Kinetics of Electron Transfer between Q_A and Q_B in Wild Type and Herbicide-Resistant Mutants of *Chlamydomonas reinhardtii*

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We have investigated the electron transfer kinetics for reduction of plastoquinone by photosystem II in six mutant strains of *Chlamydomonas reinhardtii* by following the decay of the high fluorescence state after flash activation, and compared the separate reactions of the two-electron gate with those of a wild type strain. By analysis of the electron transfer kinetics, and separate measurement of the equilibrium constant for stabilization of the bound semiquinone after one flash, we have been able to deconvolute the contributions of rate constants and equilibrium constants for plastoquinone binding and electron transfer to the overall process. Two mutations, S 264 A and A 251 V, led to a marked slowing of kinetics for reduction of plastoquinone to the bound semiquinone. In S 264 A, the second electron transfer was also slower, but was normal in A 251 V. In mutant G 256 D, the electron transfer kinetics were normal after the first flash, but slowed after the second. In mutants L 257 F, V 219 I, and F 255 Y, the electron transfer kinetics after both flashes were similar to those in wild type. We discuss the results in terms of a model which provides a description of the mechanism of the two-electron gate in terms of measured kinetic and equilibrium constants, and we give values for these parameters in all strains tested.

Introduction

Eight different amino acid substitutions that confer resistance to a variety of PS II herbicides have been identified to date. All of them are clustered between residues 211 and 271 of the D 1 subunit of PS II. The most frequent mutations, which markedly increase tolerance towards the herbicides atrazine and diuron, are localized at serine-264. In tolerant mutants of higher plants Ser-264 is replaced by glycine, whereas in the green alga *Chlamydomonas reinhardtii* and cyanobacteria the common substitution is by alanine. This repeated loss of Ser-264 is highly significant, indicating that it might play an important role in herbicide binding. Trebst [1] and Bowyer *et al.* [2] suggested that Ser-264 contributes to the binding of inhibitors of the urea-atrazine family to the Q_B site by hydrogen bonding of its side chain hydroxyl group to the NH group in the herbicide molecule, in analogy with the observed H-bonding of the herbicide terbutryne to Ser-223 in the L subunit of bacterial reaction centers. Tietjen *et al.* [3] proposed that Ser-264 would stabilize the structure of the Q_B site by H-bonding to His-252. Crofts *et al.* had previously suggested that this histidine residue could also be involved in binding of the proton that stabilizes the Q_B^- semiquinone in the pocket [4].

Bowes *et al.* [5], on the basis of kinetic measured in multiflash experiments, suggested that the amino acid substitution S 264 G in the higher plant *Amaranthus hybridus* affected the reactions of the 2-electron gate by decreasing the rate of electron transfer from Q_A^- to Q_B by an order of magnitude. The work by Ort *et al.* [6] supported the idea that the mutation led to a lower apparent equilibrium constant (K_{app}) for the reaction $Q_A^-Q_B \leftrightarrow Q_AQ_B^-$, which resulted in a higher quasi-steady state level of Q_A^-. Taoka and Crofts [7] measured a decrease in K_{app} from direct measurements of the back-reaction, and showed that this was due mainly to a much higher dissociation constant for quinone from the Q_B site in the mutant. The forward rate constant for electron transfer was 2–3 times larger in the mutant strain, but the effect of this parameter in increasing the value of K_{app} was offset by the larger effect of the quinone dissociation constant in decreasing the value. The intrinsic equilibrium constant for the sharing of an electron between $Q_A^-Q_B$ and $Q_AQ_B^-$ was of similar magnitude in mutant and wild type.

In the S 264 A mutant of *Chlamydomonas reinhardtii*, an almost unchanged equilibrium concen-

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tration of Q_A^- was observed by fluorescence, oxygen emission and thermoluminescence methods [8]. Erickson *et al.* [9] studied the effect on the PS II acceptor side of the substitutions S264A, V219I, F255Y, G256D and L275F. Only mutants S264A and G256D showed a marked decrease in the rate of Q_A^- reoxidation, measured by following the decay of fluorescence yield directly on agar plates. These results were supported by those of Govindjee *et al.* [10]. However, care should be taken in analyzing these data, since the dark redox state of the PS II quinone acceptors was not determined prior to the experiments. In dark adapted algal cells, about 50% of the PS II reaction centers have a single reduced Q_B^-, and this would lead to the observation, after one actinic flash, of the convolution of the kinetic data from two reactions: $Q_A^-Q_B \leftrightarrow Q_AQ_B^-$ and $Q_A^-Q_B^- \leftrightarrow Q_AQ_B^{2-}$, which have different half times [11]. Thus, if the initial redox state of the system is unknown, no reliable conclusions can be drawn from the experimentally measured kinetics about the discrete kinetic parameters of the individual reactions.

In the present work, wild type *C. reinhardtii* and herbicide-resistant mutant strains with substitutions in D1 of S264A, V219I, F255Y, G256D, L275F and A251V, have been studied under conditions in which the photosystem II quinone acceptors were oxidized before the experiments by treatment with benzoquinone. From the deconvolution of kinetic traces of Chl *a* fluorescence decay and the measurement of the apparent equilibrium constant for electron transfer between Q_A and Q_B, values for the intrinsic equilibrium constant, the dissociation constant of quinone from the Q_B binding site, the forward and reverse rate constants for the electron transfer between the two plastoquinone acceptors, and the on- and off-rate constants for binding of plastoquinone were calculated for wild type and resistant strains. The mutants that most significantly differed from wild type were S264A and A251V, whereas the other strains presented more minor differences.

Methods

Benzoquinone treatment of intact cells

Commercially available *p*-benzoquinone was recrystallized by sublimation. Cell suspensions containing 5–10 μM chlorophyll in the growth medium were incubated at room temperature in the presence of 100 μM benzoquinone for 5 min and then centrifuged for 5 min at 3000 × *g*. The pellet of treated cells was resuspended in benzoquinone-free medium containing 0.1 M sorbitol, 10 mM KCl, 10 mM $MgCl_2$, 100 μM NH_4Cl as uncoupler and 20 mM Hepes, pH 7.0. The treated cells were kept in complete darkness during the treatment and until the experiments were performed, since light caused an irreversible quenching of fluorescence.

Kinetics of electron transfer

Experimental methods were essentially as described by Robinson and Crofts [4, 11, 12], except that the fluorescence photometer used was an improved version. The new apparatus was equipped with three measuring flash lamps, allowing measurement of three time points in the sub-ms range in each experiment. In addition, by using a common light guide for actinic and measuring flashes, and a common reference channel for correction of variations in flash intensity, the signal to noise ratio was improved, and correction for the overlapping contribution of the actinic flash to fluorescence allowed measurement closer to the actinic flash. Cells at approx. 5 μM Chl were suspended in 0.1 M sorbitol, 10 mM KCl, 10 mM $MgCl_2$, 100 μM NH_4Cl as uncoupler and 20 mM Hepes, pH 7.0, with other additions as indicated. Other details are given in Figure legends and the text.

Experimental rationale

The equilibrium and rate constants for the forward and reverse electron transfer and for binding and dissociation of plastoquinone at the Q_B site were calculated using the following assumptions [4, 7, 11, 12]:

i) The ratio of amplitudes of fast and slow components of the rapid biphasic decay kinetics of Q_A^- following an actinic flash was assumed to reflect the ratio of the initial fraction of $Q_A \cdot Q_B$ to $Q_A \cdot$ [vacant] centers.

ii) The dissociation constant of plastoquinone from the Q_B site in the dark was assumed to remain unchanged after an actinic flash.

iii) Binding of plastoquinone to the Q_B site was assumed to follow a pseudo-first order reaction kinetics when the pool was initially oxidized (ten-

fold excess of oxidized plastoquinone over reaction center).

The two following equations are solutions of the rate equations derived from the model

$$r_1 + r_2 = k_{on}(1 + A_o/B_o) + k_{BA}\{1 + K_{app}(1 + A_o/B_o)\} \quad (1)$$

$$r_1 \cdot r_2 = k_{on} \cdot k_{BA}(1 + A_o/B_o)(1 + K_{app}). \quad (2)$$

Variables r_1 and r_2 are the slow and fast rate constants obtained directly from the experimental data by fitting the kinetic traces of fluorescence decay after one actinic flash to two exponential components, and a residual; A_o and B_o are the amplitudes of the slow and fast component, respectively, of the fitted kinetic traces, also obtained from the two-exponential fit. K_Q, the plastoquinone dissociation constant, is calculated as the ratio A_o/B_o (assumption i) above).

K_{app} is the apparent equilibrium constant for sharing an electron between Q_A and Q_B, defined (if protonated states are included) as:

$$K_{app} = \frac{[Q_AQ_B^-] + [Q_AQ_B^-(H^+)]}{[Q_A^-] + [Q_A^-(H^+)] + [Q_A^-Q_B] + [Q_A^-Q_B(H^+)]}.$$

For simplicity, we can ignore the effects of protonation, and treat K_{app} at any fixed pH as determined by the equilibrium constants for electron transfer, and the dissociation of Q from the site:

$$K_{app} = \frac{[Q_AQ_B^-]}{[Q_A^-] + [Q_A^-Q_B]} = K_E/(1 + K_Q). \quad (3)$$

Again, for simplicity we have ignored the contribution of the quinone pool, so that

$$K_Q = Q_A^-/Q_A^- \cdot Q_B = A_o/B_o = k_{off}/k_{on}.$$

Also,

$$K_E = Q_A \cdot Q_B^-/Q_A^- \cdot Q_B = k_{AB}/k_{BA}$$

so that

$$K_{app} = k_{AB}/k_{BA} \cdot (1 + A_o/B_o)^{-1}. \quad (3')$$

The apparent equilibrium constant, K_{app}, can be determined experimentally from the ratio of the half-time of the back reaction $S_2PQ_AQ_B^- \rightarrow S_1PQ_AQ_B$ to that of the back reaction $S_2PQ_A^- \rightarrow S_1PQ_A$. The measured parameters therefore allow us to determine the rate constants and equilibrium constants which define the two-electron gate mechanism. By doing the experiment at different pH values, we can also determine the pK values which describe the role of protons in the mechanism, but this more extensive data set has so far been collected only in *Amaranthus hybridus* and its S264G mutant [7].

Results and Discussion

In order to allow an analysis of the separate reactions of the two-electron gate, it was necessary to oxidize the quinone pool, and primary and secondary bound quinone acceptors, as described above. Representative kinetic curves for electron transfer through the two separate reactions leading to production of QH_2 are shown in Fig. 1 (first flash) and 2 (second flash). Parameters for the kinetics after the first flash, measured from the corrected fluorescence decays, are summarized in Table I. The rate of the back reaction from $S_2PQ_AQ_B^- \rightarrow S_1PQ_AQ_B$ was measured from the rephasing of the binary oscillation, as suggested by Robinson and Crofts [11, 12]. The results for wild type and several mutant strains are shown in Fig. 3. The kinetic parameters and equilibrium constants derived from these measurements are summarized in Table II.

The kinetic parameters obtained here for wild type *C. reinhardtii* are in agreement with those reported for pea chloroplasts [11, 12] and for *Amaranthus hybridus* [7]. If the concentration of plastoquinone in the membrane is assumed to be 5 mM [4], then the true dissociation constant for plastoquinone from the Q_B site, $K_Q' = K_Q \cdot [PQ]$, calculated from this work for wild type *C. reinhardtii* is 2 mM, which is in the range calculated by Crofts *et al.* [4].

Among the amino acid substitutions covered by this study, two clear groups can be established:

1) Mutations that confer herbicide resistance to different extents but that do not affect markedly the electron transfer between the plastoquinone acceptors of PS II. These mutations are V219I, F255Y, L275F and G256D (Table II, and see Fig. 1 and 2). It is important to note that, in the case of G256D, a slower electron transfer rate has been reported previously by two different groups [9, 10]. In both cases, the decay of fluorescence was measured with repeated illumination of the samples and short dark periods between actinic flashes,

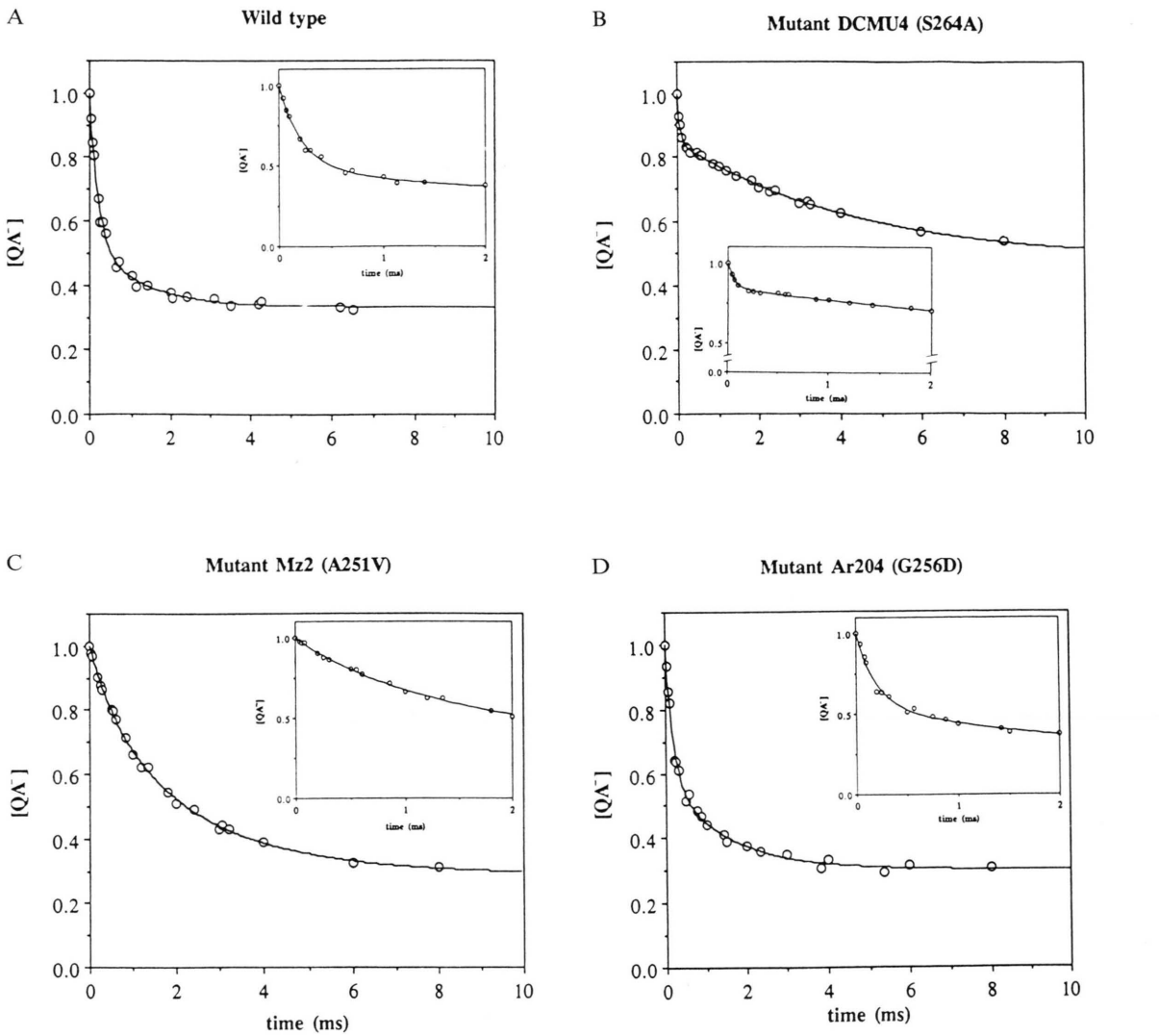

Fig. 1. Kinetics of $[Q_A^-]$ decay following the first actinic flash in dark adapted, benzoquinone treated *C. reinhardtii* cells. A: Wild type; B: S264A (strain DCMU4); C: A251V (strain Mz2); D: G256D (strain Ar204). Other strains showed kinetics similar to wild type. Insets: Same data shown in a 2 ms time scale to show the fast component. In all the traces shown, circles represent actual data and solid lines are the best non-linear squares fit of the data using the equation in Table I. Parameters obtained from the fits are given in Table I. See "Experimental rationale" for details.

which leads to a partial and uncontrolled reduction of the plastoquinone pool. In our own experiments, the kinetics after the second flash (or after scrambling the two-electron gate) are about a factor of two slower than in wild type (Fig. 2). The substitution of Gly-256 by aspartate introduces a negative charge in the Q_B region, and it could be expected that this would seriously affected the ionic environment and possibly the proton binding properties of the Q_B binding site. However, in our Q_B site model, Gly-256 is positioned facing the stromal aqueous phase rather than making contact with the plastoquinone. It should also be noted that V219I shows a significantly slower electron transfer after the second flash than wild type, and a more marked binary oscillation (not shown).

2) Mutations that confer herbicide resistance and considerably slow down the electron transfer

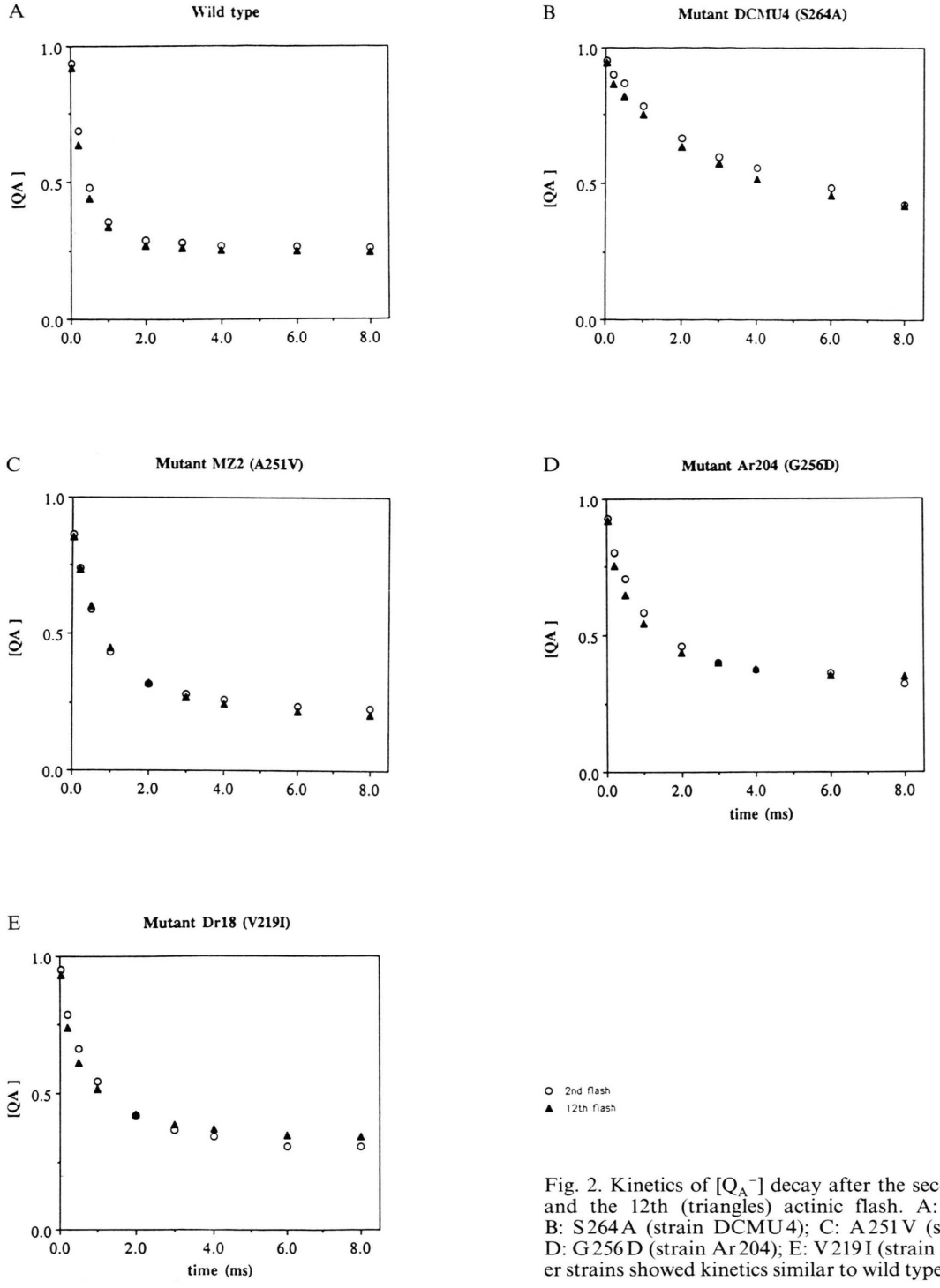

Fig. 2. Kinetics of $[Q_A^-]$ decay after the second (circles) and the 12th (triangles) actinic flash. A: Wild type; B: S264A (strain DCMU4); C: A251V (strain Mz2); D: G256D (strain Ar204); E: V219I (strain Dr18). Other strains showed kinetics similar to wild type.

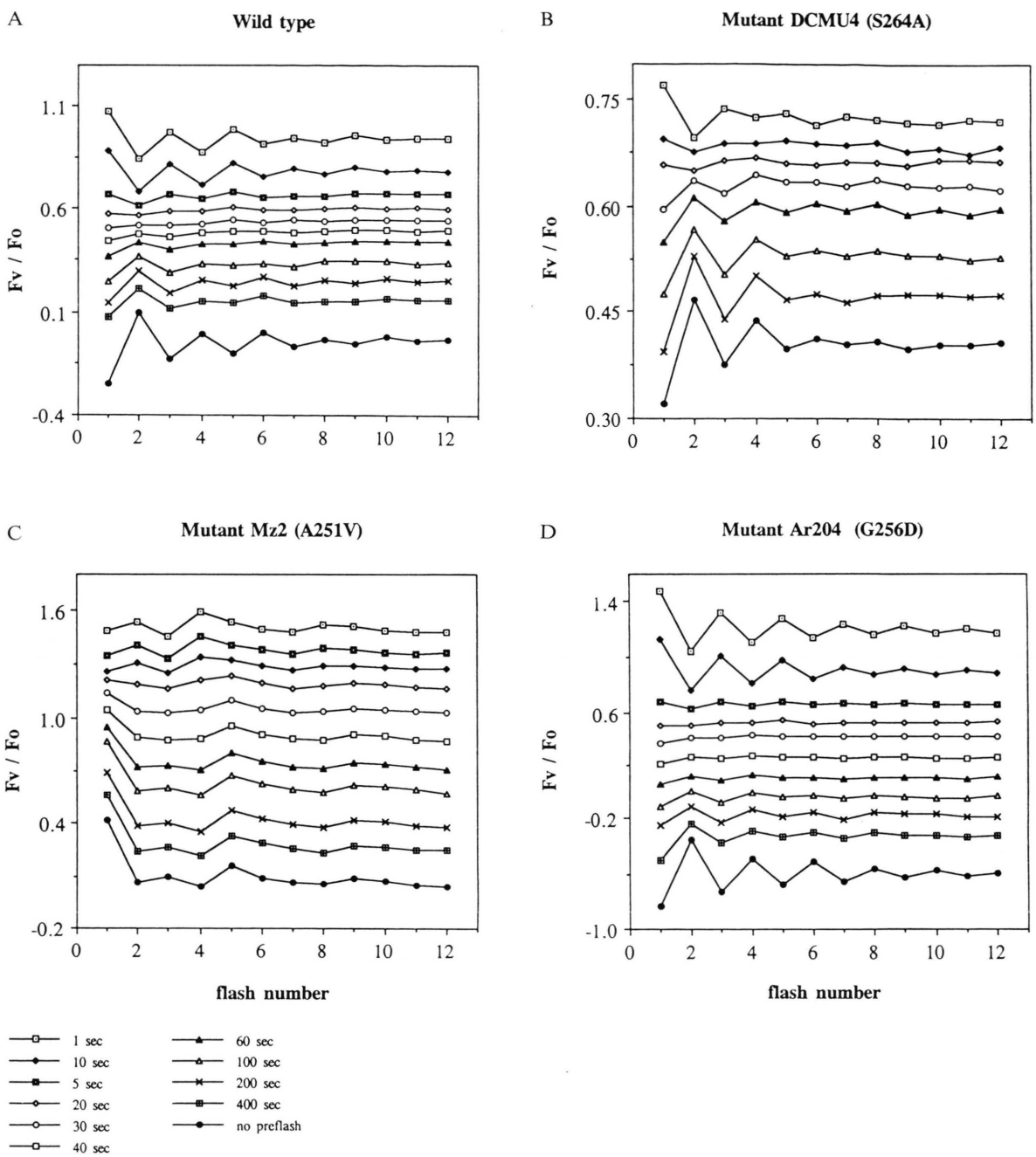

Fig. 3. Rephasing of the binary oscillations of the two-electron gate as a function of the dark time after a pre-illuminating flash. Binary oscillations of Q_A^- level, measured from normalized variable fluorescence, observed at 200 μs after each of a series of 12 actinic flashes fired at 1 Hz. Traces have been offset for clarity. The times between the preflash and the first actinic flash are shown above. A: Wild type; B: S264A (strain DCMU4); C: A251V (strain Mz2); D: G256D (strain Ar204). Other strains showed oscillations similar to wild type.

Table I. Kinetic data obtained from the deconvolution of fluorescence decay traces after correction for the non-linear relationship of $[Q_A^-]$ and the level of fluorescence [14]. Parameters shown in the table are the result of the non-linear last squares fit of the corrected fluorescence data with an equation of the form:

$$[Q_A^-] = A_o \exp(-r_1 t) + B_o \exp(-r_2 t) + 1 - (A_o + B_o),$$

where t is time, A_o and B_o are the amplitudes, and r_1 and r_2 the rate constants, of the slow and fast components, respectively. The slowest kinetic phase is considered constant in the milliseconds time scale ($t_{1/2} = 2-3$ sec, depending on strain) and is indicated here as a residual component ($1 - (A_o + B_o)$). Values t_1 and t_2 are half-times calculated directly from r_1 and r_2.

Strain	A_o (slow)	B_o (fast)	Residual $(1 - A_o - B_o)$	r_1 [ms^{-1}]	t_1 [ms]	r_2 [ms^{-1}]	t_2 [μs]
Wild type	0.19	0.47	0.34	0.82	0.85	5.05	137
S264A	0.37	0.15	0.48	0.24	2.89	16.3	42
G256D	0.28	0.42	0.30	0.67	1.03	5.51	126
V219I	0.18	0.56	0.26	0.58	1.19	6.74	102
A251V (2 comp)	0.52	0.19	0.29	0.42	1.65	1.51	459
A251V (1 comp)	0.67	–	0.33	0.65	1.06	–	–
F255Y	0.29	0.39	0.32	0.90	0.77	4.93	140

Table II. Parameters of the two-electron gate in wild type and herbicide-resistant mutants of *C. reinhardtii*. The half time of the back reaction from Q_A^- to S_2 in the presence of DCMU ($t_{1/2}(Q_A^-)$), was measured directly from the corrected fluorescence decay. The half time of the back reaction from Q_B^- to S_2 ($t_{1/2}(Q_B^-)$), was measured from the rephasing time of the binary oscillations of the two-electron gate (Fig. 3). The dissociation constant for plastoquinone from the Q_B site, $K_O = A_o/B_o$, is given without units, as a relative value for comparison between wild type and mutants. Multiplication of K_O by $[PQ_{pool}]$ (approx. 5 mM) would give the true dissociation constant in mM units.

Strain	$t_{1/2}(Q_A^-)$ [sec]	$t_{1/2}(Q_B^-)$ [sec]	K_O (*)	K_{app}	K_E	k_{AB} [ms^{-1}]	k_{BA} [ms^{-1}]	k_{off} [ms^{-1}]	k_{on} [ms^{-1}]
Wild type	2.7	25	0.40	8.3	11.6	4.29	0.37	0.34	0.86
S264A	2.4	20	2.50	7.3	25.6	15.0	0.56	0.60	0.24
G256D	2.7	25	0.67	8.3	13.8	4.66	0.34	0.47	0.70
V219I	2.0	40	0.32	19.0	25.1	6.25	0.25	0.19	0.60
A251V (2 comp)	3.0	15	2.47	4.0	13.9	n.d.	n.d.	n.d.	n.d.
F255Y	2.5	25	0.40	9.0	15.7	3.73	0.24	0.84	1.07
L275F	2.7	20	2.50	6.4	9.36	3.46	0.37	0.39	0.84

between the plastoquinone acceptors, as in the case of S264A and A251V. In S264A, only a slight change in the apparent equilibrium constant for electron transfer was observed (Table II), although a larger fraction of the fluorescence did not decay in the ms range (presumably because of a higher fraction of non-Q_B-centers). The slow electron transfer in this mutant can be ascribed to a high dissociation constant for plastoquinone rather than a change in K_{app}. The high value of K_O is associated with an increased fraction of centers in the state Q_A · [vacant] in the dark. This suggests that Ser-264, besides being involved in the binding of inhibitors, also plays an important role in plastoquinone binding. Also, both the on- and off-rate constants for binding and dissociation of plasto-

quinone are increased in this mutant (Table II). In centers in the Q_AQ_B state, however, the electron transfer reaction proceeds 3.5 times more rapidly than in wild type (Table II). Assuming structural homology with the bacterial reaction center, the loss of the Ser-264 hydroxyl group, shown to provide a H-bond to the bound quinone in *Rhodopseudomonas viridis* [13], might be expected to result in a weaker binding, or binding to an alternative site in these mutant strains. The more rapid rate for the bound quinone might reflect a shorter bonding distance to the N of His-215, which provides a H-bond to the other quinone =O group at the proximal end of the binding pocket, since reaction rates are strongly dependent on distance for electron transfer reactions. In effect, the mutations may "push" the plastoquinone molecules from the reaction center closer together, allowing for a faster electron transfer. Against this hypothesis is the fact that the electron transfer after the second flash is considerably slower than wild type (Fig. 2), though this might be explained by a rearrangement of the pocket on reduction to form the semiquinone. Within the above hypothetical framework, the difference in rate of the slow phase between S264A and S264G (compare ref. [7] and this work) might reflect a weaker binding of the quinone to the alternative H-bond donor at the distal end of the binding pocket in the former strain, due to steric hindrance introduced by a greater bulk of the alanine than the glycine side chain.

Mutant A251V had a smaller K_{app} than wild type and probably a decreased amount of non-Q_B centers, as indicated by the smaller residual fluorescence at long times after the first flash. The kinetics of electron transfer after one flash were considerably slower than that of the wild type or the other mutants (Fig. 1), but the kinetics after the second flash were normal (Fig. 2). In fact, the decay of $[Q_A^-]$ after the first flash could be deconvoluted with almost equal confidence into either two exponential components of half times 459 μs and 1.65 ms respectively, or only one exponential component of $t_{1/2} = 1.06$ ms (Table I). If the latter fit is taken, it would indicate that almost all the centers in A251V are in the $Q_A \cdot$ [vacant] state in the dark, and that K_O is correspondingly greater (and K_E also greater to keep K_{app} at the measured value) than shown in Table II. The substitution of Ala-251 by the more bulky valine residue would then lead to a much weaker binding of plastoquinone. Assuming the largest value of K_E found in the other strains ($K_E \cong 26$), K_O would be ~ 5.5 and, given the few points in each kinetic trace, we would be unable to deconvolute separate contributions from bound and vacant centers.

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