Differential Inhibition and Rephasing of Photosystem II Electron Acceptor Side by Monohalogenated Acetates of Different Hydrophobicity

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We demonstrate here that monohalogenated acetates (MFA, monofluoroacetate; MCA, monochloroacetate; MBA, monobromoacetate) are unique probes of the electron acceptor side of the photosystem II (PS II) reaction center: (1) they differentially inhibit the reoxidation of the reduced primary plastoquinone electron acceptor, Q_A^-, by the secondary plastoquinone electron acceptor Q_B, and increase the equilibrium $[Q_A^-]$ in the order: MBA $\geq$ MCA > MFA; and (2) MCA and MBA rephase the PS II electron acceptor side, a rather unusual effect. This results in flash number dependence of $[Q_A^-]$ with maxima at even flashes to change to odd flashes. Furthermore, we demonstrate a correlation between the inhibitory activity of the halogenated acetates with their hydrophobicity (*i.e.*, partition coefficient).

Introduction

The primary charge separation in the photosystem II (PS II) reaction center occurs at the D1/D2/cytochrome (Cyt)*b*-559 core complex of green plants and cyanobacteria [1] and eventually leads to the oxidation of water and the reduction of the plastoquinone pool. Electron transfer on the acceptor side proceeds through a two-electron gate mechanism [2, 3]. After light-induced formation of the primary radical pair $P680^+Pheo^-$ within 3 ps, the charge separation is stabilized by electron transfer from $Pheo^-$ to Q_A (the primary plastoquinone electron acceptor), and then from Q_A^- to Q_B (the secondary plastoquinone electron acceptor) [4]. The negative charge on the quinone is suggested to induce a pK shift of nearby amino acids, resulting in the protonation of PS II and the stabilization of the charge on Q_B^- (see *e.g.* [5]). After a second photoact, another electron transfers to Q_B^-, and two protons bind to Q_B^{2-} to form plastoquinol (PQH_2), which is replaced by a plastoquinone (PQ) from the membrane PQ pool. PS II-directed herbicides act by displacing Q_B in the D1 protein to block the PS II electron flow [6, 7].

In contrast to photosynthetic bacteria, PS II uniquely performs oxidation of water to molecular oxygen, and exhibits a bicarbonate-reversible formate/NO/acetate inhibition of the electron transfer from the Q_A^- to the quinone pool (see reviews [8–11]). Although different herbicides inhibit differentially the electron flow from Q_A^- to the quinone pool in photosynthetic bacteria and PS II [12, 13], yet they do so by a common mechanism of displacing Q_B (see *e.g.* [5]). Herbicide effects, unlike the formate effects, cannot be reversed by bicarbonate ions. In the presence of herbicides, the affinity of the thylakoid membrane for bicarbonate is decreased [14] (*cf.* [15]).

It has become obvious that bicarbonate-reversible or irreversible inhibition at the Q_A-Fe-Q_B region is a powerful probe to understand the molecular structure and function relationship of this region in the D1/D2 protein of the reaction center II [9, 10, 12]. With this aim, we have investigated the importance of a halogen substitution in acetate for the mechanism of inhibition of Q_A^- reoxidation and equilibration by using monobromo- (MBA; K_d, 2.7–2.9), monochloro- (MCA; K_d,

Abbreviations: Chl, chlorophyll; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; K_d, dissociation constant; MBA, monobromoacetate; MCA, monochloroacetate; MES, 2-[N-morpholino]-ethanesulfonic acid; MFA, monofluoroacetate; π, hydrophobicity; P, partition coefficient; Pheo, pheophytin; PQ(PQH_2), plastoquinone (plastoquinol); PS II, photosystem II; Q_A, one-electron acceptor-bound quinone; Q_B, two-electron acceptor-bound quinone.

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2.8–2.9), and monofluoro- (MFA; K_d, 2.6–2.7) acetates, which differ in their molecular geometry and hydrophobicity. The inhibitory activity was correlated with the log of the partition coefficient (P) and, thus, with the hydrophobic conatant (π), but not with the overall dipole moment of the inhibitors. Furthermore, MCA and MBA induce a novel effect of rephasing of the two electron gate of PS II.

Materials and Methods

Preparation of thylakoids

Spinach *(Spinacia oleracea)* thylakoids were isolated as in ref. [16]. Thylakoids, suspended in 0.4 M sorbitol, 15 mM NaCl, 5 mM $MgCl_2$ and 20 mM HEPES (pH 7.8), were frozen rapidly in small aliquots, and stored at 77 K until use. Chlorophyll (Chl) concentration was spectrophotometrically determined in 80% acetone (v/v) extracts of thylakoids [17]. Thylakoids, thawed immediately before use, were suspended in 0.4 M sorbitol, 50 mM NaCl, 2 mM $MgCl_2$, 40 μM hydroquinone, 40 μM benzoquinone and 1 nM gramicidin to a final [Chl] of 10 μM [16]; pH of the suspension was adjusted by using 20 mM MES (at pH 6.0 and 6.5) or 20 mM HEPES (at pH 7.5), or as specified under Results and Discussion.

Measurement and analysis of the $[Q_A^-]$ decay

Chl *a* fluorescence yields after single turnover-saturating flashes (EG & G FX-124 flash lamp, 2.5 μs duration) were measured as in ref. [16]. Using weak measuring flashes (exciting only 1% of reaction centers), the F_0 level and the decay of the variable (F_v) 685 (±10) nm fluorescence were measured with a S-20 (EMI 9558) photomultiplier. The dark interval time between actinic flashes was 1 s. When halogenated acetates (preadjusted to appropriate pH) were added, a 10 min dark incubation time was given before measurements were begun. By assuming that the probability of the intersystem energy transfer is 0.5 [19], $[Q_A^-]$ was calculated from F_v [18]. The fitting of $[Q_A^-]$ decay data into three exponential decays (see *e.g.* [20]) was carried out by the GLOBALS UNLIMITED™ global analysis software [21]. $[Q_A^-]$ is given in relative units with 1 being $[Q_A^-]_{max}$ obtained in the presence of 6 μM DCMU. Thus, the analyzed amplitudes (A's) need not add to 1 in this analysis.

Calculation of the molecular geometry, the dipole moment and the hydrophobic constant

Molecular geometries and dipole moments of (halogenated) acetic acids were calculated by using the MMX molecular mechanics (forcefield) calculation method with the PCMODEL molecular modeling program [22]. Calculation was made in an apolar solvent with a dielectric constant of 1.5 to minimize the energy of the molecular model and to get an optimal geometry of the model. The hydrophobic constants (π) for acetic acid and monohalogenated acetic acids were estimated according to Hansch [23]: $\pi = \log P_x - \log P_H$, where P_x is the partition coefficient [24] of a halogenated derivative of acetic acid and P_H that of the parent molecule, acetic acid in this study. The π for acetic acid (x = H) is defined as zero.

Results and Discussion

Halogenated acetates differentially inhibit the reoxidation of Q_A^- by Q_B as well as increase the equilibrium $[Q_A^-]$

Fig. 1 shows the concentration dependence of the change of variable Chl *a* fluorescence yield, measured at 3 ms after the first actinic flash, induced by the addition of monofluoroacetate (MFA), monochloroacetate (MCA) or monobromoacetate (MBA). These halogenated acetates

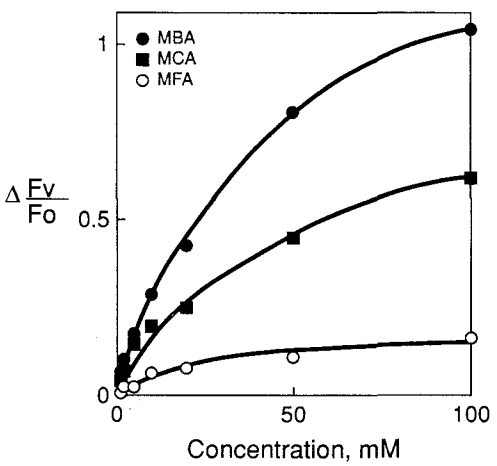

Fig. 1. Dependence of the change of variable Chl *a* fluorescence field, measured at 3 ms after the first actinic flash, as a function of concentration of monohalogenated acetates in spinach thylakoids at pH 6.0. See text for details.

induce differential increases in variable Chl *a* fluorescence, indicating differential slowing down of the Q_A^- reoxidation and/or increases in equilibrium $[Q_A^-]$ in the order MBA > MCA > MFA. Although this hierarchy can be monitored easily down to 10 mM, higher concentration (100 mM) of the chemical was chosen to further evaluate the effects because it showed the largest effect.

Fig. 2 shows $[Q_A^-]$ decays in control, 100 mM MFA-, MCA- or MBA-treated thylakoids at pH 7.5 (left panels) and 6.0 (right panels) after flash 1 (upper panels) or 2 (lower panels). The additions of the various monohalogenated acetates cause both a slowing down of the $[Q_A^-]$ decay, reflecting an inhibition of the Q_A^- oxidation, as well as an increase in equilibrium $[Q_A^-]$. All inhibitions are much stronger at pH 6.0 (right panels) than at pH 7.5 (left panels). Although it had been suggested, based on the increased inhibitory effect at low pH, that formic acid (in the micromolar range) rather than formate (in the micromolar range) is the bicarbonate-reversible inhibitory species [18], yet alternative explanation in which the reaction center protein binds increased anion at the low pH cannot be excluded. Thus, the nature of the inhibitory species remains an open question.

The time dependence of $[Q_A^-]$ after an actinic flash [20, 25, 26] is described by three major (fast, intermediate and slow) exponential processes. We analyze here results with flash 1 at pH 6.0 (Table I); it monitors mainly the $Q_A^-Q_B \leftrightarrow Q_AQ_B^-$ reaction. Both τ_1, the oxidation lifetime of Q_A^- by Q_B, and τ_2, the lifetime related to $[Q_A^-]$ equilibrium show the hierarchy: MBA $\gtrsim$ MCA > MFA. Furthermore, the ratio of the amplitudes of the slow components (A_2 + A_3) to the fast component (A_1), reflecting the equilibrium $[Q_A^-]$ in the $Q_A^-Q_B \leftrightarrow Q_AQ_B^-$ reaction, increases with MFA, MCA and MBA present. All the effects were larger after the first than after the second flash (Fig. 2). In con-

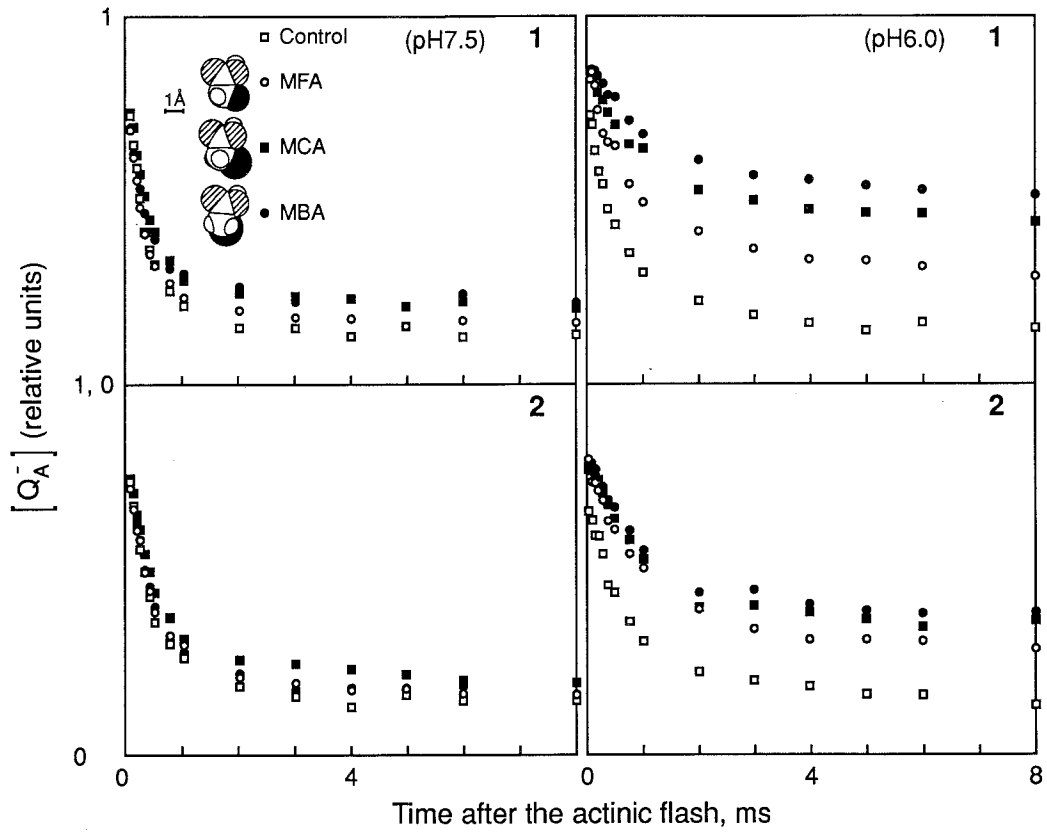

Fig. 2. $[Q_A^-]$ decays in control and 100 mM monohalogenated acetate-treated spinach thylakoids after flash 1 and 2. See text for details.

Table I. Amplitudes (A) and lifetimes (τ) of three components of the $[Q_A^-]$ decay, in control and 100 mM halogenated acetate-treated spinach thylakoids at pH 6.0 after flash 1. Amplitudes were within ± 0.05, τ_1 within ± 50 μs and τ_2 within ± 2 ms for control samples and 10–20 ms for others. τ_3 (for all samples) was 2.5 ± 0.4 s.

	A_1	τ_1 [μs]	A_2	τ_2 [ms]	A_3	$\frac{A_2 + A_3}{A_1}$	χ^2
Control	0.55	510	0.13	5	0.11	0.44	0.79
+MFA	0.50	760	0.22	20	0.16	0.76	0.44
+MCA	0.39	1200	0.29	87	0.17	1.2	0.40
+MBA	0.34	1700	0.34	104	0.18	1.5	0.44

trast to the effect of formate, that is higher only after the second and subsequent flashes [18], reflecting inhibition of protonation of Q_B^- (Q_B^{2-}), monohalogenated acetates clearly slow down electron transfer from Q_A^- to Q_B (or Q_B^-).

Furthermore, and in contrast to formate, the inhibition of the PS II reactions in the Q_AQ_B complex by the halogenated acetates was only partially (about 50%) reversed by 5 mM bicarbonate. Thus, these chemicals act in an intermediate manner to the herbicides and formate. The inhibitory hierarchy among the various monohalogenated acetates are expected to be related to the differences in their molecular geometry (see insert in Fig. 1). A key property that can affect the observed behaviour is their partition coefficient P, and, thus, their hydrophobicity. Table II shows a general correlation of log P with the inhibitory activity of the monohalogenated acetates, as monitored, *e.g.*, by the percent change in $[Q_A^-]$ at 3 ms after the 1st flash: MBA, that shows the largest effect on $[Q_A^-]$, is the largest in size and has the largest partition coefficient P and, thus, the largest hydrophobic constant (π). This is followed by MCA and MFA. No correlation with K_d or the overall dipole moment of the molecules was observed. This, however, does not mean that a correlation may not exist with the dipole moment of the head groups. Oxamic acid, that has a similar molecular weight as MCA but a lower dipole moment (MCA: 3.25; oxamic acid: 2.84), showed half-as-much effect than that observed after MCA treatment. This suggests the possibility that dipole moment in a certain geometry may also modulate the inhibitory effect on Q_A^- oxidation and equilibration.

MCA rephases the PS II acceptor side

The flash number dependence of $[Q_A^-]$ in control thylakoids (Fig. 3A) shows a binary oscillation with peaks at even flashes (2, 4 and 6). This pattern is due to the oxidation rate of Q_A^- by Q_B after odd flashes to be faster than that by Q_B^- after even numbered flashes [26]. Interestingly, we observed here that this binary oscillation of $[Q_A^-]$ is rephased by MCA, higher $[Q_A^-]$ being observed after odd than even flashes (Fig. 3B and 3C). The largest difference between $[Q_A^-]$'s after flashes 1 and 2, is seen at 3 ms. It is much larger than that measured at 300 μs. The addition of 5 mM bicarbonate does not reverse this rephasing effect. Furthermore, addition of 20 mM bicarbonate to samples, that were buffered with 200 mM MES, also was ineffective in reversing the rephasing by MCA. The above effect of MCA may imply that it modifies the ratio of Q_B to Q_B^- in darkness prior to

Table II. A comparison of the properties and effects of monohalogenated acetates.

	K_d	P	Log P	π	Dipole moment	$[Q_A^-]$ 3 ms	Bicarbonate reversibility
MBA	2.7	4.4	0.64	0.97	3.11	41 (242%)	~50%
MCA	2.8	2.09	0.32	0.65	3.25	35 (192%)	~50%
MFA	2.6	0.54	−0.27	0.06	3.14	25 (108%)	–
Control	–	–	–	–	–	12 –	–

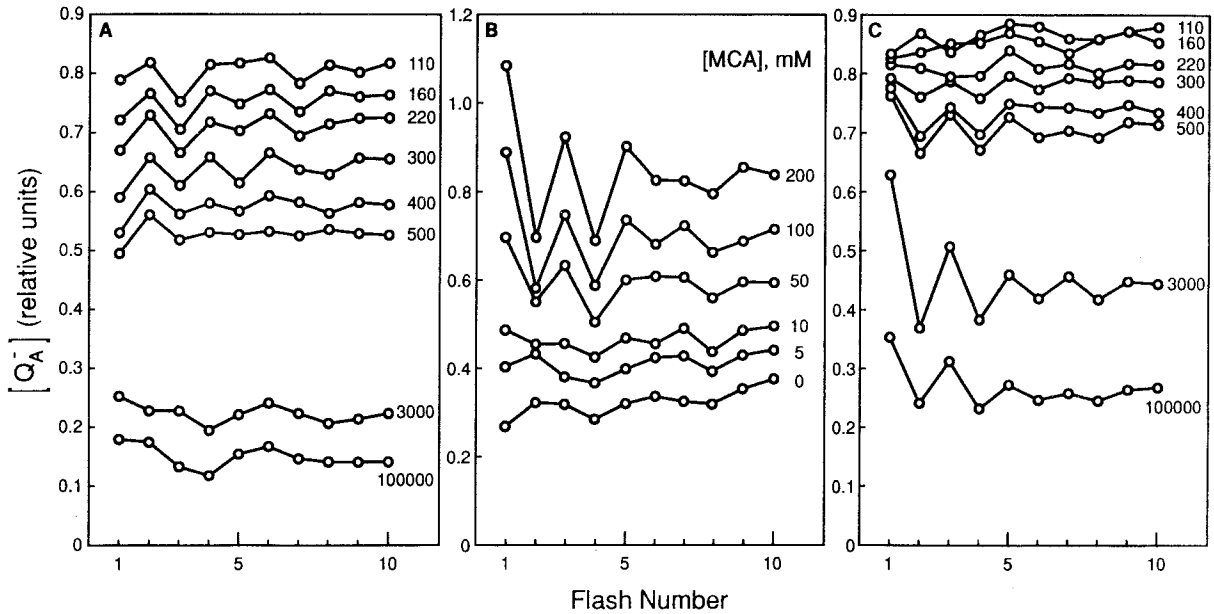

Fig. 3. Flash number dependence of the variable Chl. *a* fluorescence yield (F_v/F_o) in control (A), MCA-treated (different concentrations; measured at 3 ms after flash) (B), and 100 mM MCA-treated spinach thylakoids at pH 6 (C). The numbers in panels A and C indicate the measuring time in microseconds after the actinic flashes, but those in panel B indicate [MCA]. In A and C, F_v/F_o has been converted to $[Q_A^-]$. See text for details.

actinic flashes, the Q_B/Q_B^- ratio being normally high in dark-adapted thylakoids (see *e.g.* Wollman [27]). And, this effect is not reversed by bicarbonate although the latter partially reverses the slowing down of the reoxidation of Q_A^-.

A change in the equilibrium of $Q_A^-Q_B \leftrightarrow Q_AQ_B^-$ cannot easily explain the MCA-induced rephasing of the flash number dependence observed here since (a) trichloroacetate, that drastically changes the equilibrium of the above reaction, did not show any rephasing effect [28]; and (b) the binary oscillation persisted up to 10 flashes in the 0.3 to 100 ms range; a changed equilibrium would have dampened this periodicity.

MBA behaved in an identical manner to MCA as far as the rephasing effect on the two electron gate is concerned. Its mechanism remains to be established.

In conclusion, we have established here that monohalogenated acetates inhibit the reoxidation of Q_A^- and increase the equilibrium $[Q_A^-]$ at the plastoquinone reductase site in PS II with the hierarchy of effectiveness that follows the order: MBA $\gtrsim$ MCA > MFA. This order is related to partition coefficient of the halogenated acetates and, thus, to their hydrophobicity. This confirms the relationship observed earlier between the inhibitory activity of various herbicides and their partition coefficients (see *e.g.*, Oettmeier [12] and Bowyer *et al.* [29]). A novel observation is, however, the rephasing of the flash number dependence of $[Q_A^-]$ by HCA (and HBA). The mechanism of such an effect is not yet obvious, but we may speculate on one possibility: this could be the repulsion of the negative charge on Q_B^- by the negatively polarized chlorine atom of MCA, followed by its movement towards a positively charged niche and consequent stabilization. One known case of chemically induced rephasing of the binary oscillation on the electron acceptor side of PS II is that by 0.1 mM phenyl-*p*-benzoquinone (see Diner and Petrouleas [30]). Here, however, the mechanism is quite different: after the first flash, the extrinsic quinone is reduced to semiquinone which extracts an electron from the non-heme iron Fe^{2+} (between Q_A and Q_B) becoming doubly reduced; and after the second flash, Q_A^- delivers its electron rapidly to the oxidized Fe^{3+} leading to a faster $[Q_A^-]$ decay after even than after odd flashes. This mechanism is not considered feasible for the halo-

genated acetates that do not act as electron acceptors.

We note that the monohalogenated acetates act differently than the herbicides as (a) they do not abolish the binary oscillations, *i.e.*, they may not act by displacing Q_B and (b) their effects are partially reversed by bicarbonate. Thus, they are unique in the sense that they are between the bicarbonate-reversible formate and bicarbonate-irreversible herbicides.

Note added in proof.

At the time of correcting the proof, the authors became aware of two other cases where chemically-induced rephasing of PS II acceptor side had been earlier observed: 1) Lavergne [31] observed that reduced 2,5-dibromo-3-methyl-6-isopropyl-benzoquinone (DBMIB) inhibited the oxidation of Q_A^- more effectively after the first than after the second flash; thus, the variable Chl *a* fluorescence was higher after the first than after the second flash, as observed with MCA in this paper; 2) TaoKa *et al.* [32] also observed a similar differential effect after flash 1 and 2 upon the addition of 100 nM 3-undecyl-2-hydroxyl-1,4-naphtoquinone (UHNQ), but the flash number dependence was not shown. In our case, an explanation alternative to an increase in the intrinsic Q_B^-/Q_B ratio, must also be considered: perhaps, an equilibrium between MCA and Q_B could lead to a slowed electron flow after the 1st flash, but not after the 2nd flash as Q_B^- cannot be displaced by MCA.

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