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Changes in polyphasic chlorophyll *a* fluorescence induction curve upon inhibition of donor or acceptor side of photosystem II in isolated thylakoids

Nikolai G. Bukhov[a,b], Elena A. Egorova[a,b], Sridharan Govindachary[a], Robert Carpentier[a,*]

[a] *Groupe de Recherche en Énergie et Information Biomoléculaires, Université du Québec à Trois-Rivières, Trois-Rivières, Québec, Canada GA9 5H7*
[b] *K.A. Timiriazev Institute of Plant Physiology Russian Academy of Sciences, Moscow, Russia*

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Abstract

The action of various inhibitors affecting the donor and acceptor sides of photosystem II (PSII) on the polyphasic rise of chlorophyll (Chl) fluorescence was studied in thylakoids isolated from pea leaves. Low concentrations of diuron and stigmatellin increased the magnitude of J-level of the Chl fluorescence rise. These concentrations barely affected electron transfer from PSII to PSI as revealed by the unchanged magnitude of the fast component ($t_{1/2}$ = 24 ms) of $P700^+$ dark reduction. Higher concentrations of diuron and stigmatellin suppressed electron transport from PSII to PSI, which corresponded to the loss of thermal phase, the Chl fluorescence rise from J-level to the maximal, P-level. The effect of various concentrations of carbonylcyanide *m*-chlorophenylhydrazone (CCCP), which abolishes S-state cycle and binds at the plastoquinone site on Q_B, the secondary quinone acceptor PSII, on the Chl fluorescence rise was very similar to that of diuron and stigmatellin. Low concentrations of diuron, stigmatellin, or CCCP given on the background of *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD), which is shown to initiate the appearance of a distinct I-peak in the kinetics of Chl fluorescence rise measured in isolated thylakoids [BBA 1607 (2003) 91], increased J-step yield to I-step level and retarded Chl fluorescence rise from I-step to P-step. The increased J-step fluorescence rise caused by these three types of inhibitors is attributed to the suppression of the non-photochemical quenching of Chl fluorescence by [S_2^+ S_3] states of the oxygen-evolving complex and oxidized P680, the primary donor of PSII reaction centers. In the contrary, the decreased fluorescence yield at P step (J–P, passing through I) is related to the persistence of a ‘‘plastoquinone’’-type quenching owing to the limited availability of photochemically generated electron equivalents to reduce PQ pool in PSII centers where the S-state cycle of the donor side is modified by the inhibitor treatments.

Keywords: Chlorophyll fluorescence; Diuron; Photosystem II; Stigmatellin; Thylakoid

1. Introduction

Chlorophyll (Chl) fluorescence is a widespread noninvasive method used for the study of photosynthetic electron transport. The kinetics of fluorescence induction measured under very strong actinic light [1–3] is of special interest because the rate of the photochemical reactions is very high under these conditions and the photochemical and non-photochemical events are well resolved. Two phases are clearly distinguished in the kinetics: the photochemical phase is completed within 1–2 ms and the thermal phase lasts for 200 ms after the onset of light [3–6]. The former is attributable to the light-induced reduction of Q_A, the primary quinone acceptor of photosystem II (PS II), whereas the thermal phase is not directly related to the redox state of Q_A [5,7]. It is widely accepted that the increase in Chl *a* fluorescence from the basic level, F_o, to the maximal one, F_m or P, follows a sequence of three steps [2,3,8,9]. According to the terminology introduced by Strasser and Srivastava [8], the first step, O–J (F_o–I_1), corresponds to the photochemical phase of Chl fluorescence induction [1]. The rate of this phase increases with actinic light irradiance

Abbreviations: CCCP, carbonylcyanide *m*-chlorophenylhydrazone; Chl, chlorophyll; OEC, oxygen-evolving complex; P700, primary electron donor of photosystem I; PS, photosystem; PQ, plastoquinone; Q_A and Q_B, primary and secondary quinone acceptors of photosystem II; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine

* Corresponding author. Fax: +1-819-376-5057.
E-mail address: Robert_Carpentier@uqtr.ca (R. Carpentier).

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[2]. The second and third steps, J–I (or I_1–I_2) and I–P (I_2–F_m), respectively, constitute the first and second part of the thermal phase [3].

The rise of Chl fluorescence observed upon the onset of continuous light is largely associated with changes in the redox states of electron carriers in PSII and nonphotochemical quenching processes [3,6]. Amongst several intermediate electron carriers in PSII electron transport, the fluorescence yield is primarily determined by the redox state of Q_A as it serves as a quencher in its oxidized state. However, Q_A is not a unique fluorescence quencher in PSII. $P680^+$, the oxidized form of the primary electron donor, and reduced pheophytin, an intermediate electron acceptor, were found to quench Chl fluorescence [10–12]. The redox state of the tetra-nuclear manganese cluster also affects the Chl fluorescence yield [13]. During four successive charge separations in PSII, the sequential buildup of oxidation equivalents through one-electron oxidation steps in the manganese cluster is cycled via the S-state intermediates, $S_0 \rightarrow S_1 \rightarrow S_2 \rightarrow S_3 \rightarrow S_4$ (S_o). The S_4 state is immediately converted to S_0 with a time constant of ~ 0.750–1.0 ms during which dioxygen is evolved. The S_1 state is very stable in the dark and, therefore, the transition from S_0 to S_1 state occurs with a time constant in the range of minutes, while the decay of S_3 and S_2 states into S_1 state takes place in several seconds. For recent reviews, see Refs. [14–16]. Importantly, accumulation of S_2 and S_3 states by a predetermined number of white flashes or by treatments that inactivate the OEC is shown to quench fluorescence at the J-step of the O–J–I–P transient [3,17]. It has also been proposed that oxidized PQ molecules can act as quenchers of Chl fluorescence in isolated PSII particles [18] or whole thylakoid membranes [19,20] likely because of their static interaction with Chl molecules. Photochemical reduction of the PQ pool removes this static quenching [21].

The diversity of processes influencing Chl fluorescence complicates the interpretation of the information obtained from the induction curves. Therefore, there is a requirement for additional studies of the mechanisms that control the Chl fluorescence yield. The use of inhibitors that act at specific sites of the electron transport chain and modify the redox properties of PSII intersystem carriers would be a key to derive such supplementary information. Diuron displaces Q_B from its binding site on the D1 protein of the PSII reaction center [22,23]. Subsequently, a block in the electron transport beyond Q_A poises the PQ molecules in oxidized state under illumination. Besides, a low concentration of this herbicide also acts at a site on the donor side of PSII [24]. In spinach chloroplasts, stigmatellin intercepts the photosynthetic electron transport at two different sites [25,26]. Like diuron, this inhibitor blocks the electron transfer from Q_A to Q_B. The other inhibitory site is located on the plastoquinol oxidation site, Q_O, in the cytochrome b_6/f complex [25–28] and prevents light induced electron flow to PSI and reoxidation of PQH_2. Unlike diuron and stigmatellin, carbonylcyanide *m*-chlorophenylhydrazone (CCCP) accelerates the deactivation reactions of the OEC of PSII (ADRY-agent) [29] and diminishes the period four oscillation [30,31].

In order to examine how the activities of the oxidizing and reducing sites of PSII can influence the O-, J-, I-, and P-steps of Chl *a* fluorescence induction, respectively, we used inhibitors such as diuron, stigmatellin, and CCCP to alter the electron transport at both sides of PSII. The decay of absorbance changes at 830 nm in darkness following brief exposure of thylakoids to actinic light was used to evaluate the reduction kinetics of $P700^+$, the oxidized primary donor of PSI, as a complementary probing tool. Our experimental data revealed that inhibition of the OEC resulted in an increased magnitude of J-level up to that of I, which is attributable to a diminished contribution of the S-state and $P680^+$-mediated non-photochemical quenching of Chl fluorescence. Closure of PSII acceptor side or severe damage of the OEC led to the loss of the thermal phase of Chl fluorescence induction because of the inability of PSII to reduce the PQ pool and to remove "plastoquinone"-type non-photochemical quenching.

2. Materials and methods

Thylakoids were isolated from freshly harvested pea leaves (*Pisum sativum* L) grown in a climatic chamber under light provided by a xenon lamp at a fluence rate of 200 μmol quanta m^{-2} s^{-1}. Cooled leaves were ground in a medium containing 100 mM sorbitol, 20 mM HEPES–NaOH, 10 mM NaCl, and 2 mM $MgCl_2$. The suspension was filtered through four layers of nylon cloth and centrifuged at $150 \times g$ for 5 min at 4 °C. The supernatant was subjected to further centrifugation at $3500 \times g$ for 10 min at 4 °C. The resultant pellet was resuspended in a medium containing 20 mM HEPES–NaOH, 10 mM NaCl, and 2 mM $MgCl_2$ for the measurements concerning Chl *a* fluorescence induction and the P700 redox changes. All measurements were carried out in darkness.

Chl was assayed according to Porra et al. [32]. Chl concentration during measurements was 50 μg ml^{-1}. *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD) was oxidized prior to measurements by storage under air in the dark for 24 h. All inhibitors were dissolved in ethanol. In order to avoid solvent induced damages to photosynthetic membranes, the final concentration of ethanol in the thylakoid suspension was kept well below 0.8% (v/v) in all measurements.

Chl fluorescence was measured using a Plant Efficiency Analyser (PEA) (Hansatech, King's Lynn, Norfolk, UK) in glass cuvettes with an optical pass of 1.065 mm. Red excitation light peaking at 650 nm (intensity of 4000 μmol photons m^{-2} s^{-1}) obtained from six light emitting diodes was used for fluorescence induction. The data acquired during the first 40 μs following the onset of continuous red light are not considered as this fluorescence emission is

largely related to the artifacts attributable to the response time of the PEA.

Absorbance changes at 830 nm were assayed using a Pulse Amplitude Modulated (PAM) Chl fluorometer (Walz, Effeltrich, Germany) equipped with dual-wavelength emitter-detector unit. White actinic light was obtained from a Fiber-Lite light source (Microview, Thornhill, ON, Canada) and controlled by an electronic shutter. The signals of absorbance changes were recorded using Walz Data Acquisition System PDA100. Prior to the onset of actinic light, ascorbate (1 mM) and methyl viologen (100 μM) were added to the suspension of thylakoids unless otherwise expressively stated. The first agent served as electron donor to completely reduce P700 in the dark, while methyl viologen prevented over-reduction of the PSI acceptor side. The fast phase of the absorbance decay in darkness following illumination is associated with $P700^+$ reduction by electron transfer from PSII [33,34]. Hence, the changes in the kinetics of this fast phase are employed as a probing tool to verify the electron exit from PSII in the presence of inhibitors.

3. Results

3.1. Effects of diuron, stigmatellin, and CCCP on fast Chl a fluorescence rise kinetics and ΔA_{830} dark decay

Kinetic curves of the fast Chl fluorescence rise measured under strong actinic light in isolated thylakoids either untreated or treated with various concentrations of diuron (A) or stigmatellin (B) are shown in Fig. 1. Two clear steps were observed in the kinetics of untreated thylakoids (Fig. 1A, trace 1). The first one was the rise of Chl fluorescence from O (F_o) to a level attained in about 1 ms. The second step consisted of a slower development of Chl fluorescence to its maximum attained after about 1-s irradiation. From the position of these characteristic points on the time scale, the first point was identified as J-step (peak) and the second one as P-step [2,8]. Both diuron and stigmatellin significantly affected the O–J–P rise of Chl *a* fluorescence. At a concentration as low as 0.6 μM, diuron increased the magnitudes of O-, and J-step while the following Chl fluorescence rise to P-step was retarded (Fig. 1A, trace 2). Higher concentrations of this inhibitor readily saturated the magnitude of J-step fluorescence yield and completely suppressed the P-peak (Fig. 1A, traces 3 and 4). At concentrations of diuron above 10 μM, Chl fluorescence rapidly reached the J level and was not further modified during the 1-s irradiation (Fig. 1A, trace 5). Notably, the complete suppression of the P-step fluorescence rise by the addition of diuron above 10 μM resulted in the modification of the O–J–P transient into an O–J curve wherein the magnitudes of J- and P-steps were almost similar. The action of stigmatellin on the fast Chl fluorescence rise paralleled the effect of diuron only at higher concentrations (Fig. 1B). Fig. 1B also shows that low concentrations of stigmatellin tend

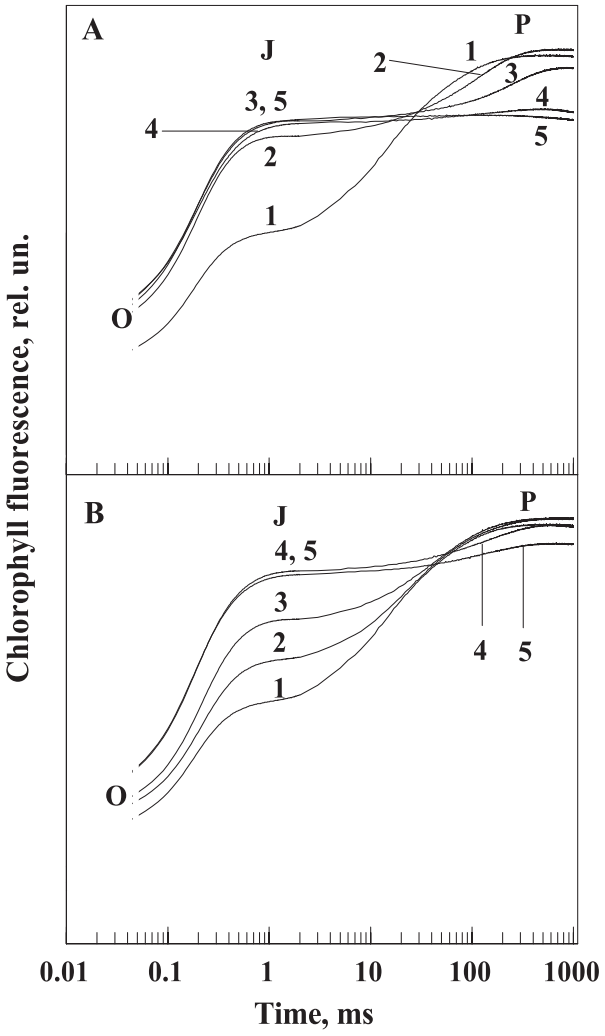

Fig. 1. Original traces of Chl fluorescence induced by red light of 4000 μmol quanta m^{-2} s^{-1} in isolated thylakoids (A) without additive (1) or in the presence of 0.75 μM (2), 1.5 μM (3), 6 μM (4) or 15 μM (5) diuron, and (B) without additive (1) or in the presence of 0.9 μM (2), 3.5 μM (3), 73 μM (4), or 535 μM (5) stigmatellin. In experiments depicted in Figs. 1 and 2, samples were incubated with the corresponding agent for 1 min before trace recordings.

to elevate the amplitude of J-step fluorescence rise. However, higher concentrations of stigmatellin failed to suppress the P-step fluorescence yield completely as observed in the case of diuron-treated thylakoid samples. In effect, the transformation of an O–J–P curve into an O–J transient was not observed even at very high stigmatellin concentration (compare Fig. 1A, trace 5 and Fig. 1B, trace 5).

It is well known that CCCP modifies the donor side reactions of PSII [29,35] whereas ferricyanide accepts electrons from both PSII and PSI. Fig. 2 shows the action of CCCP (A) and ferricyanide (B) on the polyphasic rise of Chl fluorescence. Even low concentration of CCCP enhanced the magnitude of the Chl fluorescence yield at J-step, while the P-step yield remained unaffected (Fig. 2A, traces 1–3). When CCCP concentration increased progressively, the fluorescence yield at J-step continued to increase

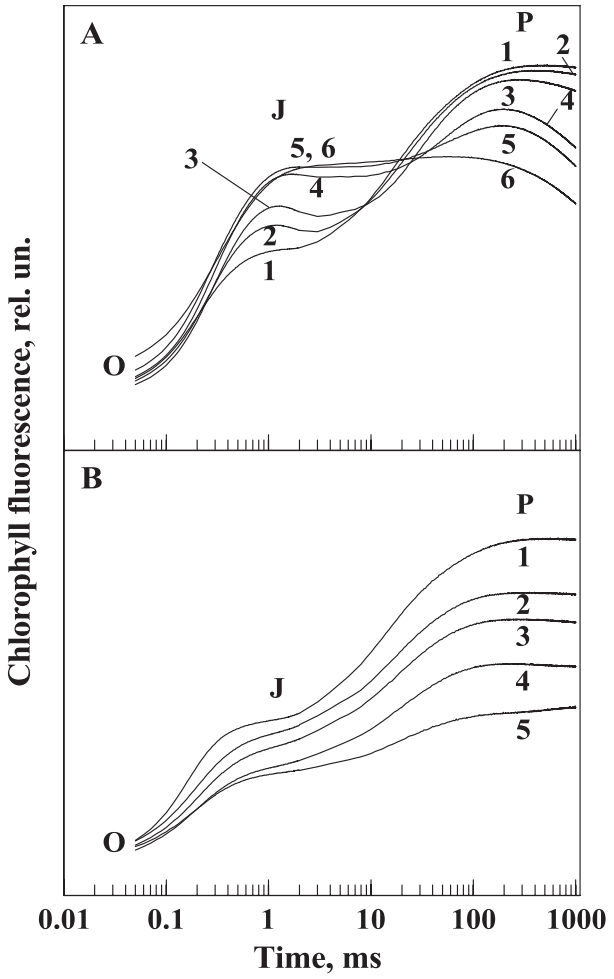

Fig. 2. Original traces of Chl fluorescence induced by red-light of 4000 μmol quanta m^{-2} s^{-1} in isolated thylakoids (A) without additive (1) or in the presence of 0.6 μM (2), 6 μM (3), 30 μM (4), 60 μM (5), or 290 μM CCCP (6), and (B) without additive (1) or in the presence of 1.5 μM (2), 3 μM (3), 6 μM (4), or 12 μM (5) ferricyanide.

but P-step rise was quenched (Fig. 2A, traces 4–6). In thylakoids treated with CCCP at a concentration of about 290 μM, Chl fluorescence continued to increase from O-step until J-step during the first 1 ms of strong illumination. This level of fluorescence yield was maintained for about 200 ms. Beyond that point, high concentrations of CCCP caused a gradual decline in Chl fluorescence. Note that this decline was not observed when high concentrations of diuron were employed (compare trace 5 in Fig. 1A and trace 5 in Fig. 2A).

The action of ferricyanide on the polyphasic rise of Chl fluorescence markedly differed from that of diuron, stigmatellin, or CCCP. Chl fluorescence decreased in all the three steps of the polyphasic rise with increasing concentrations of ferricyanide (Fig. 2B). Importantly, the O level was not quenched by ferricyanide at any concentration used. Thus, the quenching of Chl fluorescence was not related to a screening effect of the colored substance, which could decrease the actual irradiance of excitation light.

To distinguish the possible sites of action of various concentrations of diuron, stigmatellin, and CCCP on electron transport, the kinetics of P700 re-reduction after irradiation of isolated thylakoids with white light was examined. Redox changes of P700 were monitored using absorbance changes at 830 nm (ΔA_{830}). Fig. 3 shows the original traces of ΔA_{830} recorded with control, untreated thylakoids, and in thylakoids treated with various concentrations of diuron (A) and the semi-logarithmic plots of ΔA_{830} dark decay (B). The rate of ΔA_{830} dark decay decreased with increasing concentrations of diuron (Fig. 3A). In untreated thylakoids, the dark decay was much faster and its predominant part (80–85% of the total ΔA_{830} signal) was fitted by an exponential term with a half-time of 24 ms (Fig. 3B, trace 1). The remaining part of ΔA_{830} changes proceeded much more slowly (half-time of about 1 s). In fact, the fast component of ΔA_{830} decay in the dark is attributable to $P700^+$ reduction by electrons transferred from PSII, whereas the slow one originated from the PSI centers that lacked a functional connection to PSII. Indeed,

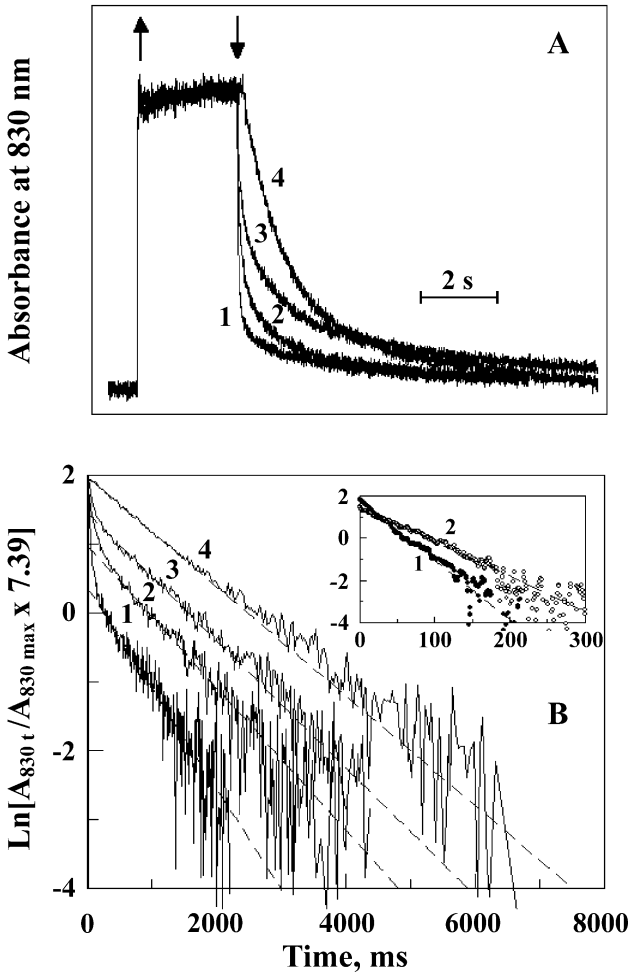

Fig. 3. Original traces of absorbance changes at 830 nm induced by white light of 730 μmol quanta m^{-2} s^{-1} (A) and semi-logarithmic plots of their dark decay (B) in isolated thylakoids without additive (1) or in the presence of 3 μM (2), 4.5 μM (3), or 15 μM (4) diuron.

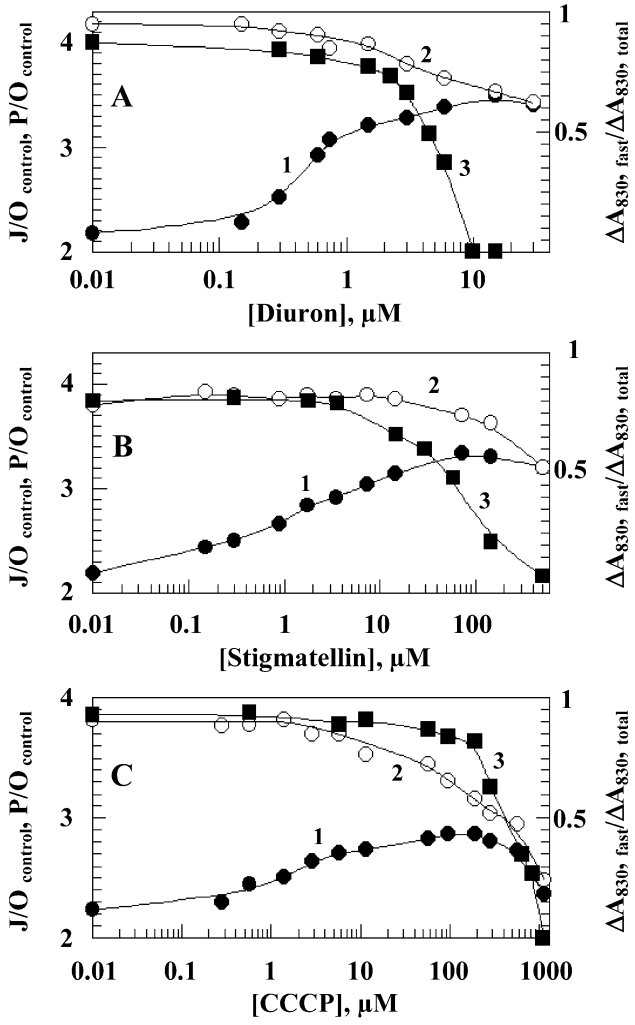

Fig. 4. The influence of various concentrations of diuron (A), stigmatellin (B) and CCCP (C) on J/O (1), P/O (2) and the relative magnitude of the fast component of dark relaxation of absorbance changes at 830 nm induced by white light of 730 μmol quanta m^{-2} s^{-1} (3).

the ΔA_{830} dark decay of the slow component progressively retarded with decreasing ascorbate concentration (data not shown). Hence, the magnitude of this slow component increased as the concentration of diuron was raised (Fig. 3B, traces 2 and 3; inset to Fig. 3B, traces 1 and 2). At high concentration of diuron, the disappearance of the fast component is explained by a complete block in the electron flow from PSII as the acceptor side of PSII is closed (Fig. 3B, trace 4). The effect of stigmatellin or CCCP on the kinetics of ΔA_{830} dark recovery was similar to that of diuron. These compounds did not affect the kinetics at low concentration but decreased the contribution of the fast component of ΔA_{830} dark decay at elevated concentration (data not shown).

Fig. 4 illustrates the action of various concentrations of diuron (A), stigmatellin (B), and CCCP (C) on the magnitudes of Chl fluorescence at J- and P-steps and the relative amplitude of the fast component of ΔA_{830} dark decay. It is clear from Figs. 1 and 2 that an increase in the concentration of anyone of the above inhibitors caused a progressive rise of Chl fluorescence at the J step. Importantly, this effect was observed at concentrations of diuron or CCCP that were insufficient to decrease the contribution of the fast phase of dark recovery of ΔA_{830} to a significant extent (Fig. 4A and C). For stigmatellin, most part of the enhanced levels of fluorescence rise at J-step was also found at concentrations that did not influence the fast phase of ΔA_{830} relaxation (Fig. 4B). Another important feature of the above concentration dependencies is that the major part of the stimulated increase in the magnitude of J level fluorescence rise proceeded at concentrations of diuron, stigmatellin, or CCCP that only weakly affected the magnitude of P-step fluorescence yield.

3.2. Changes in Chl fluorescence rise kinetics upon addition of various inhibitors together with TMPD

The ability of TMPD to initiate the appearance of I-peak in the Chl fluorescence rise kinetics of isolated thylakoids

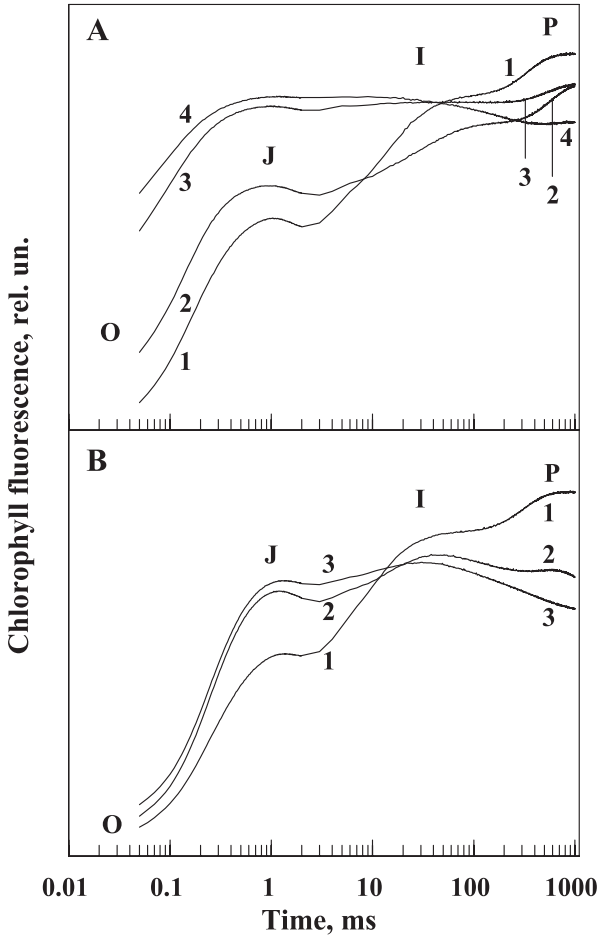

Fig. 5. Original traces of Chl fluorescence induced by red light of 4000 μmol quanta m^{-2} s^{-1} in isolated thylakoids (A) in the presence of 90 μM TMPD added alone (1) or with 0.6 μM (2), 5 μM (3), or 20 μM (4) diuron, and (B) in the presence of 90 μM TMPD alone (1) or in the presence of 50 μM (2), or 90 μM (3) CCCP.

provides a possibility to obtain additional information on the nature of different steps of that process [36]. Unlike untreated thylakoid membranes (see trace 1 of Figs. 1 and 2), the polyphasic rise of Chl fluorescence recorded in the presence of 90 μM TMPD showed the appearance of I-step following J-step (Fig. 5A, trace 1) as observed in intact photosynthetic materials. Addition of small amounts of diuron (0.6 μM) on the background of TMPD elevated the fluorescence yield at O- and J-steps while the P-step rise declined (Fig. 5A, trace 2). When the concentration of diuron was increased above 0.6 μM, the magnitudes of O- and J-step of fluorescence yield enhanced further (Fig. 5A, traces 3 and 4). However, the amplitude of J-step remained almost similar to that of I-step rise found in thylakoids treated with TMPD alone (Fig. 5A, traces 1, 3 and 4). Hence, the P-step fluorescence rise was suppressed.

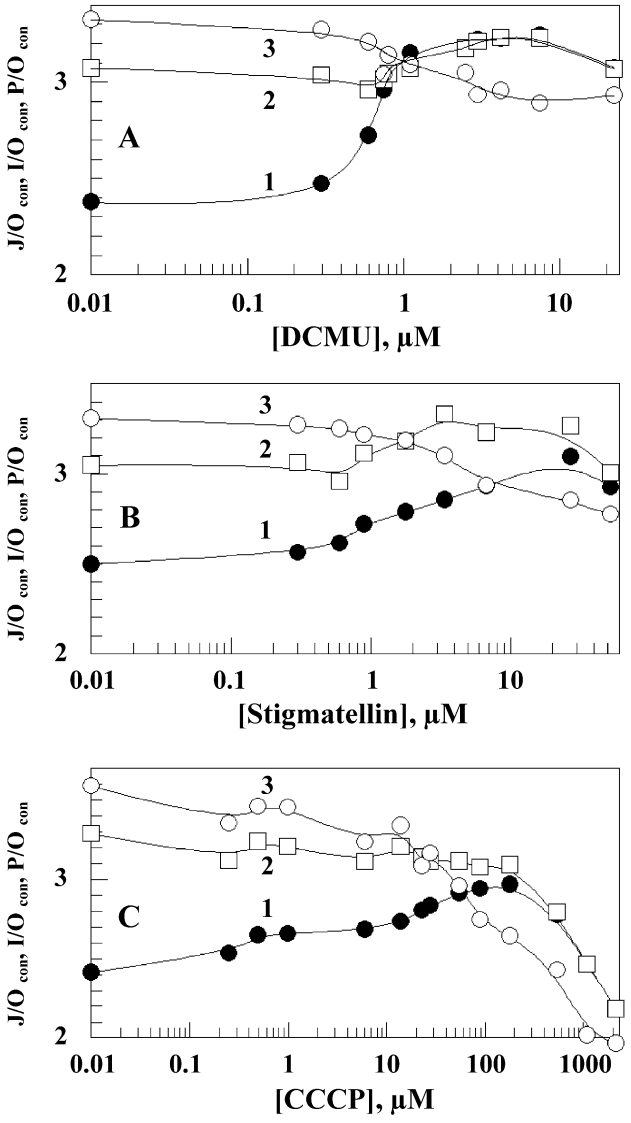

Fig. 6. The influence of various concentrations of diuron (A), stigmatellin (B), and CCCP (C) added together with 90 μM TMPD on J/O (1), I/O (2), and P/O (3).

In the presence of TMPD and 20 μM diuron, Chl fluorescence even declined after 60 ms of exposure to actinic light suggesting the enlarged size of the oxidized PQ pool as a result of plastoquinol depletion by the acceptor [36]. The action of CCCP given on the background of TMPD was similar to that of diuron (Fig. 5B). A weak concentration of CCCP increased the fluorescence emission at J-step while it quenched the fluorescence rise at I- and P-steps (Fig. 5B, traces 1 and 2). With higher concentrations of CCCP, Chl fluorescence gradually reached its maximum at I-step as the P-step rise was diminished (Fig. 5B, trace 3).

The dependencies of the magnitudes of peaks J, I, and P on the concentration of diuron (A), stigmatellin (B), or CCCP (C) added together with 90 μM TMPD are shown in Fig. 6. Similar to its action given alone, an increase of diuron concentration from 0.2 to 1 μM in the presence of TMPD was already sufficient to elevate J-peak to its maximum magnitude, which corresponded to the height of peak I (Fig. 6A, curves 1 and 2). On the other hand, the decline in the magnitude of P-peak was more pronounced if the concentrations of diuron were above 1 μM (Fig. 6A, curve 3). In the presence of TMPD, the increase in the magnitude of J-step fluorescence yield to the level of I-step rise by stigmatellin was concentration-dependent (Fig. 6B). As it exceeded 30 μM, the magnitudes of J- and P-steps were declined. However, the J-step fluorescence rise was elevated by CCCP (up to 200 μM) in the presence of TMPD (Fig. 6C). At concentrations of CCCP above 200 μM, the magnitudes of both J- and I-steps decreased. As was found for CCCP given alone, a decline in the magnitude of peak P was seen once the concentration of CCCP was raised above 1 μM (Fig. 6C). At high concentrations of CCCP, its magnitude dropped below that of peak J, which was not observed for CCCP given alone.

4. Discussion

The O–J rise of Chl fluorescence reflects the photochemical reduction of Q_A, primary quinone acceptor of PSII [5]. In this respect, the action of anaerobiosis [37] or of strong reductants [38], which provides partial or even complete dark reduction of both Q_A and the PQ pool, has been shown to stimulate the magnitude of J peak. A similar action of diuron was ascribed solely to an enhanced Q_A photoreduction owing to prevention of electron transfer from Q_A^- to Q_B, secondary quinone acceptor [1,2,4,8,39]. However, the results of our experiments provide evidence in favor of a significant influence of the modifications in the donor side of PSII electron transport on the magnitude of Chl fluorescence rise at J-step. This became particularly clear from the comparison of the changes in Chl fluorescence kinetics with the independently obtained activity of electron transfer between the two photosystems provided by ΔA_{830} measurements as discussed below.

Diuron and stigmatellin increased the magnitude of J step fluorescence rise at low concentrations (see Fig. 1). According to the literature, these inhibitors exhibit a similar mode of action on electron transport in PSII. Both diuron [24] and stigmatellin [25] affect photosynthetic electron transport at two different sites. Diuron inhibits the donor side of PSII at low concentration [24] whereas the reducing side of PSII is affected at higher concentration. Besides, the action of stigmatellin at low concentration is similar to the effect of diuron on the reducing side of PSII [25]. The data presented in Fig. 4 revealed that the weak concentrations of diuron and stigmatellin did not seem to affect the magnitude of the fast phase of $P700^+$ reduction after white light irradiation, a measure of $P700^+$ reduction by electrons released from PSII [33,40], even though J-step was increased. On the other hand, high concentration of diuron and stigmatellin completely curtailed the electron flow from PSII to PSI (Figs. 1 and 4). High concentrations of CCCP that saturated the increase in J-step fluorescence rise (Fig. 2A) also inhibited the fast phase of $P700^+$ reduction to a large extent (Fig. 4) in a manner similar to diuron or stigmatellin.

The major aspect of the present study is that diuron seems to transform the O–J–P fluorescence induction curve into O–J (J = P) kinetics (Fig. 1A, traces 4 and 5) implying that all Q_A molecules were in reduced state as shown for intact leaves where maximal fluorescence yield at P-step is shifted to lower time scale corresponding to J-step [41]. In fact, a similar conclusion can be drawn for the effect of stigmatellin at high concentrations despite the J-level rise was almost equal to P-step (Fig. 1, trace 5 and Fig. 4). Further, the action of the highest concentrations of CCCP used also showed the same trend (Fig. 2, trace 6). When these inhibitors caused the fluorescence yield to attain maximal level at J-step, a uniform feature was the quenching of the overall fluorescence yield, reminiscent of the complete inhibition of the quantum yield of PSII photochemistry. Indeed, our experimental results presented in (Figs. 1, 2 and 4) unambiguously demonstrated the existence of a close relationship between the increase in J-step rise of Chl fluorescence and decreased contribution of the fast component of P700 reduction kinetics.

The main effect of the three agents used seems to be the accumulation of photochemically reduced Q_A leading to the closure of reaction centers owing to an impaired upstream electron flow beyond Q_A, slow re-oxidation kinetics of the reduced acceptors, and electron donation from the oxidizing side of PSII. The influence of high concentrations of diuron, high enough to curtail the reduction of PQ and, therefore, the contribution of the fast component of $P700^+$ reduction, is not surprising as this herbicide is known to block the linear electron transport from Q_A to Q_B. Evidently, diuron-induced progressive decline in the maximal Chl fluorescence is explainable by the non-photochemical quenching exerted by oxidized PQ (Fig. 1, traces 3–5). As the upstream photosynthetic electron transport is being inhibited, re-oxidation of the long-lived Q_A^- ($P680Q_A^-$ state) might proceed only through a competing recombination mechanism involving the positively charged S_2 state of the OEC. This back reaction is much slower with kinetics in the order of several seconds in comparison with the recombination of Q_A^- with $P680^+$ that takes place in the microsecond timescale. Thus, the data presented here strongly suggests that light induced accumulation of oxidized P680, a quenching state, is prevented as a consequence of the accumulation of an irreversible high fluorescent non-quenching state ($S_1Y_ZP680Q_A^-$) when reduced Q_A recombines with S_2 state. The quenching of J-step fluorescence rise in PSII centers with inactivated OEC occurs only when the low fluorescent state $Y_Z^+P680^+Q_A^-$ is expected to accumulate as corollary to an imbalance between the rate of electrons reaching the reaction centers from the donor side and the speed at which the electrons are leaving the reaction centers on the acceptor side [13]. In this case, a new step, the K-step followed by a dip, appears as the J-step rise is quenched [13]. Also the dip followed by K-step rise occurs only when the reaction centers are reopened. Clearly, the observed increase in the fluorescence rise at J-step in the presence of diuron is mainly caused by slower oxidation kinetics of Q_A^-.

A progressive increase in J-step fluorescence yield caused by increasing concentrations of stigmatellin (up to 75 μM) reflected a block in the re-oxidation of plastoquinol (Fig. 1B, traces 1–4) as this inhibitor binds to the Q_o site of the cytochrome b_6/f complex. Moreover, the changes in redox state of PQ are known to affect the redox state of Q_A. Therefore, a block in the re-oxidation of PQH_2 would effectively hamper the oxidation of reduced Q_A. Notably, the half-time for the Q_A^- oxidation is around 2–3 ms if the Q_B site is empty or several tens of milliseconds if Q_BH_2 (plastoquinol) forms prevail, which is much higher when compared to the half-times for Q_A^- to Q_B or Q_B^- electron transfer [31]. However, a significant loss in the P-step fluorescence rise caused by higher concentrations of stigmatellin, which was almost equal to the effect seen with diuron, was therefore related to the quenching by oxidized PQ (Figs. 1 and 4B). Thus, this inhibitor acts like diuron at higher concentrations and tends to increase the fraction of closed PSII centers.

The data presented in Figs. 2 and 4 indicate that the changes in the kinetics of Chl fluorescence brought out by CCCP were similar to the action of diuron or stigmatellin. At concentrations as low as 0.24 μM, CCCP is known to abolish the period four oscillation by destabilizing the higher S-states [31]. This effect also segregates the period two oscillation corresponding to the two-electron gate at the PSII acceptor side from the S-state cycle. Further, CCCP efficiently competes with PQ at the Q_B binding site as a low affinity inhibitor. It has been suggested that in a fraction of PSII centers, CCCP occupies the Q_B site prior to illumination [30]. The concentration of CCCP (around 0.6 μM) used in the present study is sufficient to increase the J-step fluorescence rise without affecting the thermal phase (Fig. 2A, trace 2). When this concentration was increased by 10-

fold, the fluorescence yield at J-step elevated further (Fig. 2A, trace 2) and the P-step rise was not quenched (Fig. 2A, trace 2). A significant variation in the Chl fluorescence yield in thylakoids exposed to CCCP was closely related to the redox state of the OEC. It has been proposed that the highest magnitude of Chl fluorescence was attainable only after the first flash that corresponded to the transition of S_0 and S_1 (25:75) to S_1 and S_2 states, whereas the accumulation of S_2 and S_3 states of the OEC following additional flash decreases the Chl fluorescence yield, the so-called S-state quenching [3]. As predicted by flash experiments, if the deactivation of higher S-states to lower S-states was accelerated by CCCP, then the observed increase in the fluorescence rise at J-step (Fig. 2A, traces 2 and 3) was related to a decreased S-state quenching. Thus, the effect of CCCP included stable accumulation of reduced Q_A because the major routes of Q_A-reoxidation were suppressed.

A marked decline in the fluorescence yield after P-step caused by high concentrations of CCCP indicated the inability of thylakoids to keep the PQ pool in reduced state under light (Fig. 2A) since only four electrons located on P680, Y_Z, S_0, and S_1, respectively, could be delivered to the PSII centers against the estimated requirement of 10–12 electron equivalents per PSII reaction center for the complete reduction of the PQ pool [7].

TMPD acts as a PSII electron acceptor and modifies the O–J–P type of Chl *a* fluorescence rise into O–J–I–P kinetics in which I-step of the thermal phase is clearly resolved [36]. In the presence of a very low concentration of diuron that is insufficient to block the electron transfer from Q_A to Q_B, the elevation in the J-step fluorescence rise was unaffected (Fig. 5A, trace 2) whereas the I- and P-levels of fluorescence yield were quenched. Strikingly, this finding indicates that the diuron-induced change in the redox state of Q_A was unaltered by TMPD. Besides, this electron acceptor also enlarges the size of oxidized PQ pool when the upstream electron transfer from Q_A is partially inhibited. On the other hand, a major effect of TMPD was to fortify the magnitude of the O-step fluorescence level (Fig. 5A, traces 3 and 4) in the presence of higher concentrations of diuron which were quite enough to prevent the electron transfer from Q_A. This was followed by saturated levels of J- and I-step rise and the onset of decay after I-step. The observed increase in the fluorescence yield at O-step can be ascribed to the presence of a substantial fraction of the Q_B molecules in the Q_B^- state (reduced state) in dark-adapted thylakoids and reduced Q_B must be re-oxidized before binding of diuron in the Q_B pocket [6]. Also, the I-step fluorescence rise is characterized by the accumulation of the $Q_A^-Q_B^-$ and $Q_A^-Q_B^{2-}$ states [42]. Taken together, it is likely that the TMPD-induced large increase in the fluorescence yield at O-step is largely attributable to enhanced proportions of Q_B in its reduced state.

The S-state transitions proceed with a half-time of about 30 μs for S_0 to S_1, 110 μs for S_1–S_2, 350 μs for S_2 to S_3, and 1300 μs for S_3 to S_0, respectively [43,44], in parallel with the advancement of one-electron oxidation steps in the manganese cluster. It should be mentioned that the S-state conversions correlate with the fluorescence rise at O-step, although this is not well characterized yet [13,45,46]. According to Schreiber and Neubauer [45], the O-step rise is maximal in intact spinach chloroplasts if preilluminated with a saturating pulse. Also the amplitudes of the O-step fluorescence yield following a saturating flash and two consecutive flashes were almost similar (see Fig. 3 in Ref. [13]). The above considerations indicate that TMPD can also interfere with the redox state of the S-state cycle on the donor side of PSII. However, this remains to be clarified as the fluorimeter used in the present study does not permit the direct analysis of these two S-state transitions occurring during the first 10–100-μs time-span more precisely. We also remark that the mode of TMPD action is not fully characterized yet and the data obtained in its presence must be taken with circumspection.

An important consequence of the TMPD induced changes in the presence of diuron is the retardation of the re-oxidation of Q_A^- by several seconds in the absence of a light-induced forward linear electron flow in PSII if the recombination partners of the acceptor side or positively charged S-states of the PSII donor side are unavailable [36]. In such a case, Q_A^- might recombine with molecular oxygen as reflected by a progressive decline in fluorescence yield following I-step (Fig. 5A, trace 4).

The fluorescence rise at O-step by TMPD in the presence of CCCP was not elevated to the extent observed in thylakoids added with the former agent with high concentrations of diuron (Fig. 5B, traces 1–3). The above strongly advocates that the effect of TMPD on O-step yield is prevented if CCCP competes for the suppression of S_2 to S_3 transition and also hinders PQ binding at the Q_B pocket. Moreover, the binding affinity of this ADRY agent to Q_B is weaker than that of diuron. Therefore, the kinetics of O–J–I–P rise was not altered by TMPD added together with CCCP (compare Figs. 2A and 5B). As the period four oscillation is being abolished by CCCP, the recombination of Q_A^- is likely to proceed through an alternate route as mentioned for thylakoid samples treated with diuron in the presence of TMPD. Importantly, this reaction competes with the uptake of molecular oxygen during the photooxidation of TMPD [36].

Chl fluorescence rise from J- to I-step in intact leaves is shown to reflect the light-induced removal of non-photochemically quenched Chl fluorescence yield [8]. It has been proposed that the modulation of the J- and I-steps of Chl fluorescence rise is attributable to changes in thylakoid membrane potential [47,48]. All agents used in our experiments influenced the electron transport properties of PSII rather than directly affecting thylakoid membrane potential. However, nigericin, which is also an effective protonophorous uncoupler like CCCP, had no effect on the Chl fluorescence rise kinetics (data not shown).

The key aspect of the present study is that the action of CCCP, diuron and stigmatellin appears to enhance the fluorescence yield at J-step. In fact, CCCP can remove S-state quenching. However, it is unclear whether the increased fluorescence yield at J-step caused by diuron or stigmatellin is the consequence of a removal of S-state quenching that would indicate an inhibitory site at the donor side of PSII. Alternatively, the action of these two inhibitors on J-level fluorescence rise might be strictly due to the increased reduction of Q_A.

Inhibition of the donor side of PSII inevitably slows down and prevents the reduction of the PQ pool, which requires multiple turnover excitations of PSII centers [8]. This is accompanied by changes in I–P phase of Chl fluorescence rise. In fact, even low concentrations of diuron and stigmatellin retarded the second part of the thermal phase (Figs. 1 and 5). There are several studies in which a diuron-induced increase of Chl fluorescence at J-step to the level of P-step of untreated thylakoids has been reported [4,41,49]. In contrast, the present work shows that diuron increases Chl fluorescence only to I level as shown in the presence of TMPD (Fig. 5), which is in agreement with an early paper by Neubauer and Schreiber [2]. The latter result is explained as follows. As diuron blocks the electron transfer from Q_A^- to Q_B, no reduction of the PQ pool can occur and «plastoquinone»-type non-photochemical reduction prevents the light-induced increase of Chl fluorescence above I level. Occurrence of Chl fluorescence to the level of P-peak in diuron-treated thylakoids is in direct conflict with the notion of ''plastoquinone''-type non-photochemical quenching. This discrepancy will merit further clarification.

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