TCA. These results suggest that the D1 and D2 proteins are physically involved in the binding of chloroacetates.

3.9. A discussion of the binding niche

Table 3 lists the effects of chloroacetates on the Q_AQ_B reaction along with their various physico−chemical characteristics; the most obvious correlation is with the Hansch's hydrophobicity indicators [56,57]. This is reminiscent of a similar correlation of the inhibitory activity of herbicides with

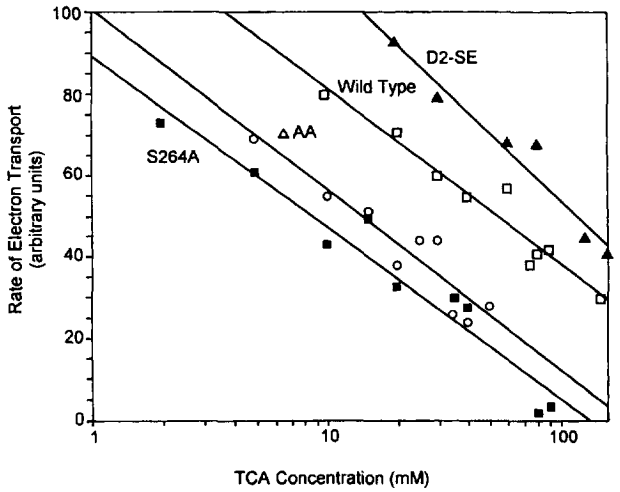

Fig. 9. A plot of the Hill reaction rate (H_2O to DCPIP electron flow) as a function of the log of TCA concentration in two D1 mutants (S264A and ΔAA), a D2 mutant (D2-SE), and wild type *Synechocystis* sp. PCC 6803. Data obtained in collaboration with H. Kless.

their hydrophobicity [7]. Of course, TCA is bulkier than DCA and DCA is bulkier than MCA as the chlorine atom is much larger than the hydrogen atom it replaces. There is, however, no obvious correlation with the overall dipole moments [55].

Several herbicides (amides, benzimidazoles, biscarbamates, carbamates, hydroxy-benzonitriles, nitrophenols, phenyl ureas, pyridazinones, S-triazines, triazinones, uracil and urea), all having hydrophobic components [7], bind to the Q_AFeQ_B region in the D1/D2 complex. The primary function of the hydrophobic component(s) is to increase the lipid solubility and to improve contacts between the herbicides and the hydrophobic surface of the binding site. In this article, we have extended this concept through our observation of a similar correlation between the hydrophobic constants of chloroacetates and their inhibitory activities on the Q_AQ_B complex (Table 3).

Positive residues such as arginine are the appropriate candidates to form the cation end of the salt bridge [17] if the anions bicarbonate and formate (and chloroacetates) are considered to be the active stimulatory and inhibitory species, respectively. The importance of specific arginine residues on the D2 protein (R233, R251, R265) in stabilizing anions has been shown through site-directed metagenesis studies [16,50]. Recent unpublished data of J. Xiong and his coworkers in our laboratory have suggested the importance of the D1 arginine 257 in bicarbonate binding. The crystallographic structure of human lactoferrin, the only other (bi)carbonate–Fe protein, reveals [58] that the oxygens of carbonate are H-bonded to neighboring amino acid residues (including arginine) in addition to a bidentate ligand to Fe^{3+}, suggesting that H-bonds may play significant roles in formate, chloroacetate, and bicarbonate binding.

Direct interaction of some exogenous ligand of inhibitors/bicarbonate with the non-heme iron in the $Q_A - Fe - Q_B$ complex is supported by EPR [59,60] and Mössbauer studies [61]. The modulation of these signals by bicarbonate/inhibitors was interpreted as implying that they could form the fifth, possibly the sixth ligand of the iron.

The bicarbonate/formate effect is specific for the PS II reaction center and has not been observed in photosynthetic bacteria [62]. TCA does not produce any inhibition of Q_A^- oxidation in purple photosynthetic bacteria (X. Wang, C. Xu, and Govindjee, unpublished data) although a dramatic inhibition of Q_A^--to-Q_B electron flow was found in PS II system (this article). The explanation for these differences must involve differences in the amino acid sequence and the molecular structure of PS II versus bacterial reaction center.

3.10. Concluding remarks

Based on the data presented in this article, we make three important points:

(1) In contrast to formate, other bicarbonate-reversible inhibitors (MCA and to some extent DCA), that act on PS II, show a large inhibition of Q_A^- reoxidation and an increase of equilibrium $[Q_A^-]$ even after the first actinic flash, demonstrating that they also block electron flow from Q_A^- to Q_B just as TCA and diuron do. A blockage of electron flow from

Table 3
Correlation of the inhibitory effect of halogenated acetates on $[Q_A^-]$ decay in spinach thylakoids with their physical properties

Calculated $[Q_A^-]$ decay				Properties of chemicals		
Chemicals	Calculated lifetime $Q_A^- \rightarrow Q_B^{(-)}$ (μs)	Nos. related to equilibrium of $Q_A^-Q_B = Q_AQ_B^-$ (Slow/fast components)	Bicarbonate reversibility	pK_a moment	Dipole (Debye) [a]	Hydrophobicity index (Log of partition coefficient) [b]
Acetate	610	0.9	+ + + + +	4.7	1.6	−0.33
Monofluoroacetate	650	0.8	+ + + + +	2.6	3.1	−0.27
Monochloroacetate [c]	845	1.8	+ + + + +	2.8	3.3	+0.32
Monobromoacetate [c]	860	1.4	+ +	2.9	3.1	+0.64
Dichloroacetate	920	3.2	+ + +	1.3	2.5	+1.33
Trichloroacetate	1,700	24.0	+	0.7	2.1	+1.54

[a] After [55].
[b] Data for the acid, not the salt, form from water/octanol systems (after [56,57]).
[c] See Xu et al. [47].

Q_A^- to Q_B was confirmed in thermoluminescence measurements, by the formation of the Q band (due to $S_2Q_A^-$ recombination) and the disappearance of the B band (due to $S_2Q_B^-$ recombination) upon the addition of TCA.

(2) The binding niche of $Q_A - Fe - Q_B$ pocket in the D1/D2 protein of PS II when probed with halogenated acetates shows a hierarchy in their ability to inhibit Q_A^- oxidation and to increase equilibrium $[Q_A^-]$. This follows the order: TCA > DCA > MCA > acetate. However, bicarbonate reversibility of Q_A^- to Q_B reaction follows the reverse order. A relationship exists between these effects, and the size and hydrophobicity of the inhibitors used.

(3) The involvement of the D1/D2 proteins was confirmed by the observation of the differential sensitivity of TCA to different D1/D2 mutants of *Synechocystis* sp. PCC 6803. The role of RC proteins in understanding the nature of the interaction with the inhibitor is emphasized; data with chloroacetates permit us to suggest that anions, not acids, are a valid alternative as the inhibitory species for blocking electron and proton transfers needed for PQ reduction.

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References

[1] W.F.J. Vermaas and M. Ikeuchi, Photosystem II, in L. Bogorad and I.K. Vasil, (eds.), *The Photosynthetic Apparatus: Molecular Biology and Operation*, Academic Press, San Diego, 1991, pp. 25–111.

[2] A.R. Crofts and C.A. Wraight, The electrochemical domain of photosynthesis, *Biochim. Biophys. Acta, 726* (1983) 149–185.

[3] Govindjee and M.R. Wasielewski, Photosystem II: from a femtosecond to a millisecond, in W.R. Briggs (ed.), *Photosynthesis*, Alan R. Liss Inc., New York., 1983, pp. 71–103.

[4] C.A. Wraight, Electron acceptors of bacterial photosynthetic reaction centers: II. H^+ binding coupled to secondary electron transfer in the quinone acceptor complex, *Biochim. Biophys. Acta, 548* (1979) 309–327.

[5] B.R. Velthuys, Electron dependent competition between plastoquinone and inhibitors for binding to photosysterm II, *FEBS Lett., 126* (1981) 277–281.

[6] C.A. Wraight, Oxidation-reduction physical chemistry of the acceptor quinone complex in bacterial photosynthetic reaction centers: evidence for a new model of herbicide activity, *Isr. J. Chem., 21* (1981) 348–354.

[7] J. Bowyer, P. Camilleri and W.F.J. Vermaas, Photosystem II and its interaction with herbicides, in N.R. Baker and M.P. Percival (eds.), *Herbicides*, Elsevier, Amsterdam, 1991, pp. 27–85.

[8] V.V. Klimov, S.I. Allakhverdiev, Y.M. Feyziev and S.V. Baranov, Bicarbonate requirement for the donor side of photosystem II, *FEBS Lett., 363* (1995) 251–255.

[9] V.A. Szalai and G.W. Brudvig, Acetate-treated photosystem II limited to two turnovers by an electron-accepting herbicide does not yield the S_3 EPR signal, *Biophys. J., 68* (1995) A92.

[10] D.J. Maclachlan and J.H.A. Nugent, Investigation of the S_3 electron paramagnetic resonance signal from the oxygen-evolving complex of photosystem 2: Effect of inhibition of oxygen evolution by acetate, *Biochemistry, 32* (1993) 9772–9780.

[11] F. El-Shintinawy and Govindjee, Bicarbonate effect in leaf discs from spinach, *Photosynth. Res., 24* (1990) 189–200.

[12] S. Demeter, T. Janda, L. Kovacs, D. Mende and W. Wiessner, Effects of in vivo CO_2-depletion on electron transport and photoinhibition in the green alga, *Chlamydobotris stellata* and *Chlamydomonas reinhardtii*, *Biochim. Biophys. Acta, 1229* (1995) 166–174.

[13] V. Petrouleas, Y. Deligiannakis and B.A. Diner, Binding of carboxylate anions at the non-heme Fe (II) of PSII. 2. Competition with bicarbonate and effects on the Q_A/Q_B electron transfer rate, *Biochim. Biophys. Acta, 1188* (1994) 271–277.

[14] R.M. Cinco, J.M. MacInnis and E. Greenbaum, The role of carbon dioxide in light-activated hydrogen production by *Chlamydomonas reinhardtii*, *Photosynth. Res., 38* (1993) 27–33.

[15] Govindjee and J.J.S. van Rensen, Photosystem II reaction center and bicarbonate, in J. Deisenhofer and J.R. Norris (eds.), *The Photosynthetic Reaction Center*, Academic Press, San Diego, 1992, pp. 357–389.

[16] B.A. Diner, V. Petrouleas and J.J. Wendoloski, The iron-quinone electron-acceptor complex of photosystem II, *Physiol. Plant., 81* (1991) 423–436.

[17] D.J. Blubaugh and Govindjee, The molecular mechanism of the bicarbonate effect at the plastoquinone reductase site of photosynthesis, *Photosynth. Res., 19* (1988) 85–128.

[18] W.F.J. Vermaas, J.J.S. van Rensen and Govindjee, The interaction between bicarbonate and the herbicide ioxynil in the thylakoid membrane and the effects of amino acid modification on bicarbonate action, *Biochim. Biophys. Acta, 681* (1982) 242–247.

[19] J.J.S. van Rensen and W.F.J. Vermaas, Action of bicarbonate and photosystem II inhibiting herbicides on electron transport in pea grana and in thylakoids of a blue-green alga, *Physiol. Plant., 51* (1981) 106–110.

[20] R. Khanna, K. Pfister, A. Keresztes, J.J.S. van Rensen and Govindjee, Evidence for a close spatial location of the binding sites for CO_2 and for photosystem II inhibitors, *Biochim. Biophys. Acta, 634* (1981) 105–116.

[21] C. Xu, S. Taoka, A.R. Crofts and Govindjee, Kinetic characteristics of formate/formic acid binding at the plastoquinone reductase site in spinach chloroplasts, *Biochim. Biophys. Acta, 1058* (1991) 32–40.

[22] J.J.S. van Rensen, W.J.M. Tonk and S.M. de Bruijn, Involvement of bicarbonate in the protonation of the secondary quinone electron acceptor of photosystem II via the non-heme iron of the quinone-iron acceptor complex, *FEBS Lett., 226* (1988) 347–351.

[23] J.J. Eaton-Rye and Govindjee, Electron transfer through the quinone acceptor complex of photosystem II in bicarbonate-depleted spinach thylakoid membranes as a function of actinic flash number and frequency, *Biochim. Biophys. Acta, 935* (1988) 237–247.

[24] Govindjee and J.J.S. van Rensen, Bicarbonate effects on the electron flow in isolated broken chloroplasts, *Biochim. Biophys. Acta, 505* (1978) 183–213.

[25] A. Trebst, B. Depka, S.M. Ridley and A.W. Hawkins, Inhibition of photosynthetic electron transport by halogenated 4-hydroxy-pyridines, *Z. Naturforsch., 40c* (1985) 391–399.

[26] R.J. Porra, W.A. Thompson and P.E. Kriedemann, Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls *a* and *b* extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic

absorption spectroscopy, *Biochim. Biophys. Acta, 975* (1989) 384–394.

[27] G.M. Cheniae and I. Martin, Studies on the mechanism of Tris-induced inactivation of oxygen evolution, *Biochim. Biophys. Acta, 502* (1978) 321–344.

[28] J.J. Eaton-Rye and Govindjee, A study of the specific effect of bicarbonate on photosynthetic electron transport in the presence of methyl viologen, *Photochem. Photobiol., 8* (1984) 279–288.

[29] S. Izawa, J.M. Gould, D.R. Ort, P. Felker and N.E. Good, Electron transport and photophosphorylation in chloroplasts as a function of the electron acceptor. III. A dibromothymoquinone-insensitive phosphorylation reaction associated with photosystem II, *Biochim. Biophys. Acta, 305* (1973) 119–128.

[30] J.J.S. van Rensen, W. van der Vet and W.P.A. van Vliet, Inhibition and uncoupling of electron tranport in isolated chloroplasts by the herbicide 4,6–dinitro–o–cresol, *Photochem. Photobiol., 25* (1977) 579–583.

[31] B.A. Zilinskas and Govindjee, Silicomolybdate and silicotungstate mediated dichlorophenyldimethylurea-insensitive photosystem II reaction: electron flow, chlorophyll *a* fluorescence and delayed light emission changes, *Biochim. Biophys. Acta, 387* (1975) 306–319.

[32] G. Schansker and J.J.S. van Rensen, Characterization of the complex interaction between the electron acceptor silicomolybdate and photosystem II, *Photosynth. Res., 37* (1993) 165–175.

[33] A. Joliot and P. Joliot, Etude cinétique de la réaction photochimique libérant l'oxygene au cours de la photosynthèse, *Compt. Rend. Acad. Sci. Paris, 258* (1964) 4622–4625.

[34] C. Xu, S.M.D. Rogers, C. Goldstein, J.M. Widholm and Govindjee, Fluorescence characteristics of photoautrotrophic soybean cells, *Photosynth. Res., 21* (1989) 93–106.

[35] R.J. Strasser, P. Eggenberg, K. Pfister and Govindjee, An equilibrium model for electron transfer in photosystem II acceptor complex: an application to *Chlamydomonas reinhardtii* cells of D1 mutants and those treated with formate, *Archs Sci. Genève, 45* (1992) 207–224.

[36] J. Cao and Govindjee, Chlorophyll *a* fluorescence transient as an indicator of active and inactive photosystem II in thylakoid membranes, *Biochim. Biophys. Acta, 1015* (1990) 180–188.

[37] A.-L. Etienne, J.-M. Ducruet, G. Ajilani and C. Vernotte, Comparative studies on electron transfer in photosystem II of herbicide resistant mutants from different organisms, *Biochim. Biophys. Acta, 1015* (1990) 435–440.

[38] A.R. Crofts, H.H. Robinson and M. Snozzi, Reactions of quinones at catalytic sites: a diffusinal role in H-transfer, in C. Sybesma (ed.), *Advances in Photosynthesis Research*, Vol. 1, Martinus Nijhoff, Dordrecht, 1984, pp. 461–468.

[39] J.M. Beechem, E. Gratton, M. Ameloot, J.R. Knutson and L. Brand, The GLOBAL analysis of fluorescence intensity and anisotropy decay data: second generation theory and programs, in J.R. Lackowicz (ed.), *Topics in Fluorescence Spectroscopy*, Vol. II, Principle, Plenum Press, New York, 1991, pp. 241–305.

[40] H.H. Robinson and A.R. Crofts, Kinetics of the oxidation-reduction reactions of the photosystem II quinone acceptor complex, and the pathway for deactivation, *FEBS Lett., 153* (1983) 221–226.

[41] S. Demeter and Govindjee, Thermoluminescence in plants, *Physiol. Plant., 75* (1989) 121–130.

[42] D.M. Kramer, R.A. Roffey, Govindjee and R.T. Sayre, The At thermoluminescence band from *Chlamydomonas reinhardtii* and the effects of metagenesis of histidine residues on the donor side of the photosystem II D1 polypeptide, *Biochim. Biophys. Acta, 1185* (1994) 228–237.

[43] H. Kless, W.F.J. Vermaas and M. Edelman, Photosystem II function and integrity in spite of drastic protein changes in a conserved region of the D2 protein, *Biochemistry, 31* (1992) 11065–11071.

[44] C. Vernotte, J.-M. Briantais, C. Astier and Govindjee, Differential effects of formate in single and double mutants of D1 in *Synechocystis* sp. PCC 6714, *Biochim. Biophys. Acta, 1229* (1995) 296–301.

[45] H. Kless, M. Oren-Shamir, S. Malkin, L. McIntosh and M. Edelman, The D-E region of the D1 protein is involved in multiple quinone and herbicide interactions in photosystem II, *Biochemistry, 33* (1994) 10501–10507.

[46] C. Xu, R. Li, Y. Shen and Govindjee, The sequential release of three extrinsic polypeptides in PSII particles by high concentrations of trichloroacetate, *Naturwissenschaften, 82* (1995) 477–478.

[47] C. Xu, Y. Zhu and Govindjee, Differential inhibition and rephasing of photosystem II electron acceptor side by monohalogenated acetates of different hydrophobicity, *Z. Naturforsch., 47c* (1992) 711–716.

[48] H.H. Robinson, J.J. Eaton-Rye, J.J.S. van Rensen and Govindjee, The effects of bicarbonate depletion and formate incubation on the kinetics of oxidation-reduction reactions of the photosystem II quinone acceptor complex, *Z. Naturforsch., 39c* (1984) 382–385.

[49] D.J. Blubaugh and Govindjee, Bicarbonate, not CO_2, is the species required for the stimulation of photosystem II electron transport, *Biochim. Biophys. Acta, 848* (1986) 147–151.

[50] J. Cao, W.F.J. Vermaas and Govindjee, Arginine residues in the D2 polypeptide may stabilize bicarbonate binding in photosystem II of *Synechocystis* sp. PCC 6803, *Biochim. Biophys. Acta, 1059* (1991) 171–180.

[51] N.E. Good, Carbon dioxide and the Hill reaction, *Plant Physiol., 38* (1963) 298–304.

[52] J. Cao, N. Ohad, J. Hirschberg, J. Xiong and Govindjee, Binding affinity of bicarbonate and formate in herbicide-resistant D1 mutants of *Synechococcus* sp. PCC 7942, *Photosynth. Res., 34* (1992) 397–408.

[53] P. Mäenpää, T. Miranda, E. Tyystjärvi, T. Tyystjärvi, Govindjee, J.-M. Ducruet, A.-L. Etienne and D. Kirilovski, A mutation in the D-de loop of D1 modifies the stability of the $S_2Q_A^-$ and $S_2Q_B^-$ states in photosystem II, *Plant Physiol., 107* (1995) 187–197.

[54] Govindjee, P. Eggenberg, K. Pfister and R.J. Strasser, Chlorophyll *a* fluorescence yield decay in herbicide-resistant D1 mutants of *Chlamydomonas reinhardtii* and the formate effect, *Biochim. Biophys. Acta, 1101* (1992) 353–358.

[55] A.L. McClellan (ed.), *Tables of Experimental Dipole Moment*, W. H. Freeman, New York, 1963, pp. 52–56.

[56] C. Hansch and A. Leo, Appendix 2. Partition coefficients, in C. Hansch and A. Leo (eds.), *Substituent Constants for Correlation Analysis in Chemistry and Biology*, John Wiley, New York, 1979, pp. 174–176.

[57] C. Hansch, Theoretical considerations of the structure-activity relationship in photosynthesis inhibitors, in H. Metzner (ed.), *Progress in Photosynthesis Research*, Vol. III, Metzner, Tübingen, 1969, pp. 1685–1692.

[58] B.F. Anderson, H.M. Baker, G.E Norris, D.W. Rice and E.N. Baker, Structure of human lactoferrin: crystallographic structure analysis and refinement at 2.8 Å resolution, *J. Mol. Biol., 209* (1989) 711–734.

[59] B.A. Diner and V. Petrouleas, Formation by NO of nitrosyl adducts of redox components of the photosystem II reaction center. II. Evidence that HCO_3^-/CO_2 binds to the acceptor-side non-heme iron, *Biochim. Biophys. Acta, 1015* (1990) 141–149.

[60] B.J. Hallahan, S.V. Ruffle, S.J. Bowden and J.H.A. Nugent, Identification and characterization of EPR signals involving Q_B semiquinone in plant photosystem II, *Biochim. Biophys. Acta, 1059* (1991) 181–188.

[61] B.K. Semin, E.R. Loviagina, A.Yu. Aleksandrov, Yu.N. Kaurov and A.A. Novakova, Effect of formate on Mössbauer parameters of the non-heme iron of PSII particles of cyanobacteria, *FEBS Lett., 270* (1990) 184–186.

[62] X. Wang, J. Cao, P. Maróti, H.U. Stilz, U. Finkele, C. Lauterwasse, W. Zinth, D. Oesterhelt, Govindjee and C.A. Wraight, Is bicarbonate in photosystem II the equivalent of the glutamate ligand to the iron atom in bacterial reaction centers?, *Biochim. Biophys. Acta, 1100* (1992) 1–8.