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KINETIC ANALYSIS OF THE LIGHT-INDUCED CHLOROPHYLL FLUORESCENCE RISE CURVE IN THE PRESENCE OF DICHLOROPHENYLDIMETHYLUREA

DEPENDENCE OF THE SLOW-RISE COMPONENT ON THE DEGREE OF CHLOROPLAST INTACTNESS

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The light-induced rise of chlorophyll fluorescence in the presence of DCMU was measured in intact chloroplasts (class A), mildly shocked chloroplasts (class D) and ruptured chloroplasts (class C). Kinetic analysis of the rise curves revealed: 1. Class A chloroplasts at $3 \cdot 10^{-6}$ M DCMU show a biphasic rise in variable fluorescence (F_v) with the slow phase comprising only 7% of F_v but 45% of the total area over the induction curve. 2. The slow rise component in class A chloroplasts is inhibited with increasing concentrations of DCMU. Only about 2% slow F_v and 10% slow area component persist at $3 \cdot 10^{-4}$ M DCMU. 3. By mild osmotic shock of class A chloroplasts the slow rise phase is substantially suppressed already at $3 \cdot 10^{-6}$ M DCMU. 4. In class C chloroplasts, with grana structure damaged, a slow component is observed which cannot be eliminated by high DCMU concentrations. The relative contribution of this slow phase increases with decreasing size of thylakoid fragments. It is concluded that the properties of the slow fluorescence rise component, and consequently the apparent PS II heterogeneity, are decisively influenced by the degree of chloroplast and grana integrity. The slow F_v appears to reflect a PS II reaction with an unusually low affinity to DCMU.

Introduction

The light-induced rise of chlorophyll fluorescence in the presence of the PS II inhibitor DCMU (also widely known as the herbicide diuron) is an indicator for reduction of the primary PS II acceptor Q [1]. The correlation between Q-reduction and the fluorescence increase is not a linear one [2]. However, it was shown by Bennoun and Li [3] that the increase of variable fluorescence is linearly correlated with the decreasing rate of photochemical charge separation upon light-induced closure of PS II centers. The existence of such a linear relationship between rate and variable fluorescence is the basis for a kinetic analysis of the PS II primary photoreaction by the 'area growth method' originally derived by Murata et al. [4] and Malkin and Kok [5]. The total 'work' that under given conditions can be performed by PS II centers is determined by the area bounded by the fluorescence rise curve and the maximal fluorescence level; and at any time during induction the area accumulated to this moment is a measure for the

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Abbreviations: DCMU, 3-(3′,4′-dichlorophenyl)-1,1′-dimethylurea; PS II, Photosystem II; PS I, Photosystem I; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-ethanesulfonic acid; Chl, chlorophyll.

relative extent of Q reduction. Hence, the 'area growth' kinetics, different from fluorescence rise kinetics, are considered a linear measure for Q reduction. Melis and co-workers [6–10] made extensive use of this method to characterize an apparent heterogeneity of PS II centers, which is suggested by the biphasic character of the fluorescence rise and area growth kinetics in presence of DCMU [6,11,12]. The slow rise comprises only 10–15% of the variable fluorescence, but contributes 30–60% to the total area above the induction curve. Provided the 'area growth' approach is also valid for the slow phase, these findings would indicate the existence of 30–60% of PS II centers with different properties as compared to the rest of the centers. However, the relative concentration of 'slow centers' may well be considerably overestimated if more than one quantum is required to close such centers, as actually suggested by Joliot and co-workers [13].

Previous kinetic studies on the properties of the slow fluorescence rise component have been performed almost exclusively with ruptured chloroplasts, although a slow fluorescence rise phase was reported for unicellular algae as well [13]. The general assumption has been that a straightforward analysis requires simple systems, and that for the study of the primary processes at the reaction centers, aspects of chloroplast or grana intactness are of little concern. The latter assumption, however, will be questioned by the results of the present investigation. It will be shown that the slow-rise component and the contribution of 'slow centers' to the total area growth are substantially suppressed in intact chloroplasts (class A following definition in Ref. 14) and in gently shocked choroplasts (class D) upon raising DCMU concentration beyond $3 \cdot 10^{-6}$ M. On the other hand, in thoroughly ruptured chloroplasts a considerable slow DCMU-insensitive rise phase does persist.

Materials and Methods

Intact chloroplasts were isolated from freshly harvested, dark-adapted spinach leaves (2 h dark period) following a modification [15] of the Jensen-Bassham method [16]. The content of chloroplasts with intact envelopes was routinely estimated with the ferricyanide method [17], and was found to be between 70 and 85%. Rates of CO_2 fixation in saturating light, as measured with an O_2-electrode, were between 50 and 80 μmol O_2/mg Chl per h. The medium used to shock the chloroplasts osmotically contained 2.5 mM Hepes brought to pH 7.6 with KOH and 5 mM $MgCl_2$. Chloroplasts were exposed for 5 s to this medium and then immediately resuspended in isotonic buffer by addition of an equal amount of double-strength reaction medium. Deviating from the original Jensen-Bassham C-medium, our reaction medium did not contain EDTA and $MnCl_2$, and the final concentration of $MgCl_2$ was 10 mM.

Ruptured chloroplasts from spinach leaves were isolated following the procedure used by Melis and co-workers [8–10], which involves thorough maceration of the leaves, centrifugation for 3 min at $10000 \times g$ and suspension of the pellet using a Potter-type homogenizer. The thylakoid preparation obtained displayed high rates of ferricyanide reduction and tight coupling of photophosphorylation (measured O_2-evolution rates at pH 7.6 were about 200 μmol O_2/mg Chl per h in the presence of 10 mM NH_4Cl and about 1/7 of this rate in absence of the uncoupler).

Chlorophyll fluorescence induction kinetics were measured with a fiber optics system described elsewhere [18,19], the signal/noise ratio of which was further improved by preamplifying the anode current of the photomultiplier within the photomultiplier housing. The induction curves were recorded on a digital oscilloscope (Nicolet Explorer III) with 12-bit (4096 points) resolution in both axes. Kinetic analysis involved reading the digital information into a Hewlett-Packard 9825 computer, automatic determination of initial and maximal fluorescence values, a curve-smoothing routine, integration of area increments and plotting of various derived functions with a Hewlett-Packard 7225 plotter. The quality of fluorescence readings and the accuracy of analysis were such that even small increments of fluorescence in the slow part of the curve (down to about 0.2%) were reliably represented in corresponding increase of the accumulated area. The presented curves were directly plotted from the computer. All curves as well as the values give for the integrated areas were normalized with respect to variable fluorescence.

Mixing of the samples and filling of the cuvette was done in darkness. DCMU was added 2 min before recording of an induction curve by injection of 2.5-μl aliquots of methanol solutions into the 750 μl cuvette; hence, methanol concentration was 0.3% throughout all experiments. The cuvette was maintained thermostatically at 17°C. Intensity of the fluorescence excitation and actinic beam (Corning 4-96) was 10 W/m^2. Chlorophyll concentration was adjusted to 8 μg Chl/ml.

Results

Electron transport between the primary PS II acceptor Q and the plastoquinone pool is inhibited by very low concentrations of DCMU. Reported I_{50} values (concentration required for 50% inhibition) are in the order of $5 \cdot 10^{-8}$ M. For complete inhibition a concentration of $3 \cdot 10^{-6}$ M DCMU is generally considered sufficient. At this concentration HCO_3^--dependent O_2 evolution was completely inhibited in the intact chloroplasts used for our fluorescence experiments (data not in the figures). Fig. 1 shows the light-induced rise of chlorophyll fluorescence in intact chloroplasts at $3 \cdot 10^{-6}$ M DCMU. In Fig. 1a variable fluorescence is plotted on two different time scales. In Fig. 1b the corresponding plots of the integrated area over the fluorescence induction curve (total area, A_{max}, minus area accumulated at given time, A_t) are shown. The fluorescence rise as well as the area growth are clearly biphasic. The slow rise component in fluorescence contributes only about 7% to the total variable fluorescence. However, being considerably slower ($t_{1/2}$ about 6-times longer) as compared to the rapid phase, the area accumulated over the slow phase is substantial, amounting to about 45% of the total area. As revealed by a logarithmic plot of the area growth (Fig. 1b), the rapid as well as the slow area growth component follow closely first-order kinetics (see, however, the deviation from first-order kinetics in the rapid phase, when the slow phase is suppressed, as will be shown in Fig. 2d).

The curves shown in Fig. 1 for intact (class A) chloroplasts are very similar to those previously observed with ruptured (class C) chloroplasts [6–13]. As with ruptured chloroplasts, there is a small slow fluorescence rise component correlated with a substantial slow area growth component, which displays exponential kinetics. The ratio of rapid to slow phase rates is about 6, which is twice that reported previously [6–10]. However, it can be shown (Schreiber and Pfister, unpublished data) that manual analysis (as in, for example, Refs. 6–10) tends to overestimate the rate of the slow phase. (In this context, please note that the computer-assisted analysis applied in this study will yield reliable plots of area growth parameters over an equivalent period of the induction curve which is about 10-times longer than with the manual analysis applied in for, example Ref. 9, Figs. 2B

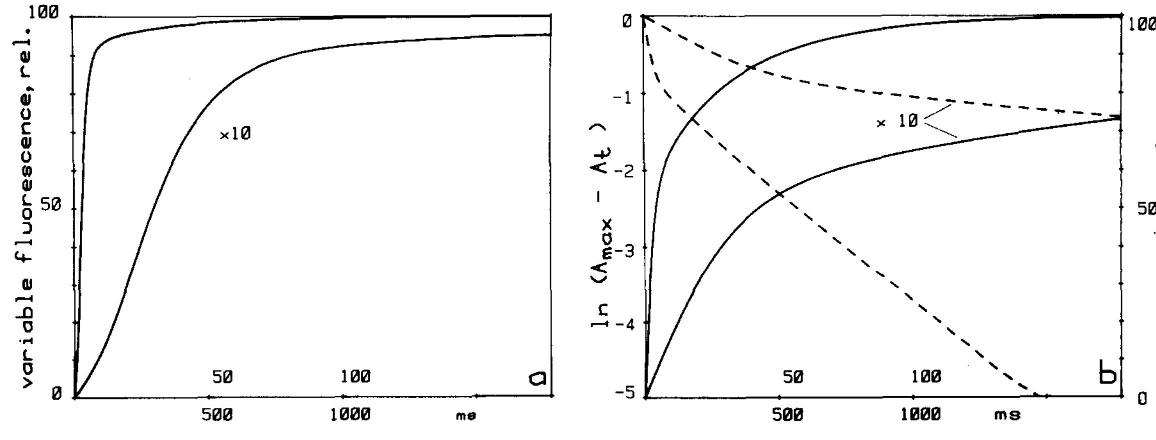
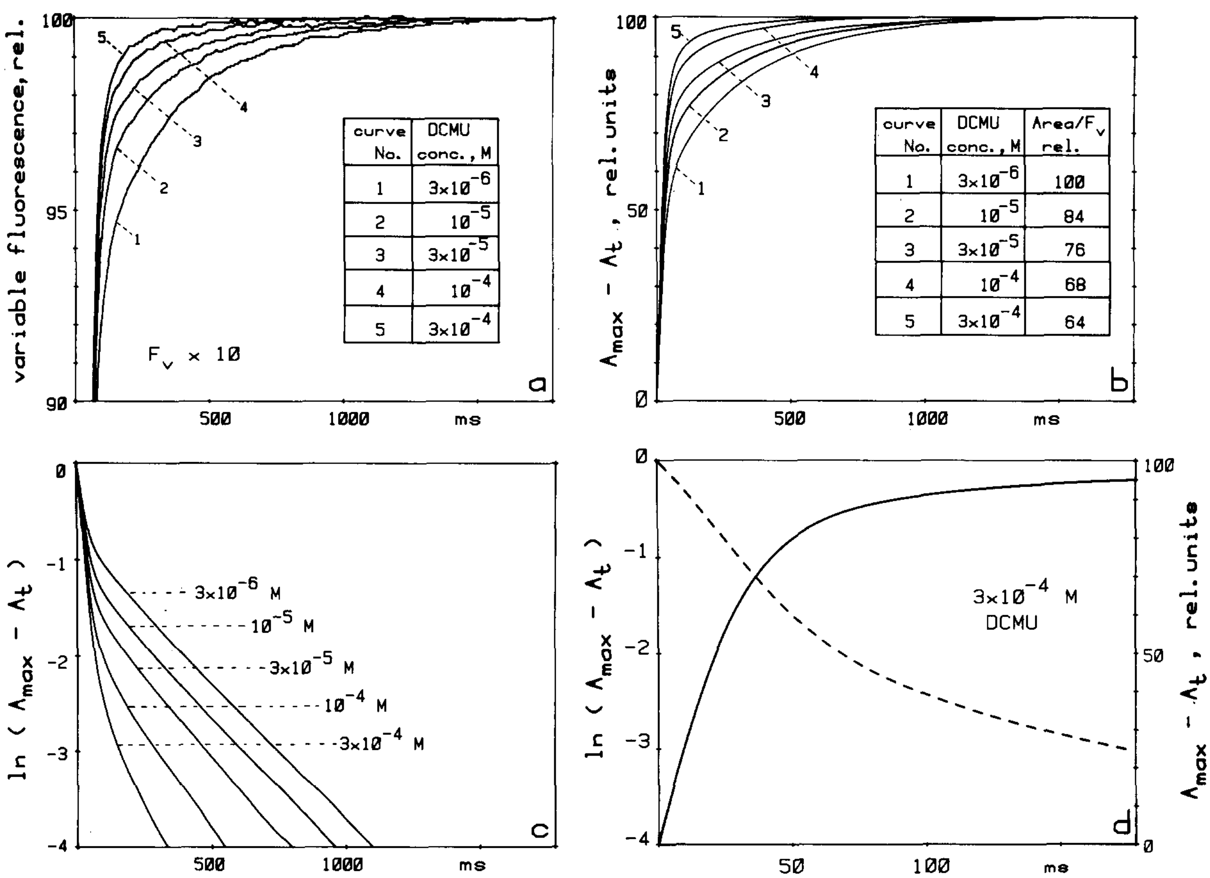


Fig. 1. Light-induced rise of chlorophyll fluorescence in presence of $3 \cdot 10^{-6}$ M DCMU in intact spinach chloroplasts. (a) Plot of variable fluorescence at two different time scales varying by a factor of 10. (b) Plots of area growth over the induction curve and of the logarithm of area growth at two different time scales. Upon extrapolation of the slow phase in the logarithmic plot to zero time, the intercept on the vertical axis gives the logarithm of the maximum value of the area accumulated due to the slow process.



curve No.	DCMU conc., M
1	3x10^{-6}
2	10^{-5}
3	3x10^{-5}
4	10^{-4}
5	3x10^{-4}

curve No.	DCMU conc., M	Area/F$_v$ rel.
1	3x10^{-6}	100
2	10^{-5}	84
3	3x10^{-5}	76
4	10^{-4}	68
5	3x10^{-4}	64

Fig. 2. Effect of DCMU concentration increasing beyond $3 \cdot 10^{-6}$ M in intact chloroplasts. Suppression of the slow rise component. (a) Variable fluorescence plotted at 10-fold sensitivity as compared to the plot of Fig. 1. For clarity of presentation, all curves are normalized to the maximal value of variable fluorescence. The original amplitudes of initial fluorescence (F_0) and of maximal fluorescence (F_{max}) for the different curves were as follows: (1) $F_0 = 29$, $F_{max} = 129$; (2) $F_0 = 31$, $F_{max} = 138$; (3) $F_0 = 30$, $F_{max} = 129$; (4) $F_0 = 29$, $F_{max} = 127$; (5) $F_0 = 28$, $F_{max} = 113$. (b) Area growth plot. The given values of area/F_v correspond to the total accumulated area normalized by the value of variable fluorescence. (c) Logarithmic plot of the area growth for different DCMU concentrations. The curves at maximal DCMU concentration are also plotted at a 5-times faster time scale, to demonstrate the deviation from first-order kinetics in the rapid area growth component (see d).

and 3B.) Hence, on first sight, it would appear that the heterogeneity of PS II centers postulated on the basis of experiments with ruptured chloroplasts [6–13] is a fundamental property also of the intact system. Such finding would not be unexpected. However, as will be shown below, the situation is more complex, as the slow component in intact chloroplasts displays several properties different from those reported for ruptured chloroplasts.

In Fig. 2 the effect of increasing DCMU concentration on fluorescence and area growth is shown for intact chloroplasts. It is apparent that the slow-rise component can be almost completely eliminated by increasing DCMU concentration from $3 \cdot 10^{-6}$ to $3 \cdot 10^{-4}$ M. The slow component of variable fluorescence is cut down to about 2% of the total, while the slow area component is suppressed from 45 to 11% (Figs. 2b, c). A similar size slow area component did also persist in 100% intact chloroplasts prepared from spinach protoplasts (data not shown) indicating that this remaining slow area component cannot be blamed on the population of non-intact chloroplasts (about

20%) present in the 'intact chloroplasts' preparations obtained following the Jensen-Bassham method [16]. As indicated in the legend to Fig. 2a, the amplitudes of initial (F_0) and of maximal (F_{max}) fluorescence are changed by different concentrations of DCMU. Increase of concentration from $3 \cdot 10^{-6}$ M to $1 \cdot 10^{-5}$ M produces equivalent increases of F_0 and F_{max}, leaving the F_{max}/F_0 ratio constant at 4.5; further increase of concentration causes F_0 and F_{max} to decline, with F_{max}/F_0 sinking from 4.5 to 4.0 at $3 \cdot 10^{-4}$ M DCMU. As shown in Fig. 2c, with increasing DCMU concentration the slope of the remaining slow phase in the logarithmic area growth plot is not much changed, suggesting that DCMU has an 'all-or-nothing' effect on the reaction reflected by the slow-rise component in intact chloroplasts. In Fig. 2d the area growth curves at $3 \cdot 10^{-4}$ M DCMU are plotted at a 10-fold extended time scale. The slow-rise component being strongly suppressed at the high DCMU concentration, the logarithmic plot of the rapid phase displays a nonlinear behavior, revealing that the rapid process cannot be described by a simple first-order reaction.

The suppression of the slow-rise component by high DCMU concentrations in intact chloroplasts is in contrast to the previously observed (or possibly sometimes only assumed) saturation of the DCMU effect on fluorescence rise properties at $3 \cdot 10^{-6}$ to $3 \cdot 10^{-5}$ M (depending on chlorophyll concentration) in the studies with ruptured chloroplasts [6–13]. Two main differences between intact and ruptured chloroplasts are apparent which could account for these different reaction center properties: (a) the stroma factor; for example, the stroma may contain substances which keep a hypothetical high potential primary acceptor [20–24] reduced; (b) the grana intactness factor; rupturing may also affect grana integrity and hence modify along with grana structure the functional properties of the reaction centers.

When intact chloroplasts are briefly (for 5 s) osmotically shocked in a hypotonic buffer containing 5 mM Mg^{2+} and then immediately resuspended in isotonic buffer, grana structure is unlikely to be much affected. On the other hand, the stroma is extremely diluted in an excess of buffer. Hence, if the stroma factor were responsible for the above difference between intact and ruptured chloroplasts, osmotic shock should enhance the slow-rise phase. As shown in Fig. 3, this is not the case. On the contrary, the pronounced slow phase observed at $3 \cdot 10^{-6}$ M is substantially suppressed by this treatment, and further eliminated by increasing DCMU concentrations. Therefore, if the stroma factor is taken into consideration, it rather seems to increase the slow-rise component in intact chloroplasts.

When class C chloroplasts are isolated following standard procedures [6–13] the original organization of the grana may be more or less modified, depending on the thoroughness of maceration, centrifugation and resuspension of the pellet by homogenization. A high content of intact grana should be favored by low-speed maceration, low-speed centrifugation and mild homogenization. Indeed, we did find considerable differences in the slow-phase contribution at $3 \cdot 10^{-4}$ M DCMU in class C chloroplasts, ranging from 15 to 60%, depending on these parameters (data not shown). When broken chloroplasts (class D) were washed in 50 mM NaCl and then, following centrifugation, resuspended in normal medium containing 10 mM Mg^{2+}, the slow-phase contribution was found to be close to 50% (data not shown). Apparently, NaCl washing removes Mg^{2+} from sites where it plays a role in stabilizing grana integrity. In general, class C chloroplasts should be assumed to contain varying populations of intact grana, grana fragments and thylakoid fragments. This aspect is emphazised by the data in Figs. 4 and 5. Fig. 4 shows an area growth analysis of fluorescence rise curves from relatively gently prepared (30 s maceration) class C chloroplasts (comparable to the chloroplast preparation used for the experiments in Ref. 9) at $3 \cdot 10^{-5}$ M and $3 \cdot 10^{-4}$ M DCMU. In this particular preparation of class C chloroplasts the relative slow area contribution amounts to 51% at $3 \cdot 10^{-5}$ M and to 27% at $3 \cdot 10^{-4}$ M DCMU. There was practically no difference between rise curves measured at $3 \cdot 10^{-5}$ M and at $3 \cdot 10^{-6}$ M DCMU, suggesting that inhibition at still higher concentrations involves a rather different type of binding site with an unusually low affinity for DCMU.

In the experiment of Fig. 5 class C chloroplasts were prepared with only 10 s maceration time. The chloroplasts obtained from a 5 min $10000 \times g$

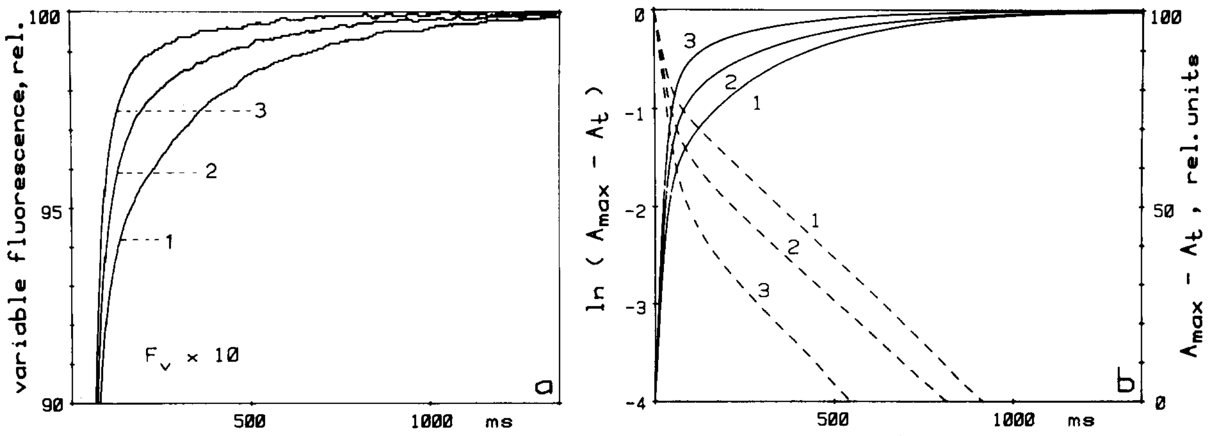


Fig. 3. Suppression of the slow-rise component observed at $3\cdot10^{-6}$ M DCMU in intact chloroplasts by mild osmotic shock and effect of a high DCMU concentration on the remaining slow component in mildly shocked chloroplasts. Curves: (1) intact, $3\cdot10^{-6}$ M DCMU; (2) shocked, $3\cdot10^{-6}$ M; (3) shocked, $3\cdot10^{-4}$ M. (a) Plot of variable fluorescence, normalized to the maximal value of variable fluorescence. Original F_0 and F_{max} values were as follows: (intact, $3\cdot10^{-6}$ M) $F_0=29$, $F_{max}=129$; (shocked, $3\cdot10^{-6}$ M) $F_0=28$, $F_{max}=125$; (shocked, $3\cdot10^{-4}$ M) $F_0=28$, $F_{max}=110$. As in Fig. 2, the upper part of the curves was enlarged by a factor of 10 with respect to the curve shown in Fig. 1; (b) Area plots and logarithmic area plots of the curves displayed in (a).

centrifugation were gently homogenized and following resuspension submitted to three consecutive centrifugation steps, yielding three preparations differing in the mean chloroplast fragment size. It is shown, that even this very mildly prepared preparation of class C chloroplasts is heterogeneous with respect to the content of DCMU-insensitive slow reaction centers. At $3\cdot10^{-4}$ M DCMU the 3 min 15000 × *g* fraction displays a slow area component of about 30%, while in the

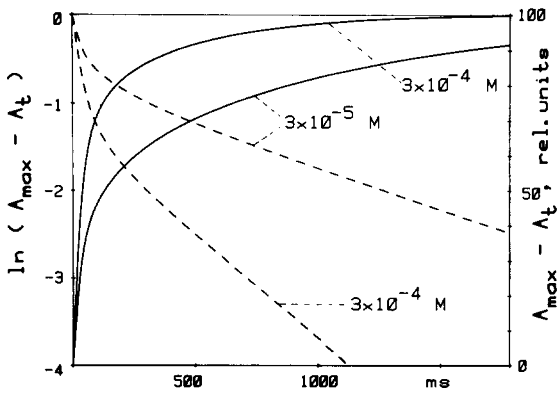


Fig. 4. Area growth plots and logarithmic area growth plots of fluorescence rise curves measured in class C chloroplasts. The curves displayed for $3\cdot10^{-5}$ M DCMU were practically identical to curves measured at $3\cdot10^{-6}$ M DCMU (not shown). Total areas normalized by the values of variable fluorescence were 164 relative units in the case of $3\cdot10^{-5}$ M DCMU and 101 relative units in the case of $3\cdot10^{-4}$ M DCMU. These relative units are directly comparable to the area values given for intact chloroplasts in Fig. 2b. Class C chloroplasts were isolated as described in Materials and Methods.

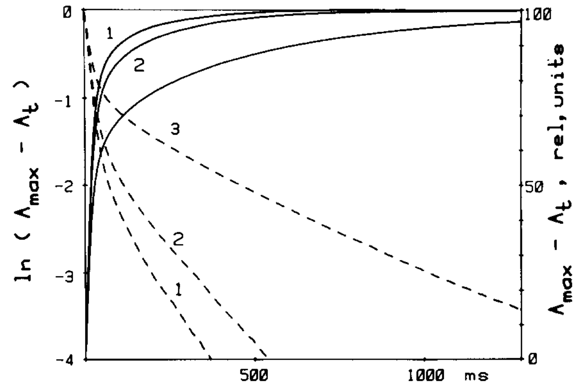


Fig. 5. Area growth plots and logarithmic area growth plots of fluorescence rise curves measured in different fractions of a class C chloroplast preparation obtained with 10 s maceration, 5 min centrifugation at 10000 × *g* and gentle homogenization of the pellet. Chloroplasts were resuspended in isolation buffer and submitted to three consecutive centrifugation steps: (1) 1 min at 4000 × *g*; (2) the supernatant 3 min at 6000 × *g* and (3) the supernatant again 3 min at 15000 × *g*. DCMU concentration, $3\cdot10^{-4}$ M.

1 min 4000 × g and in the 3 min 6000 × g fractions the slow area components are only about 13 and 20%, respectively.

Very similar results as described above for spinach chloroplasts were also obtained with chloroplasts isolated from pea leaves.

Discussion and Conclusions

The data presented in this paper demonstrate that the fluorescence rise kinetics in presence of DCMU depend on the degree of chloroplast intactness. This finding is surprising in view of the generally prevailing understanding that these kinetics reflect a very primary reaction at PS II centers and hence should be unaffected by such factors as envelope integrity and grana integrity. The dependence of the fluorescence rise kinetics on chloroplast integrity may on first sight appear confusing: Intact chloroplasts show a pronounced slow rise component at DCMU concentration generally considered saturating; this slow component is substantially suppressed upon mild osmotic shock which leaves grana structure intact; and upon harsh treatment which results in grana fragmentation, a pronounced slow rise component can be induced again. Hence, intact and fragmented chloroplasts display a very similar phenomenology. But, the question is whether the two slow phases reflect the same functional property. They appear similar in size and rate, which may argue for some common origin. On the other hand, the slow rise in intact chloroplasts is almost completely eliminated by high DCMU concentrations, which is not true for fragmented chloroplasts (Fig. 5). The notion of a common origin is also questioned by the fact that mild osmotic shock already substantially suppresses the slow phase in intact chloroplasts (Fig. 3).

The slow fluorescence rise component in presence of DCMU has been extensively studied in the past [6–13,23–26]. On the basis of careful kinetic analysis Joliot and co-workers [12,26] as well as Melis and co-workers [6–10] proposed models which involve heterogeneity at the level of the primary PS II acceptor Q. The main difference between the original Joliot model [12] and the Melis model [9,10] can be briefly summarized as follows. In the Joliot model the same PS II center can alternatingly reduce two types of primary acceptor, Q_1 and Q_2, while in the Melis model there are two types of photosynthetic unit (α and β units) with α- and β-centers operating in parallel. The existence of PS II heterogeneity has been confirmed by a number of investigations also using other than fluorescence techniques [20–28] however without having led to a generally accepted mechanistic model.

In fact, a considerable part of the data published from different laboratories seem to be in conflict with each other. We believe that most of the discrepancies found in the literature may be explained by differences in chloroplast integrity. For example, the chloroplasts used in Joliot's studies were isolated according to the method of Avron [29] which is suited to obtain a high yield of fragmented grana and free thylakoids, and were stored at −70°C in the presence of 5% dimethylsulfoxide. We found that, similar to salt treatment, freezing will increase the proportion of slow centers to rapid centers (data not shown). On the other hand, in the studies of Melis and co-workers [6–10] chloroplasts were isolated rather gently and always freshly prepared. Such chloroplasts closely correspond to the preparation used in the experiment of Fig. 4 of the present study, i.e., they are heterogeneous but may contain a substantial amount of intact grana. Unfortunately, almost all studies on PS II heterogeneity in the past were carried out with chloroplasts which, according to our present understanding, were heterogeneous with respect to grana intactness.

As shown in Figs. 2 and 3, the slow-rise phase observed in intact chloroplasts at $3 \cdot 10^{-6}$ M DCMU can be substantially suppressed by still higher DCMU concentrations. This finding suggests that at $3 \cdot 10^{-6}$ M DCMU the slow-rise component reflects more than only single turnovers at the reaction centers. Hence, the area growth associated with this rise component should not be considered a measure for the number of centers involved, but rather for the whole pool of acceptors available to these centers under the given conditions. There remains 10–15% of a slow area component even at the highest possible concentration (limited by the DCMU solubility in water), and it could be argued that this percentage corresponds to the relative content of slow centers in intact chloroplasts. However, for this type of center even $3 \cdot 10^{-4}$ M DCMU may not yet be saturating, and the true content of slow centers could be even lower.

Anderson [30] has recently proposed a new model for the structural and functional organization of the thylakoids in stroma-exposed and in appressed grana regions, derived from recent work on grana partition vesicles [31]. According to this model, almost all PS I units are located in the stroma-exposed thylakoid regions and about 80–90% of PS II units are situated in the appressed membrane regions. The remaining 10–20% of PS II are supposed to be located together with PS I in the stroma exposed membrane regions. The model involves a small heterogeneity at the level of PS II, but not of the magnitude (30–50%) suggested by the studies with class C chloroplasts [6–10]. Possibly the 10–15% slow area growth component persisting at $3 \cdot 10^{-4}$ M DCMU in intact chlororoplasts is related to this small population of stroma exposed PS II units, which then would be characterized by an unusually low affinity for DCMU. It is known that DCMU will bind with high affinity ($k_b = 1.4 \cdot 10^{-8}$ M) only to the B-protein [32,33]. A second binding site with a much lower binding constant ($k_b = 4 \cdot 10^{-7}$ M) has, however, been observed [34,35]. PS II units lacking the B-protein would be expected to show a low affinity, if any, to DCMU. In this context, recent data of Thielen and van Gorkom [36] may be important, suggesting that the slow β-units are not connected to the B-protein.

Another aspect which could be relevant in connection with a low-affinity DCMU-binding site in intact chloroplasts, is the well-known fact that reactions which depend on cyclic photophosphorylation in vivo are much less sensitive to DCMU than is O_2 evolution [37–39]. Hence, in principle the possibility should be considered that the slow-rise component observed at moderate DCMU concentrations in intact chloroplasts, is in some way related to cyclic electron flow. However, membrane energization is unlikely to be involved, as the slow rise persists in presence of uncouplers (data not shown).

The slow-rise component that persists at very high DCMU concentrations in class C chloroplasts (see Figs. 4, 5) may, like the slow phase in intact chloroplasts at moderate DCMU concentrations, reflect a population of PS II centers with an unusually low affinity for DCMU. In the case of class C chloroplasts the low affinity binding constant would be still considerably lower than with intact chloroplasts, such that for all practical purpose one may refer to 'DCMU-insensitive' centers. For technical reasons this assumption is difficult to substantiate by binding studies [40]. Grana fragmentation may affect the B-protein in a similar manner as trypsin treatment [41,42], which removes DCMU-binding sites. Alternatively, centers in fragmented thylakoids could display rapid charge recombination, resulting in an apparent low quantum yield of charge separation, as actually described by Joliot and Joliot [43]. Our findings are not in conflict with recent data published by Melis and Thielen [10] and Thielen and van Gorkum [44], who show that in class C chloroplasts prepared from tobacco mutants the slow center contribution increases with the decreasing content of grana thylakoids. It is reasonable to assume that thylakoids are more protected from fragmentation and correlated changes in DCMU-binding properties when stacked in grana.

Horton [25] very recently suggested that there are two types of PS II heterogeneity, as differentiated by redox titration measurements. He found that the slow fluorescence rise phase can be selectively removed by poising at a redox potential (+120 mV) shown to reduce only 10% of a high-potential quencher pool (comprising about 50% of the total quencher pool). Without precise information on the degree of intactness of the chloroplast preparation used in this study, the concentration of $5 \cdot 10^{-6}$ M DCMU applied should be considered too low to block all centers completely, even if the chloroplasts were intact. Possibly Horton's component with $E_{m,7}$ at +120 mV corresponds to our slow phase at $3 \cdot 10^{-6}$ M DCMU in intact chloroplasts.

In conclusion, the presented data demonstrate the decisive influence of the degree of chloroplasts and grana integrity on the apparent PS II heterogeneity as reflected by the biphasic fluorescence rise kinetics in presence of DCMU. It is suggested that there exists a population of PS II centers in intact chloroplasts which displays an unusually low affinity for DCMU. This population can, however, be almost completely blocked at $3 \cdot 10^{-4}$ M DCMU. At the same DCMU concentration in class C chloroplasts a substantial slow fluorescence rise phase does persist, suggesting still lower affinity of fragmented grana for DCMU, as compared to the intact system. With varying and unspecified contents of intact and fragmented grana in the chloroplast preparations used so far in studies of PS II heterogeneity, it is difficult to judge which of the reported findings do reflect a true PS II heterogeneity in vivo. For future work on this aspect it will be important to use class A or class D chloroplasts and to take the existence of a low-affinity DCMU-binding site into account.

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