Chlorophyll *a* fluorescence transient as an indicator of active and inactive Photosystem II in thylakoid membranes

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Upon illumination, a dark-adapted photosynthetic sample shows time-dependent changes in chlorophyll (Chl) *a* fluorescence yield, known as the Kautsky phenomenon or the OIDPS transient. Based on the differential effects of electron acceptors such as 2,5-dimethyl-*p*-benzoquinone (DMQ) and 2,6-dichloro-*p*-benzoquinone (DCBQ) on Chl *a* fluorescence transients of spinach thylakoids, we suggest that the OID phase reflects the reduction of the electron acceptor Q_A to Q_A^- in the inactive PS II (see Graan, T. and Ort, D. (1986) Biochim. Biophys. Acta 852, 320–330 [14]). In spinach thylakoids, heat-induced increase of the Chl *a* fluorescence yield is also differentially sensitive to the addition of DMQ and DCBQ suggesting that this increase is mainly on the 'I' level, and thus heating is suggested to convert active PS II to inactive PS II centers. The kinetics of decay of Q_A^-, calculated from variable Chl *a* fluorescence, was analyzed into three exponential components (365–395 μs; 6–7 ms; and 1.4–1.7 s). In heated samples, the decay rate of variable Chl *a* fluorescence is slower than the normal back-reaction rate; there is a preponderance of the slow component that may be due, partly, to the inactive centers undergoing slow back reaction between Q_A^- and the S_2 state of the oxygen-evolving complex.

Introduction

Kautsky and Hirsch [1] observed changes in the time-course of chlorophyll *a* (Chl *a*) fluorescence when a dark-adapted photosynthetic sample was illuminated with light. Since that time Chl *a* fluorescence transient (known as the Kautsky phenomenon) has been used as a non-destructive, sensitive tool for monitoring various processes in photosynthesis [2–5]. However, this application has been fraught with complexities. For example, even the nature and the meaning of the fast OIPD (see below for explanation of symbols) fluorescence changes have been debated, leading to contradictions in the literature [6].

The 'O' (for origin) level (also called F_0), the so-called constant fluorescence, is the level obtained instanteously (within 1 ns) of illumination, when all Q_A is in the oxidized state; it is the minimum fluorescence in Photosystem II (PS II) membranes. In a model in which excitation can travel randomly among all the pigments ('lake model') (however, see Eqn. 2):

$$F_0 = F_{\text{minimum}} = \phi_{f\ \text{minimum}}\ (I_{\text{abs}}) = \frac{K_f}{K_f + K_h + K_t + K_p[Q_A]}(I_{\text{abs}})$$

(1)

where $\phi_{f\ \text{minimum}}$ = minimum quantum yield of fluorescence, I_{abs} = number of photons absorbed, K_f = rate constant of fluorescence, K_h = rate constant of radiationless (heat) loss, K_t = rate constant of excitation energy transfer, K_p = rate constant of photochemistry, and $[Q_A]$, the concentration of the electron acceptor bound plastoquinone Q_A of PS II; maximum $[Q_A]$ is taken as 1. In thylakoid membranes, the measured F_0 at room temperature also includes a minor (about 20%) contribution from PS I Chl *a* fluorescence [7]. The initial fluorescence level, measured by most instruments, is called F_i (for F_{initial}) unless it is proven to be F_0. The major criterion for F_i to be F_0 is the indepen-

dence of its yield ($\phi_{f \text{ minimum}} = F_0/I_{abs}$) on exciting light intensity and the addition of electron acceptors [8]. Under these conditions, changes in F_0 can be ascribed to changes in the heat loss (K_h) or in excitation energy transfer (K_t) provided K_f remains constant, i.e., the absorption spectra remain unchanged. The OID phase (where I or F_I stands for fluorescence intensity at the first inflection or intermediate peak, and D stands for dip or plateau; ID is alternatively called pl for plateau [9]) was suggested [10,11] to arise from the interplay of PS II and PS I in intact algal cells, due to the transient changes in the $[Q_A^-]$ in the main pathway of photosynthesis. Furthermore, the following D to P (P for peak) rise reflects the net accumulation of $[Q_A^-]$ as the plastoquinone pool is reduced, and, there is a 'traffic' jam in the flow of electrons all the way up to the electron acceptors of PS I [10]. Addition of the electron acceptor methyl viologen, that accepts electron from PS I, abolishes the D to P rise, but not the OID phase [10] (see Ref. 12 also). Melis [13] suggested, without proof, that the initial chloroplast fluorescence rise from F_0 to F_I was identified as the variable fluorescence yield controlled by a type of Photosystem II center called PS IIβ. Graan and Ort [14] showed that the number of PS II centers capable of active water oxidation was increased by a chloroquinone 2,6-dichloro-p-benzoquinone and closely matched the number of herbicide binding sites. However, the number of PS II centers active in water oxidation was much lower when methyl-substituted benzoquinone, 2,5-dimethyl-p-benzoquinone (DMQ) was used as an electron acceptor. These experiments were explained by the hypothesis of the existence of two types of PS II center, the active and the inactive centers, the inactive centers being the ones that are ineffective in electron flow to plastoquinone and, thus, inactive in net electron flow and water oxidation. The nature of these active and inactive PS II centers has been recently examined, by independent means, in Refs. 15 and 16. In spinach thylakoids, the majority of the PS II reaction centers (68%) recover in less than 50 ms. However, approx. 32% of the PS II reaction centers require a halftime of 2–3 s to recover. In spinach leaves, the halftime of the oxidation of Q_A^- in the inactive PS II centers is 1.5 s [16].

In this paper, we used DCBQ and DMQ to investigate their effects on the OID phase of fluorescence transient in order to assess their relationship to the active and inactive centers, as defined earlier [14]. This has allowed us to obtain information on the nature of the OID phase that had remained controversial. We show here that DCBQ is able to quench the F_I much more effectively than DMQ, while F_0 remains unchanged. Since DCBQ was shown [15] to intercept electrons from the 'inactive' PS II centers, we suggest that the OID phase reflects the reduction of Q_A to Q_A^- in the inactive PS II centers. We also show that in

spinach and soybean thylakoids, high temperature (55°C) induced increase in fluorescence yield (e.g., F_{50ms}, fluorescence at 50 ms) is quenched differentially by the electron acceptor DCBQ and DMQ. The amplitude of F_{50ms} in 55°C-treated samples decreases proportionally by DCBQ addition, but it is much less affected by DMQ. Thus, we suggest that the heat-induced increase in fluorescence yield is due mainly to the increase in F_I level owing to the conversion of active PS II to inactive PS II centers. In addition, decay kinetics of Q_A^-, which is calculated from the Chl a fluorescence decay data, with and without DCMU after one flash is analysed in terms of a multiple exponential decay model. In the heated chloroplasts, we observed a slower decay than the normal slow component, which is suggested to reflect the back reaction between Q_A^- and the S_2 state. We attribute it to an additional heat effect on an inhibition of the S-state turnover in the oxygen-evolving system.

Material and Methods

Chlorolast thylakoid membranes were isolated from commercial spinach and growth chamber soybean as described [14,17]. Soybean plants were grown at 30°C/20°C day/night temperatures and 10 h/14 h day/night photoperiod. The Chl concentration of chloroplast suspension was determined by measuring absorbance at 664 nm and 647 nm [17,18].

Thylakoids were suspended in a reaction medium containing 0.4 M sorbitol, 5 mM Mes-KOH (pH 6.5), 20 mM KCl, 2 mM $MgCl_2$, and 1 μM nigericin when fluorescence measurement was made. The Chl concentration was 39 μM. The suspension buffer for heat treatment contained 0.4 M sorbitol, 0.5 mM Mes-KOH (pH 6.5), 20 mM KCl and 2 mM $MgCl_2$. The (55°C) heat treatment time was 1 min for spinach thylakoids and 6 min for soybean thylakoids in a water-bath (Beckman, Lauda RC3) kept in the dark.

The Chl a fluorescence transient was measured in a home-built spectrofluorometer [19] with modifications. The light from the exciting lamp (Kodak Carousel 4200 projector) was filtered with Corning CS 5-56 and CS 4-76 blue filters. The fluorescence was filtered with a Corning CS 2-61 red filter before it entered a monochromator set at 685 nm (slit widths: 4 mm; band pass: 13.2 nm). A EMI (9958 B) photomultiplier (S-20 response) was used as a photodetector. The output signal was digitized with 8 bit precision by a Biomation Model 805 waveform recorder and stored on an LSI 11 minicomputer (Digital Equipment Corporation) (see details in Ref. 19).

The kinetics of decay of variable Chl a fluorescence was measured at 685 nm (10 nm bandwidth) by a weak measuring light. The measuring light was fired at variable times after each actinic flash. The actinic (FX-124,

EG and G) and the measuring flashes (Stroboslave 1593A, General Radio) were filtered with Corning blue (CS 4-96) glass filters; both had a 2.5 μs duration at half-maximal peak (see details in Ref. 20).

Q_A^- concentration was calculated from variable Chl a fluorescence according to Joliot and Joliot [21] using the formula given in Ref. 22; there is a hyperbolic relationship between the fluorescence yield $F(t)$ and the fraction $q(t)$ of closed reaction centers at time t:

$$\frac{F(t) - F_o}{F_{max} - F_0} = \frac{(1 - p)q(t)}{1 - pq(t)} \tag{2}$$

where F_{max} is the maximum fluorescence yield when all Q_A is in the reduced state, F_o is the minimum fluorescence yield when all Q_A is in the oxidized state, and p is the connection parameter which reflects the probability of the intersystem energy transfer from a closed unit to any other unit in the system. In this work, we assumed that the p was 0.5, as measured earlier for chloroplasts [23]. Thus, $q(t)$ could be presented explicitly as:

$$q(t) = \frac{2 F_v (t)}{F_v^{max} + F_v (t)} \tag{3}$$

where $F_v (t) = F(t) - F_0$ and $F_v^{max} = F_{max} - F_0$.

In our multiple exponential decay model, the Q_A^- concentration at time t, $q(t)$ is given by a sum of N exponential components:

$$q(t) = q(0) \sum_{i=1}^{N} \alpha_i \exp(- t/\tau_i) \tag{4}$$

where $q(0)$ is the Q_A^- concentration at time 0, α_i is the amplitude and τ_i the lifetime of the ith component. The fitting of the above Eqn. 4 with the experimental data was carried out by a least-squares program using iterative method (Marquard search algorithm) [24]. Quality of fits were judged by the reduced χ^2 (Ki^2) criterion and the plot of the weighted difference between theoretical fit and the experimental data, normalized to the square root of the data points [25].

Results

Fig. 1 shows the Chl a fluorescence transients in the spinach thylakoids in the presence of different concentrations of DMQ (top graph) and DCBQ (bottom graph). In the control without exogenous quinones, the curve shows clearly the various characteristic points, namely OIDP [2–6]. I and P levels occur at 50 ms and 3 s after the start of illumination, respectively. In both spinach and soybean (data not shown) thylakoids, increasing (2.5 to 20 μM) concentrations of DMQ and DCBQ affect Chl a transient differently. DMQ
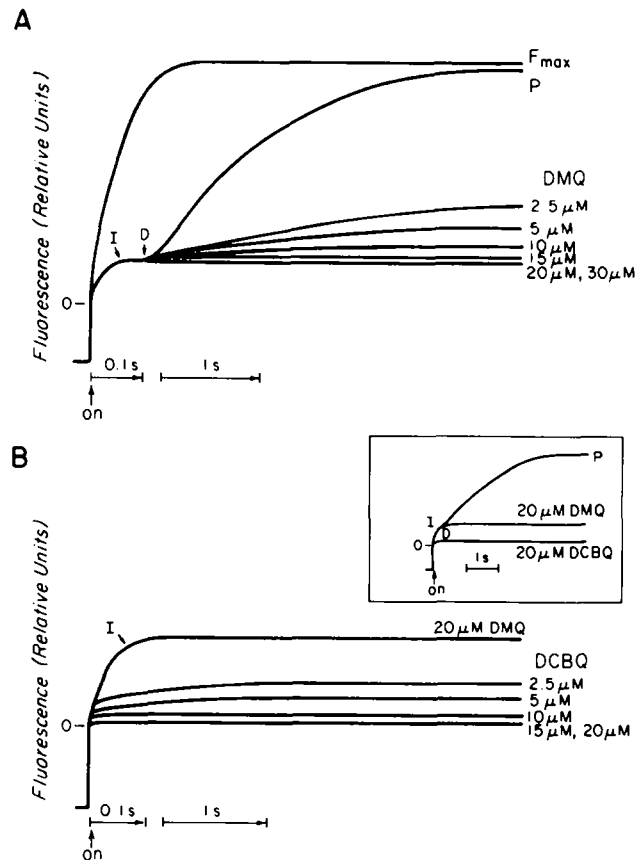

Fig. 1. Chlorophyll a fluorescence transients of spinach thylakoids in the presence and absence of different concentrations of DMQ and DCBQ at 20 °C. The thylakoids were suspended in a reaction medium containing 0.4 M sorbitol, 5 mM Mes-KOH (pH 6.5), 20 mM KCl, 2 mM MgCl$_2$ and 1 μM nigericin. The Chl concentration was 39 μM. The inset shows the fluorescence transient of control and 20 μM quinone-treated thylakoids. The thylakoids were dark-adapted for 10 min before the fluorescence measurement. O, constant fluorescence; I, intermediate peak fluorescence; D, fluorescence yield at the plateau or the dip; P, fluorescence yield at the peak (maximum). In (A), the fast-rising curve is with 1 μM DCMU; F_{max} is the maximum fluorescence level. See list of abbreviations for the full forms of DMQ and DCBQ.

quenches only the DP rise, where DCBQ is able to quench, in addition, OI phase. Fig. 2 is a plot of the dependence of the fluorescence yields at 50 ms and 3 s after the start of illumination on quinone concentration. DMQ quenches the P (or F_P) level greatly, but does not affect the I (or F_I) level (Figs. 1 and 2A). The quenching effect on F_P is saturated at 20 μM DMQ. On the other hand, DCBQ not only quenches F_P but the F_I level also (Fig. 1 and Fig. 2B). This quenching effect is saturated at 15 μM DCBQ. However, the saturated concentration of DCBQ does not affect the O (or F_o) level at all. In these calculations, the values of F_0 was obtained by the value of F_i at 30 μM DCBQ; this value was confirmed by measurements of F_0 by two other independent methods [6] (extrapolation of F_i rise to
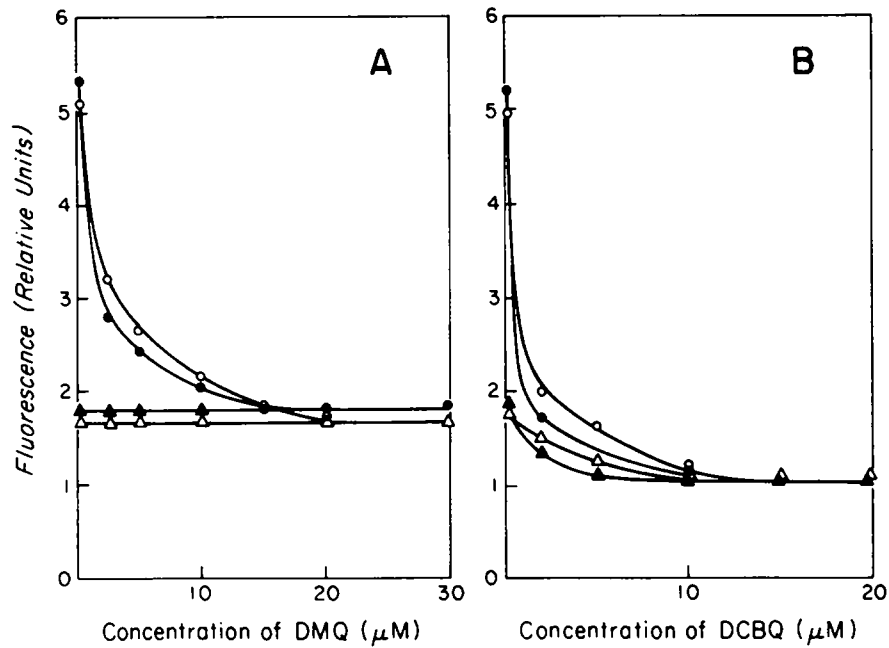
Fig. 2. Effects of increasing concentrations of DMQ (A) and DCBQ (B) on Chl a fluorescence yields at 50 ms and 3 s in thylakoid membranes. Fluorescence at 50 ms (▲,△) and 3 s (●,○) in spinach (▲,●) and soybean (△,○) thylakoid membranes. F_0 level is normalized to 1.

zero time and independence of fluorescence yield with light intensity, data not shown).

In order to quantify the quenching effect of the quinones on fluorescence yield at 50 ms (F_{50ms}), the amplitude of ($F_1 - F_0$) is normalized as 100 units and the quenching of fluorescence F_{50ms} by quinone is

calculated (Fig. 3). Quenching is absent when no exogenous quinone is added. In DCBQ treated thylakoids, the concentration of DCBQ at which $F_1 - F_0$ is half
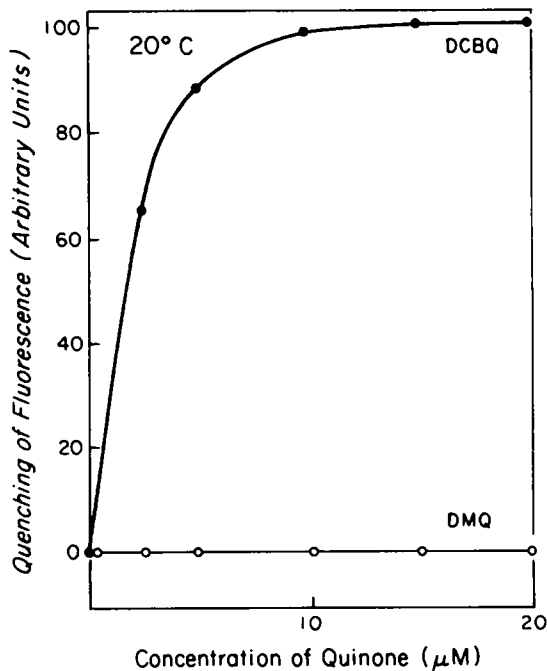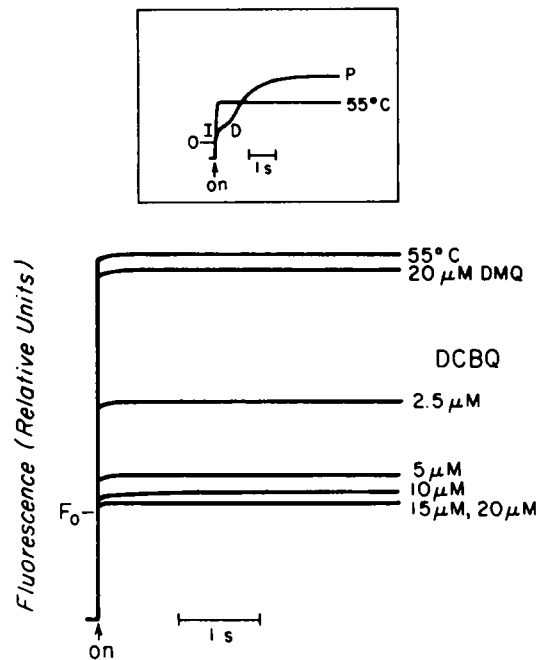

Fig. 3. Quenching of Chl a fluorescence at 50 ms by DCBQ (solid circle) and DMQ (open circle) in spinach thylakoid membranes. The amplitude difference ($F_1 - F_0$) is normalized to 100. The quenching of fluorescence yield is the decrease in fluorescence yield from the F_1 level.

Fig. 4. Chlorophyll a fluorescence transients of heat-treated thylakoid membranes of spinach and the effects of DMQ and DCBQ on the fluorescence yield. Inset (at top) shows fluorescence transients at 20 °C and 55 °C. The thylakoid membranes were heated for 1 min in a medium consisting of 0.4 M sorbitol, 5 mM Mes-KOH (pH 6.5), 20 mM KCl and 2 MgCl₂. Then the thylakoids were resuspended in the same medium with added 1 μM nigericin when the fluorescence transient was measured at 20 °C. F_0 is the constant fluorescence yield. See the legend of Fig. 1 for other abbreviations.
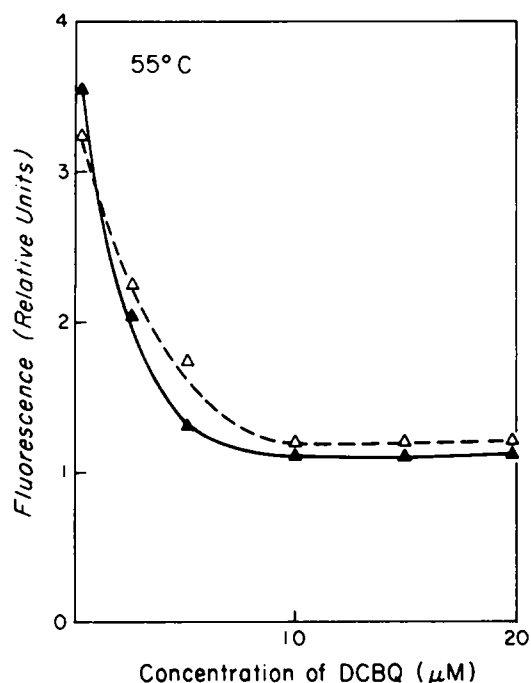
Fig. 5. Fluorescence yield at 50 ms as affected by DCBQ in heat-treated spinach (▲) and soybean (△) thylakoid membranes. F_0 level is normalized to 1.

quenched is 2.5 μM for spinach; however, F_{50ms} does not decrease with increasing concentration of DMQ in the micromolar range. This concentration dependence of F_{50ms} on DCBQ is in quantitative agreement with the earlier concentration dependence data [14] on the increase in the number of PS II centers capable of water-oxidation in the presence of DCBQ in spinach thylakoids. In soybean thylakoids, 3.5 μM of DCBQ is needed in order to quench 50% of the amplitude from F_0 to F_1 (data not shown).

Heat treatment caused a significant change in Chl a fluorescence transient in comparison to the control at 20°C in spinach (Fig. 4; measurements at 20°C). The normal clear OID characteristic points disappeared and instead a fast rise of fluorescence yield occurred with treatment at 55°C (see Methods). Fig. 5 (see also Fig. 4) shows the quenching effect of DCBQ on F_{50ms} in heat-treated chloroplasts. When DCBQ was added to the heat-treated samples, we found that the heat-induced fluorescence yield declined as [DCBQ] increased and that the quenching effect of fluorescence by DCBQ is proportional to its concentration as in the thylakoids at 20°C (cf. with Fig. 3). However, in heat-treated samples, even 15–20 μM DCBQ could not totally quench fluorescence yield to its F_0 level as in the samples at 20°C. These residual small increases in fluorescence yield may reflect heat-induced rise in F_0 level. Again DMQ was an ineffective quencher here (Fig. 4). Thus, based on the present data on both DCBQ and DMQ and comparison with data at 20°C,

we suggest that the increase in fluorescence yield due to heating is mainly due to the rise in 'I' level, 97% in spinach thylakoids. Soybean thylakoids give similar results (data not shown). This heat-induced rise in the 'I' level is due to an increase in the inactive PS II centers. Only 5% or less of heat-induced fluorescence rise is due to increase in the 'O' level.

The top graph (A) in Fig. 6 shows the data on decays of the variable Chl a fluorescence after one flash (flash 5) in spinach thylakoids. The first data point is at 70 μs. The F_0 levels used in these calculations are true F_0 levels, not the measured F_i levels. When 10 μM DCMU is added, the forward reaction of electron flow from Q_A^- to Q_B is known to be totally blocked and a slow decay of variable Chl a fluorescence is observed (see curves with upside-down triangle, and in Refs. 26, 27). Heat treatment (55°C, 1 min; spinach) causes an even slower decay (curve with (triangle). Furthermore, in agreement with the conclusion that 55°C treatment does not simply increase the 'O' level, we observed a significant
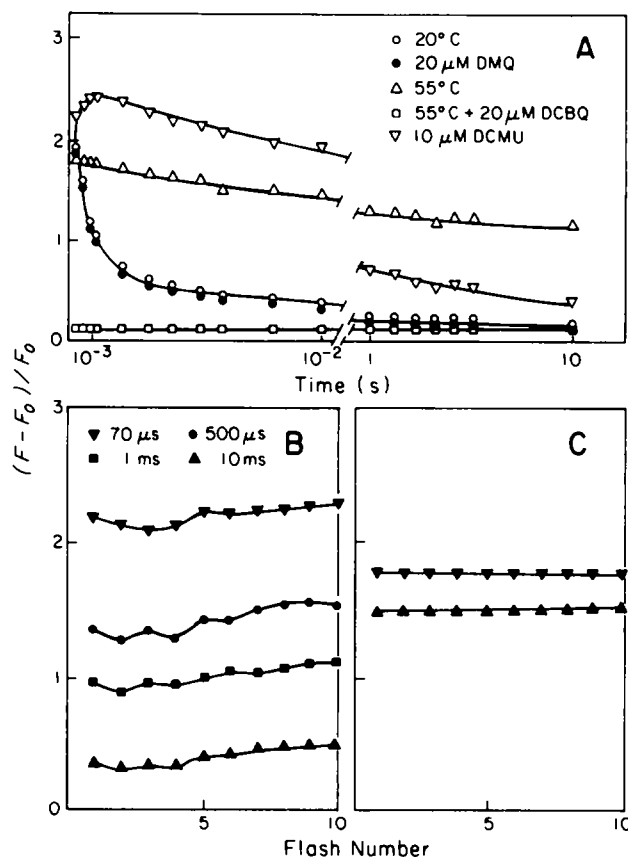

Fig. 6. Decay of variable Chl a fluorescence of spinach thylakoids after the fifth flash (A). Fo is the Chl a fluorescence yield from the measuring flash with all Q_A oxidized, and F is the fluorescence yield at the indicated time after the actinic flash. The actinic flash frequency was 1 Hz. (○): 20°C; (●): 20 μM DMQ; (△): 55°C; (□): 55°C + 20 μM DCBQ; (▽): 10 μM DCMU. (B) Variable Chl a fluorescence as a function of flash number at 20°C; (C) Variable Chl a fluorescence as a function of flash number at 55°C. The times indicated are when the measuring flash was fired.

variable fluorescence (curves with triangle). 20 μM DMQ has little effect on variable fluorescence decay at 20°C (cf. curves with open and closed circles) as is observed with the addition of electron acceptor methyl viologen [20]. Both abolish the DP fluorescence rise during fluorescence induction, but show no effect on fluorescence decay after an actinic flash. This is simply because the electron flow from Q_A^- to Q_B is faster than the electron acceptance by these acceptors at a more distant site. However, the addition of 20 μM DCBQ quenches the fluorescence level ($t \leqslant 70$ μs) to be close to the F_0 of the control (20°C). This suggests an efficient electron acceptance by DCBQ from close to the Q_A^- site.

The fluorescence as a function of flash number, at various times after the flash, is shown in the bottom graph (B) of Fig. 6. In a control sample (20°C), a complex oscillation pattern that is superimposition of a binary oscillation (due to the operation of the two electron gate on the electron acceptor side of PS II) and an oscillation of period four (due to the turnover of the S state in the oxygen-evolving system) is observed (see, for example, Ref. 20). Heat treatment, however, damps those oscillations significantly (see Fig. 6C).

Q_A^- decay involves multiple processes (see, for example, Ref. 28). By using exponential decay models, we quantitate our results by describing the decay of Q_A^- in terms of three decay components representing different electron transport pathways. We realize that in reality these processes may not be necessarily independent of each other or even follow the first-order reaction. The experimentally measured Chl a fluorescence data were converted to Q_A^- using Eqn. 3. Q_A^- decay data as a function of time were fitted by Eqn. 3 with $N = 3$ (see Material and Methods). In Fig. 7, the best fit for the three-exponential model is superimposed on the experimental decay curve in spinach thylakoids. A plot of the
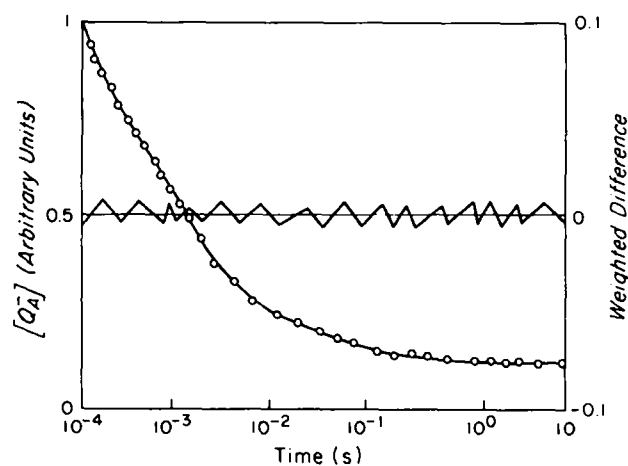
weighted difference between the experimental data and the best fit is also shown. The calculated amplitudes and halftimes are summarized in Table I. The residual plot and K_i^2 show that the best fit by a three exponential decay match the Q_A^- data very well and the deviations are due only to statistical noise. In both spinach and soybean thylakoids, large portion (60–62%) of Q_A^- decay is through the fast component (halftime 365–395 μs). The amplitudes of the intermediate component (halftime 6–7 ms) and the slow component (halftime 1.4–1.7s) are 18% and 20%, respectively. In DCMU-treated samples, reduced Q_A recombines with S_2 state after one actinic flash; the halftime of Q_A^- decay is 1.5–1.6 s. This value fits the slow component in the DCMU-free samples. In addition, an initial rise in variable fluorescence is observed in the presence of 10 μM DCMU (Fig. 6). In spinach and soybean chloroplasts, the lifetimes for the rise component are 26 μs and 28 μs, respectively (see Table I); its origin will be discussed later.

Discussion

A heterogeneity among PS II centers has been known for quite sometime [29]. Doschek and Kok [9] found that single first-order reaction did not fit fluorescence rise under continuous illumination in DCMU-treated chloroplasts. Melis and Homann [30,31] observed a biphasic first-order growth of area curve and identified the existence of fast-rising PS II centers, labelled as PS IIα, and slow-rising centers, labelled as PS IIβ. The rate of reduction of electron acceptors in PS II is about 3-times slower in the PS IIβ than in PS IIα centers [31]. The PS IIβ centers are suggested to have smaller chlorophyll antenna [32,33]; electron transport in these centers does not proceed via the two-electron-accumulating

Fig. 7. The decay of the calculated Q_A^- concentration (○) and the best fit (———) of a three exponential decay model. The weighted difference (the saw-tooth curve) is the difference between experimental data and the best fit, weighted by the square of the data points.

plastoquinone [34]. Although PS IIβ centers may be able to oxidize water, it appears that they do so slowly [35,36]; thus PS IIα centers are the dominant suorce of electrons for the reduction of NADP.

Lavergne [37] has found another heterogeneity: that of the non-Q_B (or B) centers; these are impaired at the Q_B site and they do not transfer electrons efficiently to the plastoquinone pool. These may be considered inactive PS II centers. Lavergne [38] showed that these non-Q_B centers can be induced to turnover by exogenous quinone, although neither di- nor tetramethylquinone seemed to fully relieve the acceptor side limitation. As noted earlier, Graan and Ort [14] showed that micromolar concentrations of a chloroquinone DCBQ can support flash-induced water oxidation in what they called inactive PS II centers much more effectively than DMQ. From our Chl a fluorescence transient experiments (Fig. 1–3), done under similar experimental conditions, we found that DCBQ is much more effective than DMQ in quenching the ID (F_I) fluorescence level. The absence of any effect of DCBQ on true F_0 indicates that the observed quencing is due to a diminished Q_A^- concentration, but not due to nonphotochemical quenching.

Based on the differential effects of micromolar concentrations of electron acceptors DMQ and DCBQ on F_I quenching (Figs. 1 and 2), we suggest that OID phase reflects the reduction of the electron acceptor Q_A to Q_A^- in the inactive PS II centers. An earlier study by Melis [13] on the quenching of fluorescence rise (F_0 to F_I) was done by adding 100–200 μM DMQ and TMQ to spinach thylakoids. The interpretation given was that the initial fluorescence rise from F_0 to F_I is due to the prompt reduction of the primary electron acceptor Q_A of PS IIβ and the consequence of the inability of PS IIβ to donate electron, on a fast scale, to secondary electron acceptors. Although this interpretation is apparently similar to that given in this paper, it is based on the use of high concentrations of quinones that affect the F_0 level as well and is not based on the differential effects of low (μM) quinones DCBQ and DMQ on inactive and active centers, respectively. Our results in this paper justify our conclusions, but those in the earlier paper [13] do not. Furthermore, it is not clear whether the inactive centers are identical to PS IIβ centers [30,31]. Inactive PS II centers are defined by the interaction between Q_A and the PQ pool, whereas PS IIβ centers are defined by their antenna size. Thus, they represent two different criteria for classifying PS II centers [16]. A recent work [39] suggests that PS IIβ centers exist in both active and inactive forms. Thus, it is equally likely that PS IIα centers also exist in inactive and active forms.

Schreiber and Armond [40] measured heat-induced (up to 60°C) changes of Chl fluorescence in *Larrea divaricata* chloroplasts and observed the F_0 level to rise

greater than 3-fold from that at 25°C; a concomitant F_{max} decline was also observed. It was postulated that heat-induced increase in F_0 reflects inhibition of energy conversion at system II centers, which may be caused by a functional separation of the electron acceptor Q_A from the primary electron donor P680. This implies that $K_p[Q_A]$ in Eqn. 1 that is maximum at F_0 is no longer maximum; furthermore, this increase in fluorescence could also be due to decrease in K_t due to other physical changes. Sane et al. [41] suggested that heating causes an increase in excitation energy transfer (K_t) from PS II to PS I, or a change from the 'state I' to 'state II' (see Ref. 42); this, however, would explain the decline in fluorescence. Weis [43] observed almost parallel decline of variable fluorescence, F_v, and rise of F_0 versus temperature by measuring Chl fluorescence transient in heat-pretreated spinach thylakoids. F_0 rise at above 50°C was 3–4-fold. Sundby et al. [44] concluded that at 30°C there were 30% PS IIβ and 70% PS IIα centers, whereas at 45°C, as much as 84% of centers were PS IIβ.

Based on the Chl a fluorescence transient in the absence of PS II inhibitor (Figs. 4,5), our results show that F_{50ms} rise in heat-treated thylakoids can be diminished by 15–20 μM of DCBQ, while only small quenching was obtained by 20 μM DMQ under our experimental conditions. In this work, we have shown that 20 μM DMQ is able to quench F_P to F_I, while 20 μM DCBQ quenches F_P to F_0 (Figs. 1 and 2). Thus, the fluorescence yield differences between the effects of 20 μM DMQ and 20 μM DCBQ could be attributed mainly to heat-induced rise in the amplitude of F_0 to F_I. Only a minor change occurs in the F_0 level upon heat treatment, in contrast to data in Refs. 40 and 43. This residual small effect, however, could be interpreted by changes in excitation energy transfer [40]. The differential effect of quinones DCBQ and DMQ on heat-induced fluorescence rise leads us to suggest that the significant increase of fluorescence in heat-treated thylakoids is on F_I level, not F_0 level. Thermal conversion from active PS II centers to inactive PS II centers increases F_I level, as expected from our interpretation of the OID phase. DCBQ is able to draw electrons from inactive PS II centers and therefore is able to quench heat-induced fluorescence rise, while DMQ is less effective. The increase in F_I level is accompanied by a dramatic decrease in the rate of O_2 evolution (cf. Ref. 45). The small quenching by 20 μM DMQ may be due to the absence of most of the active PS II centers (Fig. 4).

From freeze-fracture studies, Gounaris et al. [46,47] showed reorganization of the thylakoid membrane at moderately elevated temperatures. Sundby et al. [44] found that above 30°C there is a disassociation of peripheral light-harvesting Chl a/b complex (LHC II) from PS II and a migration of LHC II to the PS-I-rich

non-appressed thylakoid regions. We speculate that the heat-induced reorganization of thylakoid membranes may also alter the reaction center II (D1 and D2 protein) comformation and, thus, the previously active PS II centers cannot perform efficient electron transfer from Q_A^- to the plastoquinone pool. Those centers, therefore, would act like inactive PS II centers in which electron transfer to plastoquinone pool is impaired and a quick rise in initial fluorescence would occur as in the DCMU-inhibited PS II centers.

After one flash, the initial first order component of variable Chl a fluorescence reflects electron flow from Q_A^- to Q_B in reaction centers which have Q_B bound before the flash [28]. In spinach chloroplasts, the half-time of the fast component is 320 μs at pH 6.5 [21]. In our samples at pH 6.5, halftimes of this component are almost the same: 365 μs for spinach and 395 μs for soybean, respectively. The intermediate component has a halftime of 6–7 ms. This time may reflect the process of movement of plastoquinone molecules from the plastoquinone pool to the reaction centers which have no Q_B bound in the dark [28]. The third component, the slow component, is suggested to reflect the back-reaction between Q_A^- and the S_2 state of the oxygen evolving complex, since its halftime matches that of Q_A^- decay in the presence of DCMU [48] (Table I). It has been known from Bennoun's work [26] that the reoxidation of the acceptor Q_A^- after an illumination in the presence of DCMU is a recombination between it and S_2, one of the redox states of the oxygen evolving complex. In the presence of 10 μM DCMU Q_A^- is oxidized with a halftime of 1.5 s after one flash [27]. This back reaction is pH-dependent. At pH 6.5, the time at which $[Q_A]$ is 50% of the maximum Q_A^- for S_2 and Q_A^- back-reaction is 1.3 s and at pH 7.5 it is 2 s [48]. Results in this paper are in agreement with those mentioned above (see Table I).

In the DCMU-treated thylakoid membranes, an initial rise in variable Chl a lfluorescence is observed (Fig. 6) before it declines. This rise component corresponds to a process with a lifetime of about 26 μs in spinach. This can be compared with an increase of F_v with a halftime of 50 μs [49] and of 250 μs range [50]. This was suggested to reflect the re-reduction of the oxidized reaction center P_{680}^+; the observed times were related to the equilibration of electrons on the donor side of PS II [49,50]. The reduction of P_{680}^+ follows multiphasic kinetics [51]. Eckert and Renger [52] measured the relaxation of absorption changes at 820 nm versus time, which reflects the P_{680}^+ reduction kinetics. They found three decay components of lifetimes 20 ns, 330 ns and > 1.4 μs after the first saturating flash. It has been known that the inhibition of Q_A^- reoxidation by Q_B at low temperature [52], or in the presence of DCMU [53] alters the kinetics of P_{680}^+ decay. In order to explain the F_v rise with 26 μs lifetime in the presence of DCMU in

this work, we assume that the effect of blockage of electron transport between Q_A^- and Q_B by DCMU is to decrease the fast components in the nanosecond range and increase the slow component in the microsecond range for P_{680}^+ decay; the halftime values of the slow component(s) in the microsecond range varies in the literature [52–54]. It is also considered likely that the 26–28 μs rise component observed in our work may originate in the recombination of Q_A^- with P_{680}^+; if so, we will have to postulate that the Chl a fluorescence yield in the presence of P_{680}^+ Q_A^- is smaller than in the presence of P_{680} Q_A.

Chylla and Whitmarsh [16] showed that in spinach leaves, oxidation of Q_A^-, monitored by fluorescence induction measurement, displays a halftime of about 1.5 s, which matches the halftime of the recombination between Q_A^- and S_2 state. As a result, one could speculate that the major decay pathway of Q_A^- in the inactive PS II centers may be through the recombination between Q_A^- and the S_2 state. Since, as we have shown, heat alters the ratio of the active PS II centers to inactive PS II centers, it is expected that a larger portion of Q_A^- may be oxidized through this back reaction route in heated samples. A slow decay of Q_A^- is indeed observed (Fig. 6). This reaction is further slowed in heat-treated samples, because of additional heat-induced effects (cf. Refs. 43, 45, 55).

During the revision of this paper, we became aware of independent measurements of Bukhov et al. [56] in heat-treated *Amaranthus* chloroplasts in which preheating was suggested to shift the redox state of Q_A in weak light.

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