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Polyphasic rise of chlorophyll *a* fluorescence in herbicide-resistant D1 mutants of *Chlamydomonas reinardtii*

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Abstract

Chlorophyll (Chl) *a* fluorescence transient, a sensitive and non-invasive probe of the kinetics and heterogeneity of the filling up of the electron acceptor pool of Photosystem II (PS II), was used to characterize D1-mutants of *Chlamydomonas reinhardtii*. Using a shutter-less system (Plant Efficiency Analyzer, Hansatech, UK), which provides the first measured data point at 10 μs and allows data accumulation over several orders of magnitude of time, we have characterized, for the first time, complete Chl *a* fluorescence transients of wild type (WT), cell wall less (CW-15) *C. reinhardtii* and several herbicide-resistant mutants of the D1 proteins: D1- V219I* A251V, F255Y, S264A G256D and L275F. In all cases, the Chl *a* fluorescence induction transients follow a pattern of O–J–I–P where J and I appear as two steps between the minimum Fo (O) and the maximum Fmax (Fm, P) levels. The differences among the mutants are in the kinetics of the filling up of the electron acceptor pool of PS II (this paper) in addition to those in the re-oxidation kinetics of Q_A^- to Q_A, published elsewhere (Govindjee et al. (1992) Biochim. Biophys. Acta: 1101: 353–358; Strasser et al. (1992) Archs. Sci. Genève 42: 207–224) and not in the ratio of the maximal fluorescence Fm to the initial fluorescence Fo. The value of this experimental ratio is Fm/Fo = 4.4 ± 0.21 independent of the mutation. At 600 W m^{-2} of 650 nm excitation, distinct hierarchy in the fraction of variable Chl *a* fluorescence at the J level is observed: S264A > A251V ~ G256D > L275F ~ V219I > F255Y ~ CW-15 ~ WT. At 300 and 60 W m^{-2} excitation, a somewhat similar hierarchy among the mutants was observed for the intermediate levels J and I. Addition of bicarbonate-reversible inhibitor formate did not change the O to J phases, slowed the I to P rise, and in many cases, slowed the decay of fluorescence beyond the P level. These observations are interpreted in terms of formate effect being on the acceptor rather than on the donor side (S-states) of PS II. The formate effect was different in different mutants, with L275F being the most insensitive mutant followed by others (V219I, F255Y, WT, A251V and S264A). Further, in the presence of high concentrations of DCMU, identical transients were observed for all the mutants and the WT.

The quantum yield of photochemistry of PS II, calculated from 1 – (Fo/Fm), is in the range of 0.73 to 0.82 for the WT as well as for the mutants examined. Thus, in contrast to differences in the kinetics of the electron acceptor side of PS II, there were no significant differences in the maximum quantum yield of PS II, among the mutants tested. We suggest that earlier photochemistry yield values were much lower (0.4–0.6) than those reported here due to either higher measured values of Fo by instruments using camera shutters, or due to the use of cells grown in less than-optimal conditions.

* The mutants are labeled as follows: the single letter code for the wild type amino acid, followed by the residue number, then the code for the mutated amino acid.

Introduction

A variety of herbicides of agricultural importance inhibit photosynthesis by displacing Q_B, the secondary

plastoquinone acceptor of Photosystem II (PS II) (see Velthuys 1981; Trebst 1991; Oettmeier 1992). Different amino acid substitutions that confer resistance to a variety of PS II herbicides are clustered in the Q_B-binding region between helices IV and V from residue 211 to 275 of the D1 subunit of PS II (see a review by Diner et al. 1991). One of the mutants (D1-S264A) of *Chlamydomonas reinhardtii* markedly increases tolerance (5000 times) towards the herbicide metribuzine, where D1 serine 264 is changed to alanine. The loss of the D1 serine 264 is highly significant in herbicide tolerance, indicating that it plays an important role in herbicide binding. In addition to affecting herbicide binding, these alterations also modify, to different degree, certain functional properties of PS II (Erickson et al. 1989) including sensitivity to bicarbonate-reversible formate on chlorophyll *a* (Chl *a*) fluorescence transients (Govindjee et al. 1991), and on the kinetics of Chl *a* fluorescence decay yield, after single-turn over flashes (Govindjee et al. 1992; Strasser et al. 1992; Crofts et al. 1993). Govindjee et al. (1992) suggested that the amino acid substitutions at S264 and at G256 in *C. reinhardtii* altered the equilibrium for $Q_A^- Q_B \rightleftharpoons Q_A Q_B^-$ reaction and the ratio of slow to the fast PS II centers showing a modification at the Q_B binding site. By the analysis of the electron transfer kinetics, Crofts et al. (1993) showed a marked decrease in the rate of reduction of bound plastoquinone to the bound semiquinone in A251V and S264A mutants. In the S264A mutant, the second electron transfer was also slower but was normal in the A251V mutant. However, the G256D mutant showed normal electron transfer after the first flash but slower after the second flash. These results suggest differential involvement of S264 and G256 with protonation events (Crofts et al. 1993). On the other hand, other amino acids like D1 V219, F255, L275 were of marginal importance for the reactions at the $Q_A Q_B$ complex except that these reactions in the mutant D1 L275F are insensitive to bicarbonate-reversible formate effect (Govindjee et al. 1991, 1992; Strasser et al. 1992).

In contrast to Chl *a* fluorescence decay measurements, that measure Q_A^- to $Q_B^{(-)}$ kinetics, Chl *a* fluorescence induction transients measure the kinetics of the filling up of the plastoquinone (PQ) pool and the heterogeneity associated with it (see reviews by Govindjee and Papageorgiou 1971; Papageorgiou 1975; Lavorel and Etienne 1977; Govindjee and Satoh 1986). During Chl *a* fluorescence rise, the electron acceptor pools (Q_A, Q_B and PQ, etc.) of PS II are filled with electrons originating from water on the donor side of PS II; its kinetics represent all the PS II steps. The inflections in the transients represent 'quasi-steady states' not only related to reduced Q_A but related as well to the heterogeneity in the PQ (a fast versus slow reducing) pool. Three different rise components have been distinguished when Chl *a* fluorescence induction curves are measured in vivo (Schreiber and Neubauer 1987; Neubauer and Schreiber 1987; Strasser and Govindjee 1991, 1992; Strasser et al. 1995). The O to J phase, that reflects mainly the reduction of Q_A to Q_A^- (photochemical phase), also includes the influence of the S-states (Delosme 1967; Schreiber and Neubauer 1987; Hsu 1993). The J to I and I to P phases are due to the filling up of the heterogeneous fast and slow PQ-pool (Strasser et al. 1995). P is followed by a decay, via a level S, to the terminal steady state T. The entire phenomenon is, as expected, dependent upon the intensity of excitation light as shown for plants and cyanobacteria (Strasser et al. 1995).

In this paper we present (i) for the first time, the characterization of complete fluorescence transients of several (F255Y, V219I, A251V, L275F, S264A, G256D) D1-herbicide resistant mutants, (ii) the maximum quantum yield of PS II ($\Phi_P$max), calculated from the ratio of variable to maximal fluorescence yield ((1 –Fo/Fm) equivalent to $(F_P–Fo)/F_P$), and (iii) the characterization of the Chl *a* fluorescence transients of the D1 mutants in the presence of DCMU and formate which differently block electron transfer on the electron acceptor side of PS II (see e.g. Blubaugh and Govindjee 1988; Govindjee and Van Rensen 1993).

Our work has revealed a high quantum yield of PS II but differences in the kinetics of the filling up of the electron acceptor pool of PS II among the six D1-herbicide mutants of *C. reinhardtii*. Fluorescence transient data presented here allows one to distinguish the different sites of action of inhibitors on the acceptor side of PS II.

Materials and methods

Growth conditions of the algae

Wild type *Chlamydomonas reinhardtii* 137C and nine mutants ((Dr-2 (V219I #1), DR-18 (V219I #2); Ar-207 (F255Y); MZ-2 (A251V); DCMU-4 (S264A); AR-204 (G256D #1); BR-24 (G256D #2); Br-202 (L275Y) and CW-15 (a cell wall less)) were grown mixotrophically in tris-acetate phosphate medium at pH 7 (Gorman and Levine 1965) on agar plates. Cultures were grown in

growth chamber at 20 °C and illuminated for 12 h with fluorescent white light. The physiological condition of the mother cultures on the agar plates were regularly checked by fluorescence induction measurements directly on the growing colonies on the plate. From these mother cultures, cells were transferred to liquid culture flask, kept in the growth chamber with constant shaking. The cells were harvested in the second hour of the light phase during the log phase of their optimal growth conditions. All the experiments were done with 3-day-old cultures in liquid medium. The cells were harvested and then diluted with 1/2 strength autotrophic tris-HCl phosphate medium at pH 6.5, 1 h prior to measurements, as described before (Govindjee et al. 1991).

Chlorophyll determination

Chlorophyll content of the cell sample was measured at 680 nm (A680) by a spectrophotometer (Perkin–Elmer, Lambda 3UV/VIS) which was set at zero absorbance at 800 nm to correct for scattering. The empirical relation between absorbance and chlorophyll content for wild type cells as well as for all the mutant cells was μg total chlorophyll/ml suspension = 29 ± 0.5 × A680; the error includes the variation in the relationship among the mutants. At the low concentration (3–5 μg) of Chl, used in our experiments, differences in [Chl], measured among the mutants, had no effect on the transient recorded since all data are normalized at the Fo level.

Fluorescence induction kinetics

Chlorophyll *a* fluorescence transients were measured by a Plant Efficiency Analyzer (PEA, Hansatech, Ltd, King's Lynn, Norfolk, England) with 60, 300 or 600 W m^{-2} light intensity. Illumination was provided by an array of 6 light emitting diodes (peak of emission, 650 nm), focused on to the sample surface to provide a homogeneous illuminated light spot of about 4 mm in diameter. This excitation wavelength was appropriate for *C. reinhardtii* since energy transfer from Chl *b* to Chl *a* is known to be close to 100% (Govindjee and Satoh 1986).

The Chl *a* fluorescence signals were detected using a PIN photocell after passing through a long pass filter (50% transmission at 720 nm). The optical characteristics of the PEA instrument are comparable with most of the instruments commercially available. The use of long wavelength range (720–760 nm) emission is appropriate for *C. reinhardtii* since (1) most of this fluorescence at room temperature is from PS II; and (2) the measured fluorescence is free from reabsorption of fluorescence; it is the mirror image of a major vibrational band of the first singlet excited state, S1. All the experiments were done with aliquots of 500 μl cell suspension (3–5 μg Chl) in 1 cm vials. Here, the optical thickness of the sample was 5 mm, and the diameter of the sample area irradiated was 4 mm. The exciting beam was very homogeneous as judged, on a piece of a white paper, by eyes. Samples were dark adapted for 5 min before fluorescence transients were monitored. Chl *a* fluorescence transient was recorded in a time span from 10 μs to 1 min with data acquisition rate of 10 μs for the first 2 ms and 12 bit resolution. The fluorescence signal at 40 μs (the fourth measured data point) was considered as Fo as it was within 3% of the automatically extrapolated value obtained by the instrument. Calibration of the PEA instrument with a high speed digital oscilloscope (Nicolet No. 410) reveals that the data point at 40 μs is a reliable value with no detectable artifacts from the electronics of the instrument. The J and I levels were determined by a computer as the points with the lowest slope on a log time scale. The time of appearance of steps J and I are very reproducible at a given light intensity. The results are plotted on a logarithmic time scale over 5 orders of magnitude (40 μs–4 s) as well as on a linear time scale (0–1 s) to show the differences in the appearance of the transients, and to allow for a comparison with the published data on the linear scale. The use of a high sampling rate which allows a presentation on the log time scale was the key to visualize the intermediate steps J and I in the fluorescence transient between the initial (Fo) and the maximum (Fm). In WT and F255Y, 5 μM DCMU was enough to inhibit electron flow beyond Q_A^-. However, to inhibit this electron flow in S264A, L275F A251V and V219I, higher concentrations of DCMU (25 or 100 μM) had to be used.

Results and discussion

Characterization of chlorophyll a fluorescence transient of herbicide-resistant D1 mutants of Chlamydomonas reinhardtii

Chlorophyll *a* fluorescence transients provide information on the filling up of the plastoquinone (PQ) pool and the associated heterogeneity, but they are affected by both the electron donor and acceptor sides of PS

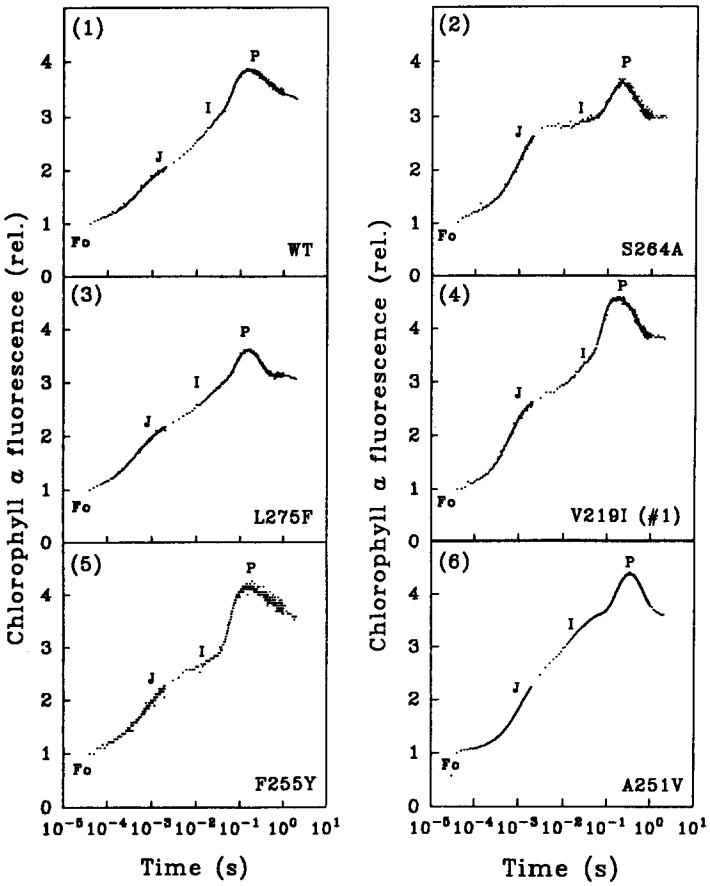

Fig. 1. Chlorophyll (Chl) *a* fluorescence transients of *C. reinhardtii* cells of (1) WT and of D1 mutants: (2) S264A, (3) L275F, (4) V219I, (5) F255Y and (6) A251V grown mixotrophically in liquid culture (Tris-acetate phosphate medium). The cells were harvested and then diluted with 1/2 strength autotrophic tris-phosphate medium (pH 6.5), 1 h prior to measurement; illumination, 300 W m^{-2} 650 nm light (see text for details). The fluorescence signal at 40 μs is Fo. The data are plotted on a logarithmic time scale. Note the J and the I inflections between the Fo and the P levels.

II of oxygen evolving organisms (Papageorgiou 1975; Lavorel and Etienne 1977; Krause and Weis 1991; Hsu 1993; Shinkarev and Govindjee 1993). The initial Chl *a* fluorescence yield at O level reflects the minimal fluorescence yield when all Q_A is in the oxidized state. Here, the photochemical yield is maximum. Measurement of the true Fo level is a problem with instruments using camera shutters that have an opening time of one or more ms (Brewer et al. 1979), as has been the case with earlier measurements on herbicide-resistant mutants (Govindjee et al. 1991). However, this difficulty is avoided with the PEA (Hansatech) or other LED-based instruments which use optoelectronics with a time resolution in the 10 μs range.

Figure 1 shows the Chl *a* fluorescence transients (40 μs to 1 s) of 5 min dark adapted WT *C. reinhardtii* cells as well as of five mutants (S264A, L275F, V219I, F255Y and A251V), exposed to 300 W m^{-2} 650 nm light and plotted on the logarithmic time scale. These curves show two steps J and I between O and P, with J more clearly observed than I at the light intensity used. At lower light intensities, I predominates over J. The first rise from O to J is attained in the 2 ms range. It is seen only when the data, obtained with a digitization rate of 10 μs, are plotted on the logarithmic time scale or on a linear fast time scale from 0 to 10 ms. Figure 2 shows the data of Fig. 1 on a linear time scale. Although the location of Fo is clear, the inflection J is no longer seen as it hides in the O to I rise. This explains why 'J' level was not observed earlier since all data had been thus far presented on a linear time scale. The intermediary point I levels in the 20 to 30 ms range (Figs. 1 and 2); and the total time in reaching the final peak (P) is about 150 ms at 300 W m^{-2}. The time for

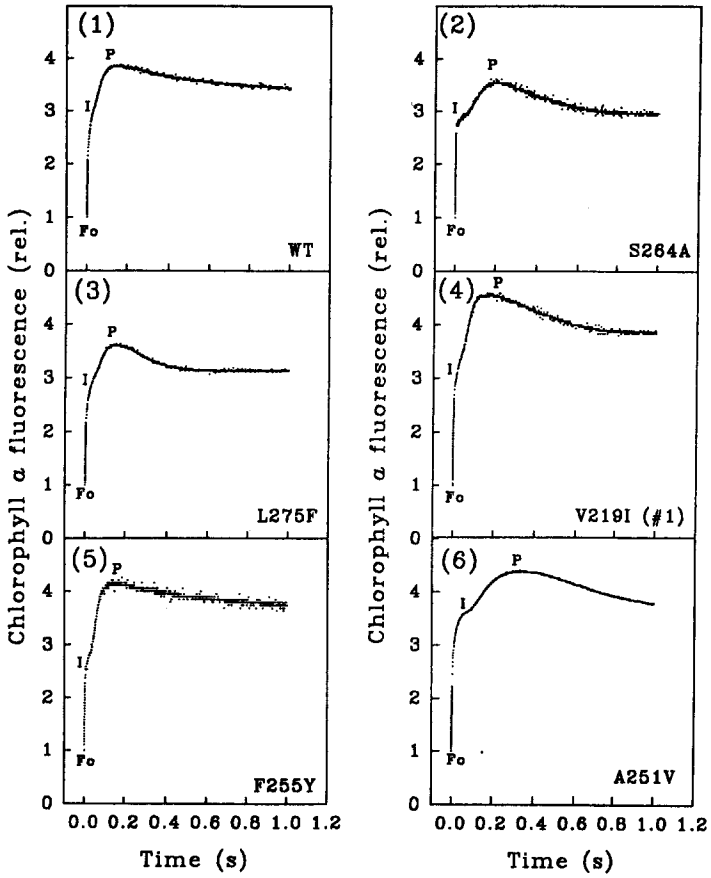

Fig. 2. Chl *a* fluorescence transients as in Fig. 1, but plotted on a linear time scale. Here, only one inflection I is visible between Fo and P.

leveling of each intermediatary step varies depending on the light intensity used (Strasser et al. 1995). With 60 W m^{-2}, the P levels off at about 250 ms.

Figure 3A shows the variable Chl *a* fluorescence (V = (Ft–Fo)/(Fm–Fo)) at different levels, J and I. Quantitatively speaking, the relative variable fluorescence intensities which level at J are quite different in different mutants. As compared to WT cells, the D1 S264A mutant shows a very rapid rise from Fo to the J level (Figs. 1 and 2). There is a distinct hierarchy in the relative variable Chl *a* fluorescence for each step. V_J decreases and V_I increases in the following order: S264A > A251V ~ G256D > L275V ~ V219I > F255Y ~ CW-15 ~ WT (Fig. 3A). These differences reflect differences on the electron acceptor side of PS II related to (1) the rate (or equilibrium) of electron transfer in the Q_AQ_B complex (Govindjee et al. 1992; Crofts et al. 1993); and (2) the PS II heterogeneity (Strasser et al. 1995) related either (a) to the Q_B^- reducing and Q_B non-reducing centers (Guenther et al. 1990) or (b) to the heterogeneity in the PQ pool (Lavergne and Joliot 1991).

Quantum yield of PS II photochemistry

Table 1 and Fig. 3B show the ratios of the Fm/Fo and the Fv/Fm (representing maximum quantum yield of PS II photochemistry) for one representative set of data. Within parenthesis are shown the earlier data measured with a camera-shutter instrument (where F at 4 ms was taken to reflect Fo). The Fv/Fm in this work ranges from 0.73 to 0.82, whereas it ranged between 0.44 to 0.62 from the camera-shutter data reported earlier (Govindjee et al. 1991). Spalding et al. (1984) had obtained still smaller values, such as 0.38 and 0.52 for air grown and 5% CO_2 grown *C. reinhardtii* WT cells, respectively. On the other hand, Guenther et al. (1990) had also obtained small values of 0.52 and 0.62 for dark grown and light grown *C. reinhardtii* WT cells. However, Erickson et al. (1989) had obtained a Fv/Fm

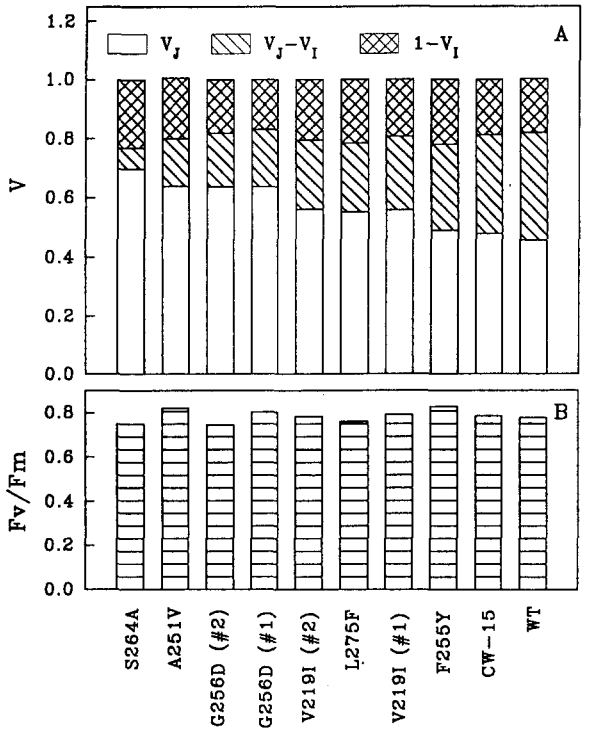

Fig. 3. (A) The relative variable Chl *a* fluorescence of WT and different D1 mutants of *C. reinhardtii* cells at J (V_J), I (V_J–V_I) and P ($1–V_I$) levels. V = (Ft–Fo)/(Fm–Fo). (B) Ratio of Fv/Fm of WT and D1 mutants of *C. reinhardtii* cells. These are the average data (±0.015) obtained over a year during the growth of the cells examined. For all the measurements, the PEA head was positioned directly 1 mm above the surface of agar plate containing the algal strain, excited with 600 W m^{-2} of 650 nm light.

Table 1. Ratios of Fm/Fo and of Fv/Fm (representing quantum yield of PS II photochemistry) of the WT and D1 mutants of *C. reinhardtii* cells grown mixotrophically in tris-acetate phosphate medium (pH 6.5) and then transferred to 1/2 strength autotrophic tris-HCl phosphate medium 1 h prior to measurement. Intensity of 650 nm light, 300 W m^{-2}. Shown in parentheses are data obtained with a camera shutter instrument (Govindjee et al. 1991)

Mutants[a]	Names	Fm/Fo	Fv/Fm
	WT	3.95 (2.6)	0.75[b] (0.61)
F255Y	AR-207	4.25 (2.4)	0.76 (0.59)
V219I #1	DR-2	4.5	0.77
V219I #2	DR-18	4.58 (2.6)	0.78 (0.62)
A251V	MZ-2	4.3 (2.0)	0.77 (0.50)
L275F	BR-202	3.70 (1.9)	0.73 (0.439
S264A	DCMU-4	3.66 (1.9)	0.73 (0.56)
G256D #1	AR-204	5.58	0.82
G256D #2	BR-24	5.05	0.80
	CW-15	4.63	0.78

[a] Named as follows: single letter code of the amino acid of the wild type (WT) followed by the residue number and the mutated amino acid.

[b] The values presented here are representative values obtained in this experiment. However, over a year, we have obtained even higher values of Fm/Fo and Fv/Fm ratios (see Fig 3).

of 0.68 in WT and G256D mutant, using a shutter with 1 ms opening time. Using the Chl *a* fluorescence decay method, monitoring Q_A^- to $Q_B^{(-)}$ steps, and cells grown differently than used here, Crofts et al. (1993) also reported lower values of 0.52 (WT), 0.43 (S254A), 0.62 (A251V) and 0.58 (G256D) after one pre-illuminating flash. Although the cause of these particular low values must be different, our present results show that most of the earlier measurements underestimated the quantum yield of PS II photochemistry in *C. reinhardtii*. The control mother cultures on agar plates over a long period (more than 1 year) showed that all the mutants used had high quantum yields of photochemistry (Fv/Fm = 0.77 ± 0.05). However the value of Fv/Fm during growth of the cells on the agar plate varied differently from one mutant to another. Some mutants hold higher value for several days which decreases suddenly and strongly. Other mutants show a high Fv/Fm value only for a short period (2–4 days) and then the value decreases slowly and steadily (data not shown). This different physiological behaviour of the mutants during growth may also explain why often lower Fv/Fm ratios are reported.

Effect of formate on the Chl a fluorescence transients of D1 mutants

It has been shown earlier that the Hill reaction is inhibited by formate treatment and is uniquely and fully restored by the addition of CO_2 under conditions where no reduction of CO_2 occurs (Blubaugh and Govindjee 1988; Govindjee and Van Rensen 1993). It has been suggested that one of the functions of bicarbonate is to regulate the reduction and protonation of plastoquinone beyond Q_A, the one electron primary plastoquinone acceptor of PS II (Govindjee et al. 1976; Eaton-Rye and Govindjee 1988a,b; Xu et al. 1991). Thus formate treatment can be used to modify the steps of the filling up of the PQ pool and used as a tool to further characterize the fluorescence transients of D1 mutants. Different D1 mutants of the cyanobacteria *Synechocystis* 6714 (Govindjee et al. 1990) and *Synechococcus* sp. PCC 7942 (Cao et al. 1992) and

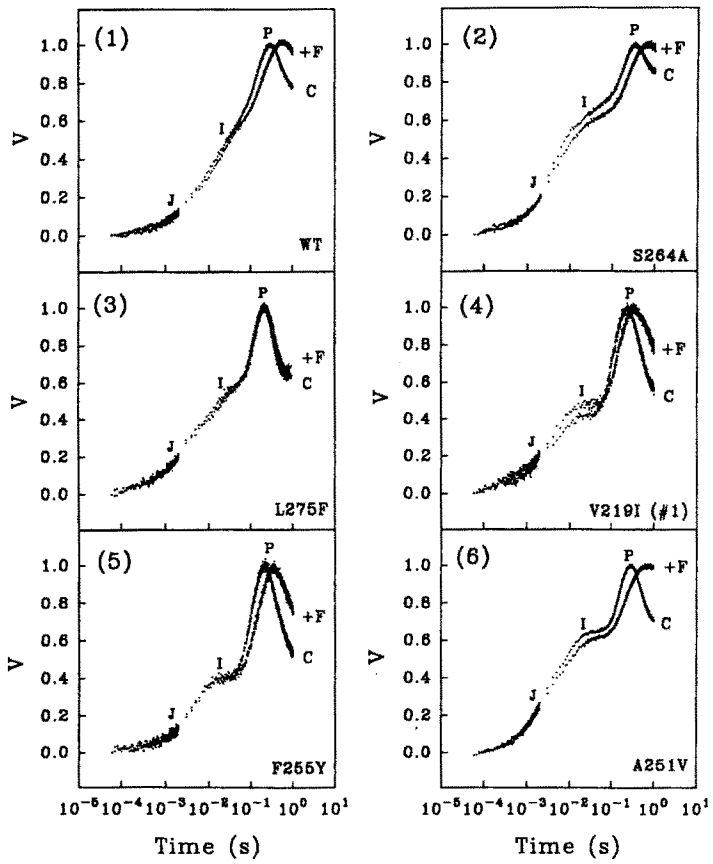

Fig. 4. Time course of changes in the relative variable Chl *a* fluorescence V (= (Ft–Fo)/(Fm–Fo)) of (1) WT and different D1 mutants: (2) S264A, (3) L275F, (4) V219I, (5) F255Y and (6) A251V before (C, for control) and after 20 mM formate treatment for 5 min in dark (+F). The intensity of the 650 nm light was 60 W m^{-2}. Other experimental details are as in Fig. 1. Data have been plotted on a logarithmic time scale

eukaryotic alga *C. reinhardtii* (Govindjee et al. 1991) are already known to be differentially sensitive to bicarbonate-reversible formate effect suggesting a possible interaction of HCO_3^- with the D1 protein. Previous results on Chl *a* fluorescence transients with the D1 mutants of *C. reinhardtii* cells show (Govindjee et al. 1991) that formate slows down the Chl *a* fluorescence rise indicating a slowing down in the overall accumulation of Q_A^-, possibly due to an effect prior to Q_A (El-Shintinawy et al. 1990). However, this is followed immediately by higher yield of fluorescence in the P to S region suggestive of a block beyond Q_A. This was further confirmed by a slowing down of Chl *a* fluorescence decay after light flashes in D1 mutants of *C. reinhardtii* (Govindjee et al. 1992).

Thus, we have reinvestigated the effects of formate on Chl *a* fluorescence transients here since we are now able to monitor and further characterize the complete fluorescence transient from the 'true' O (40 μs) level to the P level, i.e., the O, J, I, P transient.

Figure 4 shows the fluorescence transients, measured in the absence and presence of 20 mM formate, at 60 W m^{-2} of 650 nm light, plotted as normalized (relative) variable Chl *a* fluorescence V = (Ft–Fo)/(Fm–Fo), in WT and five D1 mutants. Superposing the individual curves of all the control mutants without formate revealed that V at the J phase (V_J) is higher in S264A (0.22), A251V (0.22), L275F (0.19), V219I (0.19) and F255Y (0.18) than in the WT (0.15). These results further confirm the differences between the mutants in the kinetics and the heterogeneity of electron acceptors of PS II. At the low light intensity (60 W m^{-2}) used here, the step J is quite low relative to the step I. This is consistent with the light intensity dependence on the J and

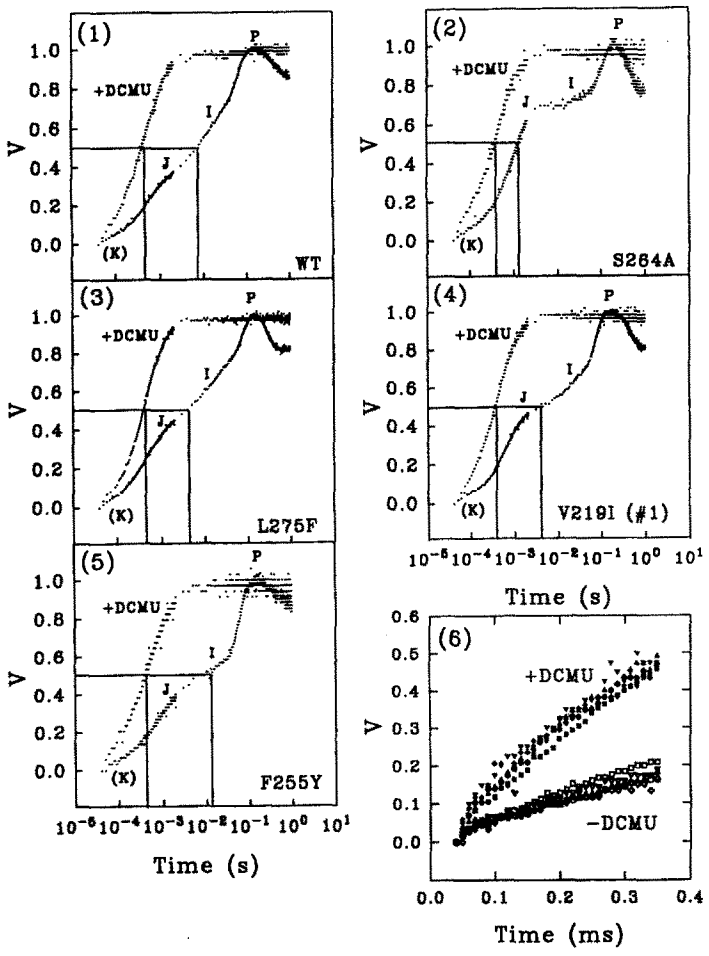

Fig. 5. Changes in the level of relative variable Chl *a* fluorescence V = (Ft–Fo)/(Fm–Fo) of (1) WT and of D1 mutants: (2) S264A, (3) L275F, (4) V219I and (5) F255Y of *C. reinhardtii* cells before and after DCMU treatment. The panel (6) shows the relative variable Chl *a* fluorescence rise in the first 0.4 ms in control (hollow symbols) and DCMU (filled symbols) treated cells. Different symbol types represent different mutants. Formate treated cells showed the same kinetics as those of the control cells both in presence and absence of DCMU. The DCMU concentrations used were according to their tolerance to DCMU, i.e., for WT, S264A, L275F, V219I and F255Y, the concentration used was 5, 100, 25, 100 and 5 μM, respectively. Cells were excited with 300 W m^{-2} of 650 nm light. The drawn vertical lines on the graphs are the half rise times for P. A small band (K) (not easily seen in the figure) at about 100 μs remains after treatment with DCMU.

I levels in higher plants and cyanobacteria (Strasser et al. 1995).

It has been shown earlier that different effects of formate on Chl *a* fluorescence can be observed depending on the concentration of formate used (Xu et al. 1991). In this study we have used a rather mild formate treatment (20 mM for 5 min) which provides typical changes in the fluorescence transient without loosing the maximum yield of photochemistry (ΦPo = 1 – Fo/Fm). The typical changes are (1) a slower rise to the maximum fluorescence intensity Fp followed by (2) slower decrease from the P level (see Fig. 4).

To localize closer the action of formate in the electron transport chain from the water splitting side to PQ, we must compare the kinetics of the relative variable fluorescence V = (Ft–Fo)/(Fm–Fo). On purpose we have chosen a condition of formate treatment in which the ratio Fo/Fm remains unaffected. Therefore, only the shape of the fluorescence transient between Fo and Fp needs to be analyzed. V can be taken as a measure of the fraction of the closed reaction centers $[Q_A^-]/[Q_A](\text{total})$. The variation of V in time (dV/dt) is, therefore, a measure of the fractional variation of $[Q_A^-]$ in time. Thus,

$$dV/dt = \frac{d[Q_A^-]/[Q_A](\text{total})}{dt}$$

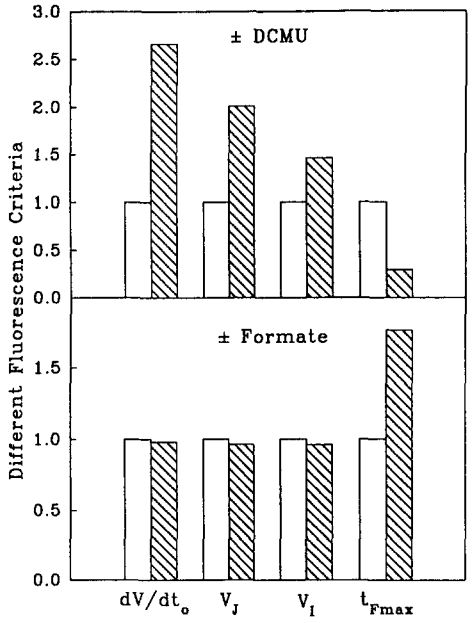

Fig. 6. Different patterns of action of DCMU (saturating concentration) and formate (non saturating concentration, 20 mM incubation for 5 min) on the fast fluorescence transient. Four criteria are indicated: (1) d V/dt$_o$; the slope at the origin of the relative variable fluorescence V = (Ft–Fo) / (Fm–Fo); this expression is a measure for the rate of photochemistry. (2) and (3) V_J and V_I; the relative variable fluorescence at the intermediate steps J and I at 2 ms and 30 ms, respectively. (4) t_{Fmax}; the time needed to reach the maximum fluorescence intensity. The effect of DCMU (top panel) and formate (lower panel) (filled bars) are normalized to their respective control values (empty bars). Note the large increase in dV/dt$_o$ and strong decrease in t_{Fmax} in the DCMU treated sample whereas only an increase in t_{Fmax} is observed in the formate treated samples. What is not shown is the effect on the P to S decay: it is totally abolished in the case of DCMU, and is showed in the case of formate (Fig. 4; Govindjee et al. 1991).

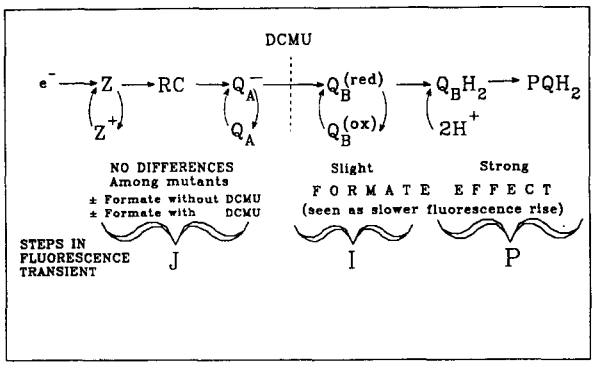

Fig. 7. A summary of PS II events (± formate and DCMU) and its relationship to the fluorescence transient (O–J–I–P).

However, the variation in time of the $[Q_A^-]$ is the difference between the reduction flux of Q_A to Q_A^- and the oxidation flux of Q_A^- to Q_A, where Q_B or Q_B^- acts as the subsequent electron acceptor. At the J level (~ 2 ms) the dV_J/dt is practically zero. That means at the J level the reduction rate of Q_A and the oxidation rate of Q_A^- are equal. The initial slope dV/dt$_o$ (measured between 50 and 300 μs) shows the difference between the Q_A reduction rate and the Q_A^- oxidation rate after the onset of illumination. In the presence of DCMU, the reoxidation of Q_A^- is mainly blocked. Thus, here, dV/dt$_o$, after the illumination, represents only the electron flux from the donor side to the acceptor side. No changes were observed by 20 mM formate addition in the initial relative variable fluorescence at the J level, V_J (2 ms) (Fig. 4) or in the original slope dV/dt$_o$ whether DCMU was present or not (see Fig. 5 and 6). Thus under our experimental conditions formate did not have any effect on the electron donation to Q_A. This was further supported by the lack of formate effect on the entire O to J phase (Fig. 4). It has, however, been reported elsewhere (El-Shintinawy et al. 1990) that formate slows down the electron donation from Z to Q_A in *C. reinhardtii* cells. We cannot contradict this observation but from the results presented here we cannot make this conclusion. In previous results (El-Shintinawy et al. 1990) stronger formate treatment had been used which decreased heavily the variable Chl *a* fluorescence allowing the earlier authors to observe an action of formate on the electron donor side.

Formate only slightly lowers the I level (about 30 ms) of the fluorescence induction curve. But after the addition of 20 mM formate in the suspension medium a typical slowing down of the fluorescence rise is observed between the J and the P phases in all cases except the L275F mutant (Fig. 4). The time to reach the maximum fluorescence intensity t_{Fmax} is increased in WT, S264A, V219I, F255Y and A251V (Fig. 4) but there is a hierarchy in the effect. S264A showed the maximum increase in t_{Fmax} (about 147%) and L275F was unaffected. Others showed intermediate effects: A251V (129%), WT (94%), F255Y (70%) and V219I (48%). Previous results (Govindjee et al. 1991) showed that formate inhibits the P to S decay and have been interpreted to be due to the inhibition of the reoxidation of Q_A^- to Q_A The same trend is observed here in S264A, A251V, WT, F255Y and V219 mutants, but since the complete P to S decay was not recorded, we do not make quantitative comparison. In summary, 20 mM formate provokes (1) no change in dv/dt$_o$, and in the OJ phase (V_J) (2) only a slight or no change in

the I level, (3) an increase in t_{Fmax}; and (a) slowing down of the P to S decay (Fig. 7, also see Govindjee et al. 1991). Further, a hierarchy of these effects is observed in the D1 mutants examined with L275F being insensitive and S264A and A251V being quite sensitive. We interpret these effects to be mainly due to effects on the kinetics and the heterogeneity of the PS II acceptor side, the nature of which remains to be still deciphered.

Chlorophyll a fluorescence transient of D1 mutants of C. reinhardtii *in the presence of DCMU*

Figure 5 shows fluorescence transients, plotted as V = (Ft–Fo)/Fm–Fo) versus time, also on a logarithmic time scale. With DCMU, there is a fast O to P rise and there is no P to S decay. DCMU is known to block electron flow beyond Q_A by displacing Q_B (Velthuys 1981). Here, the phases J and I merge with P. What is of interest to this paper is that after treatment with saturating [DCMU] the differences between the mutants disappear. A generalized effect of DCMU is shown in Fig. 6. Superposition of the WT curve on the individual mutant curve gives, within the experimental error, only one curve exhibiting the same rate of photochemistry (Fig. 5, Panel 6). The expression dV/dt_o can be taken as a measure for $d[(Q_A^-]/dt_o$ which is the same for all the mutants (Fig. 6). With DCMU the transient levels off at the step J and the half rise time in each case, for 300 W m^{-2} light exposure, is identical (350 μs). A small inflection, of unknown origin, labeled as (K) (Eggenberg et al. 1992; Strasser et al. 1995) persisted upon DCMU treatment. It is possible that (K) reflects the accumulation of PS IIs which behave (in terms of fluorescence) like closed ones before Q_A has been reduced. Further work is needed to examine the nature of (K).

Concluding remarks

Chlorophyll *a* fluorescence transients, obtained by the method used in this work, provide the full O, J, I and P transients with true 'O' level. The relative intensity of J and I are dependent upon light intensity. There is a remarkable hierarchy in the fluorescence transient characteristic with S264A having the fastest O to J (or I) rise with or without formate present. Several effects of formate (no change in OJ phase, slowed J to P rise (i.e., increased t_{Fmax} and decreased P to S decline) indicate its action on the PS II acceptor side. Differential effect of 20 mM formate on these mutants and the lack of its effect on L275F mutant confirms the importance of D1 protein in bicarbonate-reversible formate effect in PS II.

All the D1 mutants examined here have identical kinetics in the presence of DCMU, but a (K) band in the transient persists. Our data show that the WT as well as the D1 herbicide-resistant mutants of *C. reinhardtii* cells investigated by us have high Fv/Fmax, thus high quantum yield of photochemistry of PS II (0.7–0.8), just as in higher plants.

We conclude that the fast polyphasic rise (Fo–J–I–P) of chlorophyll *a* fluorescence is a useful tool to screen the behaviour of mutants, and to localize the sites of stress such as that by the inhibitors (e.g. DCMU) and regulators (e.g. formate/bicarbonate) of PS II. An extension of these measurements to temperature and light stress is underway.

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