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Non-photochemical quenching of chlorophyll *a* fluorescence by oxidised plastoquinone: new evidences based on modulation of the redox state of the endogenous plastoquinone pool in broken spinach chloroplasts☆

Pierre Haldimann[a,*], Merope Tsimilli-Michael[b]

[a]*Ruelle du chalet 4, CH-1796 Courgevaux, Fribourg, Switzerland*
[b]*Ath. Phylactou str. 3, CY-1100 Nicosia, Cyprus*

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Abstract

Twenty-five years ago, non-photochemical quenching of chlorophyll fluorescence by oxidised plastoquinone (PQ) was proposed to be responsible for the lowering of the maximum fluorescence yield reported to occur when leaves or chloroplasts were treated in the dark with 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), an inhibitor of electron flow beyond the primary quinone electron acceptor (Q_A) of photosystem (PS) II [C. Vernotte, A.L. Etienne, J.-M. Briantais, Quenching of the system II chlorophyll fluorescence by the plastoquinone pool, Biochim. Biophys. Acta 545 (1979) 519-527]. Since then, the notion of PQ-quenching has received support but has also been put in doubt, due to inconsistent experimental findings. In the present study, the possible role of the native PQ-pool as a non-photochemical quencher was reinvestigated, employing measurements of the fast chlorophyll *a* fluorescence kinetics (from 50 μs to 5 s). The about 20% lowering of the maximum fluorescence yield F_M, observed in osmotically broken spinach chloroplasts treated with DCMU, was eliminated when the oxidised PQ-pool was non-photochemically reduced to PQH_2 by dark incubation of the samples in the presence of NAD(P)H, both under anaerobic and aerobic conditions. Incubation under anaerobic conditions in the absence of NAD(P)H had comparatively minor effects. In DCMU-treated samples incubated in the presence of NAD(P)H fluorescence quenching started to develop again after 20–30 ms of illumination, i.e., the time when PQH_2 starts getting reoxidised by PS I activity. NAD(P)H-dependent restoration of F_M was largely, if not completely, eliminated when the samples were briefly (5 s) pre-illuminated with red or far-red light. Addition to the incubation medium of $HgCl_2$ that inhibits dark reduction of PQ by NAD(P)H also abolished NAD(P)H-dependent restoration of F_M. Collectively, our results provide strong new evidence for the occurrence of PQ-quenching. The finding that DCMU alone did not affect the minimum fluorescence yield F_0 allowed us to calculate, for different redox states of the native PQ-pool, the fractional quenching at the F_0 level (Q_0) and to compare it with the fractional quenching at the F_M level (Q_M). The experimentally determined Q_0/Q_M ratios were found to be equal to the corresponding F_0/F_M ratios, demonstrating that PQ-quenching is solely exerted on the excited state of antenna chlorophylls.
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Keywords: Chlorophyll fluorescence quenching; Mercury; NAD(P)H; Photosystem II; Plastoquinone; Thylakoid

1. Introduction

At ambient temperature, chlorophyll (Chl) *a* fluorescence emitted by higher plants, algae and cyanobacteria is generally accepted to originate basically from photosystem (PS) II. The fluorescence transient, induced in vivo by illumination of a photosynthetic sample with continuous actinic light, consists of light intensity dependent changes in fluorescence emission. In dark-adapted samples, the

Abbreviations: Chl, chlorophyll; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; F_M, maximum fluorescence yield; F_0, minimum fluorescence yield; I and J, intermediate steps in the Chl *a* fluorescence transient appearing between F_0 and F_M; PQ, plastoquinone; PQH_2, plastoquinol; PS, photosystem; Q_A, primary quinone electron acceptor of PS II; RC, reaction centre

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* Corresponding author. Present address: Institute of Plant Sciences, University of Bern, Altenbergrain 21, CH-3013 Bern, Switzerland. Tel.: +41 31 631 4959; fax: +41 31 631 4142.
E-mail addresses: pierre.haldimann@bluewin.ch (P. Haldimann), tsimicha@spidernet.com.cy (M. Tsimilli-Michael).

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fluorescence transient, also known as Kautsky effect [1], is characterized by a fast rise in fluorescence intensity from an initial low level F_0 to a high level F_P, followed by a slower decline towards a steady-state level F_S. Because the light absorption of a sample can be considered as constant during the fast fluorescence rise, the changes of fluorescence intensity are equivalent to changes of the fluorescence yield. It is generally accepted that Chl *a* fluorescence rise kinetics from F_0 to F_P reflects the conversion of Q_A to its reduced form Q_A^- (closure of the reaction centres (RCs) of PS II), and can therefore provide information on the photochemical activity of PS II and the associated filling up of the plastoquinone (PQ) pool [2–6]. The level of F_P depends on the achieved Q_A^-/Q_A balance, which is the net result of Q_A reduction due to PS II activity and Q_A^- reoxidation via the PQ-pool, driven by PS I activity. F_P becomes equal to the maximum fluorescence yield F_M when Q_A is fully reduced, as happens under strong actinic illumination (above 200 W m^{-2} for red light with peak at 650 nm [7]). When dark-adapted leaves are illuminated at ambient temperature with such a strong actinic light, the Chl *a* fluorescence rise they exhibit, accomplished within less than 1 s, is polyphasic with two intermediate steps between the minimum yield F_0 (O-level) and the maximum yield F_M=F_P (P-level), labelled as I_1 and I_2 [8,9] or J and I [7,10]—hence the notation O-J-I-P for the fluorescence transient. The J- and I-step, more clearly revealed when the fluorescence kinetics is plotted on a logarithmic time scale [7,10], appear at about 2 and 30 ms, respectively; the corresponding fluorescence intensities are denoted as F_J and F_I. The O-J-I-P transient has been attributed to the dynamic variation of the concentrations of the various redox states of the PS II units, with $Q_A^-Q_B$ and $Q_A^-Q_B^-$ predominating at the J-step and $Q_A^-Q_BH_2$ at the I- and P-step (see, e.g., Ref. [11]).

When the electron flow beyond Q_A^- is inhibited by 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), the fluorescence rise reflects only photochemical events leading to the complete reduction of Q_A. Hence, the maximum fluorescence yield in the presence of DCMU ($F_{M,DCMU}$) is expected to be as high as the F_M exhibited by untreated samples. Therefore, the finding that $F_{M,DCMU}$ is lower than F_M [12,13] raised questions about the origin of the unpredicted difference. Vernotte et al. [14] attributed the phenomenon to a non-photochemical quenching of Chl fluorescence by oxidised PQ. However, the extent of the $F_M-F_{M,DCMU}$ difference was not consistent among different studies, varying from about 20% down to even zero [14–21]. The inconsistency of the experimental findings, which has not yet been resolved, has put the notion of PQ-quenching in doubt. On the other hand, this notion has recently received new support from results obtained with in vitro experiments, where enlargement of the PQ-pool via the incorporation of exogenous plastoquinone molecules in thylakoid membranes and PS II membrane fragments resulted in enhanced fluorescence quenching [18,19]. A non-photochemical quenching of Chl fluorescence was also demonstrated to occur when artificial quinones, like 2,2-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone [22] and 2-methyl-1,4-naphthoquinone [19], were added to DCMU-treated chloroplasts.

We here reinvestigated the possible role of oxidised plastoquinone as a fluorescence quencher in DCMU-treated broken spinach chloroplasts, however, dealing only with the native PQ-pool, brought to different redox states by appropriate treatments (see also Ref. [23]). The reduction of the PQ-pool in higher plant chloroplast is a well-documented light-driven process. Normally, the PQ molecules that become reduced by the PS II activity during illumination are reoxidised at a high rate when the sample is transferred to darkness. On the other hand, there are reports showing that PQ accumulates in its reduced form when leaves are exposed to anaerobic conditions in the dark [24–26] or following a light-to-dark transition [27–29]. Actually, a large body of evidence has recently accumulated showing that in the dark the redox state of the PQ-pool can be modulated by the components of a putative chlororespiratory electron transport pathway that involves the non-photochemical reduction of PQ by an NAD(P)H-PQ oxidoreductase and the reoxidation of reduced PQ by a terminal plastid oxidase; alternative pathways of non-photochemical reduction and oxidation of the PQ-pool are also likely to occur (for recent reviews, see Refs. [30–32]). In open cell preparation of *Chlamydomonas reinhardtii* [33] as well as in broken chloroplasts and thylakoid membranes [34–37], non-photochemical reduction of PQ can be induced by incubating the samples in the dark in the presence of NAD(P)H. Incubation under anaerobic conditions enhances NAD(P)H-dependent non-photochemical PQ reduction, while mercury was found to inhibit it [37].

In the present study, based on our previous findings [37], we investigated how the $F_{M,DCMU}$ level is affected when the PQ-pool is brought to different redox states by the following treatments and combinations of them: (a) addition of NAD(P)H, which has been shown to induce dark reduction of PQ; (b) anaerobiosis, which results in the inhibition of O_2-dependent dark reoxidation of reduced PQ; (c) addition of $HgCl_2$, which inhibits the NAD(P)H-dependent PQ reduction; (d) pre-illumination (red or far-red light) which results in the reoxidation, due to PS I activity, of the non-photochemically accumulated plastoquinol (PQH_2). The utilization of a high time-resolution fluorimeter, which permits a precise detection of F_0, allowed us to determine the quenching at this level of the transient and to compare it with that on the F_M level. The PQ redox state was probed by the O-J-I-P transient in the absence of DCMU, based, as in previous reports [26,37], on evidences [11] that the higher the PQH_2 fraction, the higher the J-level is.

2. Materials and methods

2.1. Chloroplast isolation

Chloroplasts were isolated under dim light at 4 °C from spinach (*Spinacia oleracea* L.) purchased from local markets. Sliced leaves were homogenised for 10 s in a Waring Blender in a grinding medium containing 330 mM sorbitol, 50 mM MES-KOH (pH 6.1), 30 mM KCl, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$ and 0.5 mM KH_2PO_4. The slurry was filtered through two layers of Nylon (36 μm) plus two layers of muslin cloth. The pellet obtained after centrifugation at 2000×*g* for 5 min and 4 °C was suspended in 2 ml of the same medium and layered on 40% Percoll (v/v) in the same medium. The pellet with intact chloroplasts, obtained after centrifugation of the Percoll-containing tubes at 4000×*g* for 10 min and 4 °C, was resuspended in a medium containing 330 mM sorbitol, 5 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA and 50 mM HEPES–KOH (pH 7.5) and centrifuged to remove the Percoll. The pellet was resuspended in 1–2 ml of the same medium. The chloroplasts were osmotically ruptured by resuspension in 30 mM $MgCl_2$ for 5 min followed by the addition of an equal volume of double strength suspension medium. The chloroplast suspension was then kept on ice in the dark. Measurements were made at a chlorophyll (Chl) concentration of 50 μg Chl ml^{-1}. The Chl concentration was measured according to the method of Arnon [38].

2.2. Treatment of the chloroplasts with NAD(P)H, DCMU and mercury

For the treatment with reduced pyridine nucleotides, NADPH or NADH was added, at a final concentration of 250 μM, to 500-μl aliquots of chloroplast suspension (50 μg Chl ml^{-1}), in 1-cm diameter vials with a total volume of 2 cm^3. Thereafter, the samples were incubated for 60 min at room temperature in the dark either under aerobic or anaerobic conditions. Anaerobic conditions were induced by flushing the samples with N_2 gas and adding 10 mM glucose, 50 units ml^{-1} glucose-oxidase and 1000 units ml^{-1} catalase to the chloroplast suspension. The vials were then immediately sealed with air tight caps. When NAD(P)H-treated samples were additionally treated with the inhibitors DCMU (50 μM) and/or $HgCl_2$ (100 μM), the inhibitors were added to the chloroplast suspension 5 min before applying the NAD(P)H treatment. DCMU was dissolved in water instead of ethanol to avoid possible effects of ethanol on the fluorescence transients. All chemicals were purchased from Sigma or from Boehringer Mannheim.

2.3. Chlorophyll a fluorescence measurements

Chlorophyll *a* fluorescence transients were recorded at room temperature using a PEA-fluorimeter (PEA, Hansatech, King's Lynn, Norfolk, UK) as described elsewhere [7]. The measurements were performed on 500-μl aliquots of chloroplast suspension in 1-cm diameter vials. The optical thickness of the sample was 5 mm and the diameter of the irradiated area 4 mm. The Chl *a* fluorescence signal was detected using a PIN photocell after passing through a long pass filter (50% transmission at 720 nm) and recorded in a time span of 10 μs to 5 s at 12-bit resolution, with data acquisition every 10 μs for the first 2 ms, every 1 ms between 2 ms and 1 s and every 100 ms thereafter. All fluorescence transients were induced with saturating red actinic light (600 W m^{-2}, equivalent to about 3200 μE m^{-2} s^{-1}; peak at 650 nm), provided by an array of six light-emitting diodes. When applied, pre-illumination of the samples with far-red light (3 W m^{-2}) was provided by a Schott lamp (KL 1500; Schott Glasswerke, Mainz, Germany) equipped with a fiber optic and a far-red light filter (RG 730; Schott). Irradiance was measured with an YSI-Kettering 65A light-meter (Yellow Springs Instrument Co.).

3. Results

We first checked whether and to which extent DCMU addition affected the maximum Chl *a* fluorescence intensity exhibited by osmotically broken spinach chloroplasts upon illumination with saturating red light (600 W m^{-2}). The Chl *a* fluorescence transients of untreated (denoted as "control") and DCMU-treated chloroplasts, plotted on a logarithmic time scale, are depicted in Fig. 1. The fluorescence values for each transient are expressed as $F_t/F_{M,control}$, where F_t is the fluorescence intensity at time *t* and $F_{M,control}$ the maximum fluorescence intensity of the transient exhibited by the control sample. This normalisation was applied throughout the entire paper, for all presented transients, in order to facilitate the comparison of fluorescence intensities within a transient and/or among transients. The control transient is a typical O-J-I-P polyphasic rise exhibited by broken chloroplasts and thylakoids under strong red actinic illumination, with the characteristic absence of a clear I-step [26,37,39–41]. The initial O-level in the presented transients is set at 50 μs because, due to limitations related with the response time of the detector (about 30 μs), $F_{50\mu s}$ is the earliest reliable measurement [7]. However, when referring to the fluorescence values at the O-level, we will use the notation F_{50s} to distinguish them from the F_0 values that can be determined, as will be shown later in this study, with a higher precision. The J-step (F_J) appears at about 2 ms and the P-level (F_P=F_M) is reached within 500 ms. The shape of the transient in the presence of DCMU is also characteristic of the treatment, with no intermediate steps between the O- and P-level.

Fig. 1 clearly shows that the F_M level in DCMU-treated chloroplasts ($F_{M,DCMU}$) is lower than the F_M of the control sample ($F_{M,control}$) by about 19%. DCMU-induced lowering of F_M was found in all preparations used in this study; its

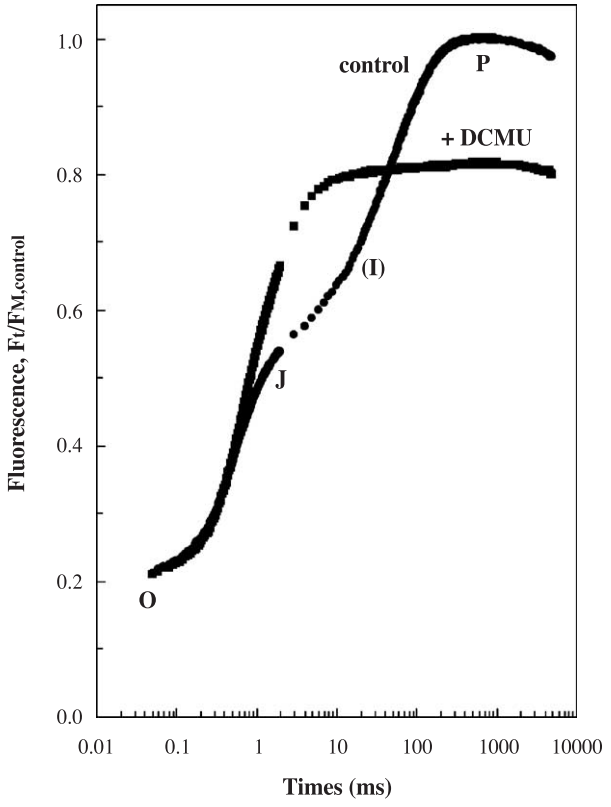

Fig. 1. Chl *a* fluorescence rise kinetics of osmotically broken spinach chloroplasts incubated for 60 min at room temperature in the dark in the absence (●) or presence of 50 μM DCMU (■). The transients, induced by saturating red actinic light (600 W m^{-2}, peak at 650 nm, duration 5 s), are plotted on a logarithmic time scale. The fluorescence values for both transient are expressed as $F_t/F_{M,control}$, where F_t is the fluorescence intensity at time t and $F_{M,control}$ the maximum fluorescence intensity of the transient exhibited in the absence of DCMU (control sample). The chlorophyll concentration was 50 μg Chl ml^{-1}.

extent showed minor variations among preparations (17–20%; i.e., $F_{M,DCMU}/F_{M,control}$ between 0.83 and 0.80). The DCMU treatment had no effect on $F_{50\mu s}$.

In a previous paper [37], we showed that dark incubation of osmotically broken spinach chloroplasts with NAD(P)H (250 μM) induces a non-photochemical reduction of PQ to PQH_2, reflected in a wide increase of F_J, the fluorescence yield at the J-step (shifted from 2 to 5 ms). It was also shown that the NAD(P)H-dependent PQ reduction is enhanced by anaerobiosis, as revealed by a further F_J increase. Anaerobiosis alone was found to have minor effects on the fluorescence transient. It was therefore of interest to apply these treatments for the case of DCMU-treated broken chloroplasts and investigate whether the thereby caused modifications of the PQ redox state would affect the $F_{M,DCMU}$ level.

The results depicted in Fig. 2A show unequivocally that the NADPH-induced reduction of PQ restores the $F_{M,control}$ level in DCMU-treated samples. The restoration was always found to be complete under anaerobic conditions ($F_{M,DCMU}/F_{M,control}=1$), while in the presence of O_2 it was also complete or almost complete ($0.97<F_{M,DCMU}/F_{M,control}\leq 1$). Fig. 2A also shows that anaerobiosis alone results in a comparatively minor increase of $F_{M,DCMU}$. Hence, the $F_{M,control}-F_{M,DCMU}$ difference is found to decrease when the PQ redox state is shifted towards higher PQH_2/PQ ratios and to vanish in the presence of NADPH under anaerobic conditions. As shown in Fig. 2A, not only F_M, but any point F_t is affected by the modifications of the PQ redox state, though at a different extent, as theoretically predicted for quenching effects [14]. Comparing, for example, the transient exhibited by DCMU-treated samples in the presence of NADPH under anaerobic conditions, which can well be considered as expressing the completely unquenched case, with the transient exhibited by DCMU-treated samples under aerobic conditions, which shows the maximum extent of quenching, we can see that the quenching, amounting to about 19% at the F_M-level, is only about 9% at $F_{50\mu s}$.

The same effects were observed when using NADH instead of NADPH. This holds true not only for the findings depicted in Fig. 2A, but also for all the other results obtained in this work. Hence, though we present only the experiments with NADPH to avoid repetitions, we keep the notation NAD(P)H.

The fluorescence transients recorded in non-DCMU-treated samples from the same chloroplast preparation are presented in the insert of Fig. 2. All four transients exhibit the same maximum fluorescence intensity ($F_M/F_{M,control}=1$), which indicates that the PQH_2/PQ ratio reaches, in all four cases, the same maximum value as that induced non-photochemically by NAD(P)H under anaerobic conditions in the presence of DCMU. This means that the photo-reduction of PQ under the strong red actinic light used is complete, independently of the initial differences of the PQ redox state established by the different treatments applied prior to illumination. It is worth noting that in the absence of DCMU (insert of Fig. 2) the NAD(P)H-induced increase of $F_{50\mu s}$ is wider than in the presence of DCMU (Fig. 2A). This may indicate that, besides the abolishment of quenching due to the reduction of PQ by NAD(P)H, a partial reduction of Q_A also occurs, probably due to an equilibration between Q_A^-/Q_A and PQH_2/PQ in the dark. Though in DCMU-treated samples NAD(P)H completely abolished the F_M quenching both under anaerobic and aerobic conditions (Fig. 2A), indicating that full reduction of PQ was achieved in both cases, in the corresponding non-DCMU-treated samples F_J was lower than $F_{M,control}$ by about 5% for anaerobic and 14% for aerobic conditions (insert of Fig. 2 and [37]). This observation may suggest that the fluorescence signal at the J-step, though basically determined by the PQ redox state, is also affected by other factors, whose impact, though enhanced in the presence of O_2, is still comparatively minor. On the other hand, the treatment with anaerobiosis alone resulted in a minor increase both of F_J in the non-DCMU-treated sample (insert of Fig. 2) and F_M in the DCMU-treated sample (Fig. 2A). This might be due to the presence of native reductants in the chloroplasts suspension, which, in the absence of O_2, resulted in a minor reduction of PQ.

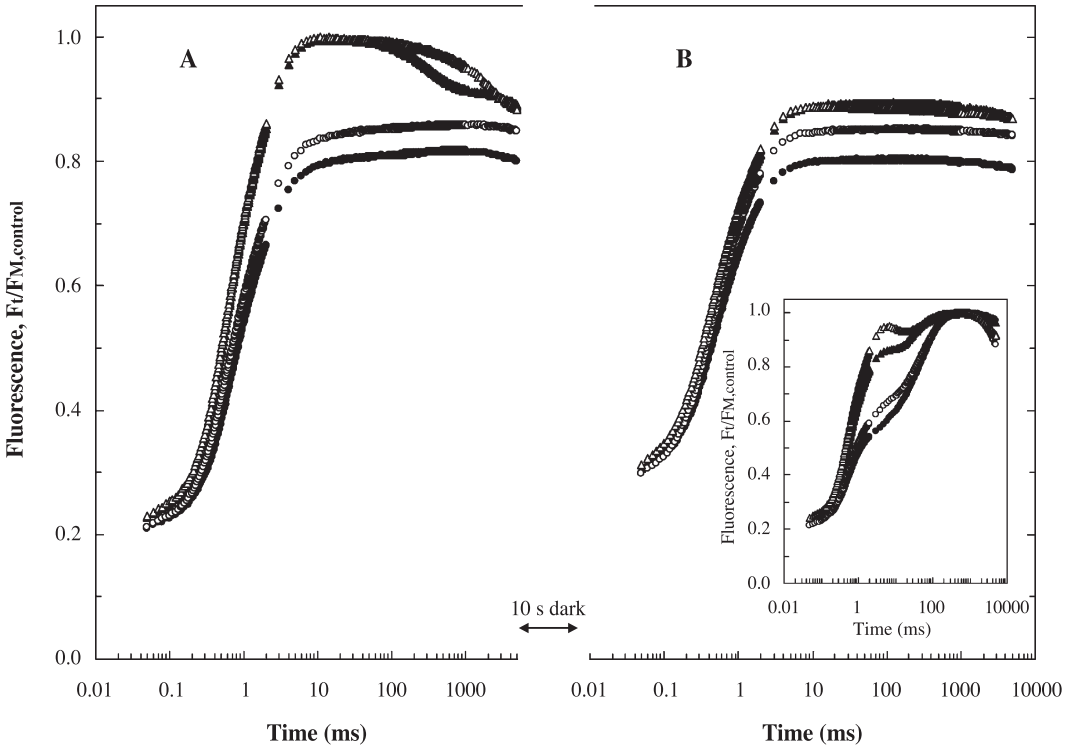

Fig. 2. Chl *a* fluorescence rise kinetics of DCMU-treated osmotically broken spinach chloroplasts incubated for 60 min at room temperature in the dark under aerobic (●, ▲) or anaerobic (○, △) conditions, in the absence (●, ○) or presence (▲, △) of 250 μM NADPH. (A) First pulse transients, induced immediately after the dark incubation; (B) second pulse transients, induced after a 10-s dark interval following the completion of the first pulse transients. The insert shows the first pulse transients obtained from non-DCMU-treated samples. For other details, see legend of Fig. 1.

From Fig. 2A, which depicts the fluorescence transients of DCMU-treated samples, we see that, in the presence of NAD(P)H, fluorescence attains its maximal value at about 10 ms, while after 30 ms a pronounced decline is observed. It can be reasonably speculated that the observed decline reflects a restoration of fluorescence quenching, due to a PS I-driven reoxidation of PQH_2 that had been formed non-photochemically prior to illumination. Though restoration was not complete at 5 s, we avoided prolongation of illumination that would possibly result in additional effects. The attribution of the observed fluorescence decline to the reoxidation of PQH_2 is supported by a previous finding that, under the same actinic illumination as in our experiments, the initiation of electron transfer from PQH_2 to PS I occurs at 20 to 30 ms after the onset of illumination [20,42]. As depicted in Fig. 2A, the decline is faster under aerobic than under anaerobic conditions, most probably because of the role of O_2 as an electron acceptor after PS I.

In order to further test whether the observed decline is reflecting a reoxidation of PQH_2, the samples, after the completion of the transients shown in Fig. 2A, were illuminated with a second light pulse of 5 s, allowing a 10-s dark interval between the two pulses. The 10-s dark interval is too short to permit modification of the PQH_2/PQ ratio either by further reoxidation of PQH_2 or by re-reduction by NAD(P)H of the fraction of the plastoquinone pool that had been oxidised during the first pulse. Thus, for each sample, we could obtain a second fluorescence transient, after the PQH_2/PQ ratio had been lowered by the first pulse. As demonstrated in Fig. 2, for each treatment, the F_M value of the second pulse transient (Fig. 2B) is identical with the final value (at 5 s, F_{5s}) of the first pulse transient (Fig. 2A). The second pulse transients show a slight decline from F_M to F_{5s} that may reflect a further oxidation of PQH_2.

We also investigated the effect of far-red light pre-illumination in DCMU-treated samples that had been incubated in the dark either in the presence or absence of NAD(P)H, both under aerobic and anaerobic conditions. The fluorescence transients exhibited after the pre-illumination, which was given for 5 s and followed by a 10-s dark period, are presented in Fig. 3A. Compared with the transients recorded without pre-illumination (Fig. 2A), they reveal a restoration of fluorescence quenching, in accordance with the expected oxidation of PQH_2 by PS I activity. Moreover, when compared with the second pulse transients of Fig. 2B, they show a further lowering of F_M, which can be reasonably attributed to the more effective reoxidation of PQH_2 by PS I activity, when driven by far-red light. A minor decline is observed in all transients of Fig. 3A, starting, however, only after about 1 s of illumination. Similarly to the results depicted in Fig. 2, we observe again that, for each treatment, the F_M value of the second pulse transient (Fig. 3B) is identical with the F_{5s} value of the first pulse transient (Fig. 3A).

Comparing Fig. 2A with Fig. 2B, we observe that the $F_{50\mu s}$ values of the second pulse transients (red light pre-illuminated samples) are higher than the F_0 values of the

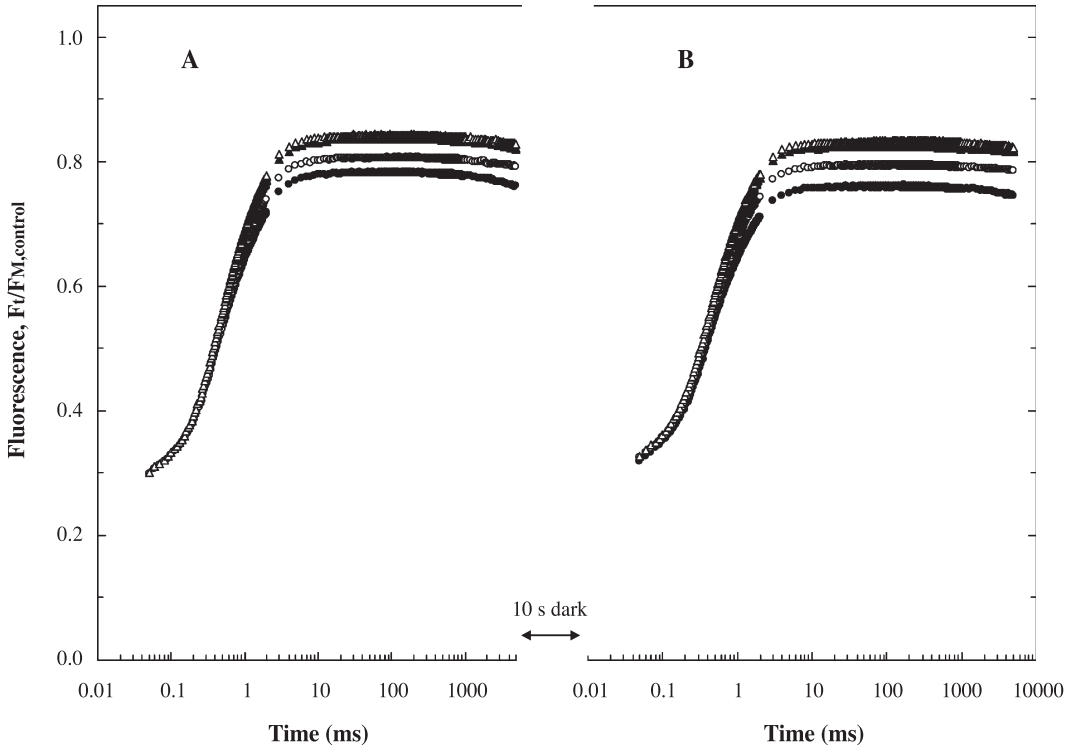

Fig. 3. Chl *a* fluorescence rise kinetics of DCMU-treated osmotically broken spinach chloroplasts incubated for 60 min at room temperature in the dark under aerobic (●, ▲) or anaerobic (○, △) conditions, in the absence (●, ○) or presence (▲, △) of 250 μM NADPH. At the end of the dark incubation, the samples were illuminated for 5 s with far-red light. (A) First pulse transients, induced after a 10-s dark interval following the pre-illumination; (B) second pulse transients, induced after a 10-s dark interval following the completion of the first pulse transients. For other details, see legend of Fig. 1.

first pulse transients (dark-adapted samples). This means that the 10-s dark interval was not long enough to fully reoxidise Q_A^-. The same is also observed concerning the samples pre-illuminated with far-red light (Fig. 3A), indicating that the far-red light used was not exclusively exciting PS I. The maximum difference among the $F_{50\mu s}$ values of the samples subjected to the different treatments is smaller after red and far-red pre-illumination (about 4%) than without pre-illumination (about 9%).

We then addressed the question whether the elimination of fluorescence quenching upon addition of NAD(P)H is indeed occurring because of the thereby induced modification of the PQ redox state and not arising from redox state changes of other components of the electron transport chain. We utilised our previous finding that the NAD(P)H-induced PQ reduction in the dark is achieved via a mercury-sensitive pathway [37]. As then reported, mercury (100 μM $HgCl_2$, with a Chl *a* concentration of 50 μg ml^{-1}) inhibits the NAD(P)H-induced PQ reduction, fully under aerobic and partially under anaerobic conditions. With the same $HgCl_2$ concentration, we investigated the fluorescence behaviour of dark-adapted DCMU-treated chloroplasts. The fluorescence transients of samples subjected to the same treatments as in Fig. 2A, but in the presence of 100 μM $HgCl_2$, are depicted in Fig. 4A. The transients are almost identical for all treatments. This provides strong evidence that elimination of fluorescence quenching by NAD(P)H is exclusively related to the NAD(P)H-induced PQ reduction. Fig. 4A shows also that in the presence of $HgCl_2$ the $F_{50\mu s}$ value is the same for all treatments. For each treatment, the difference between F_M and F_{5s} (Fig. 4A), the latter being identical to the F_M of the corresponding second pulse transient (Fig. 4B), is very minor, if not existing at all. We observe that in the presence of mercury (Fig. 4), like in its absence (Fig. 2), the $F_{50\mu s}$ values of the second pulse transients are higher than the $F_{50\mu s}$ values of the first pulse transients.

Focusing only on the transients exhibited by DCMU-treated samples under aerobic conditions and in the absence of NAD(P)H, we observe that in the presence of mercury (Fig. 4A) F_M is lower and F_0 is higher than in its absence (Fig. 2A). Similar effects are observed for non-DCMU-treated broken chloroplasts (insert of Fig. 4), as also previously reported [37]. The transients presented in the insert of Fig. 4 reveal that the extent of F_M decrease (by about 6%) and of F_0 increase (by about 15%) is the same as in DCMU-treated samples, which indicates that we are witnessing, both in the absence and presence of DCMU, an additional effect of mercury, independent of its inhibitory role on NAD(P)H-induced PQ reduction.

Fig. 5 summarises our findings concerning the F_M values in DCMU-treated broken chloroplasts at different PQ redox states. The results are expressed by the fractional quenching $Q_M=(F_{M,uq}-F_M)/F_{M,uq}$, where the $F_{M,control}$, identical in all dark-adapted samples in the absence of DCMU and mercury (insert of Fig. 2), was taken as the $F_{M,uq}$ (subscript "uq" stands for "unquenched"). The data presented in Fig. 5 were

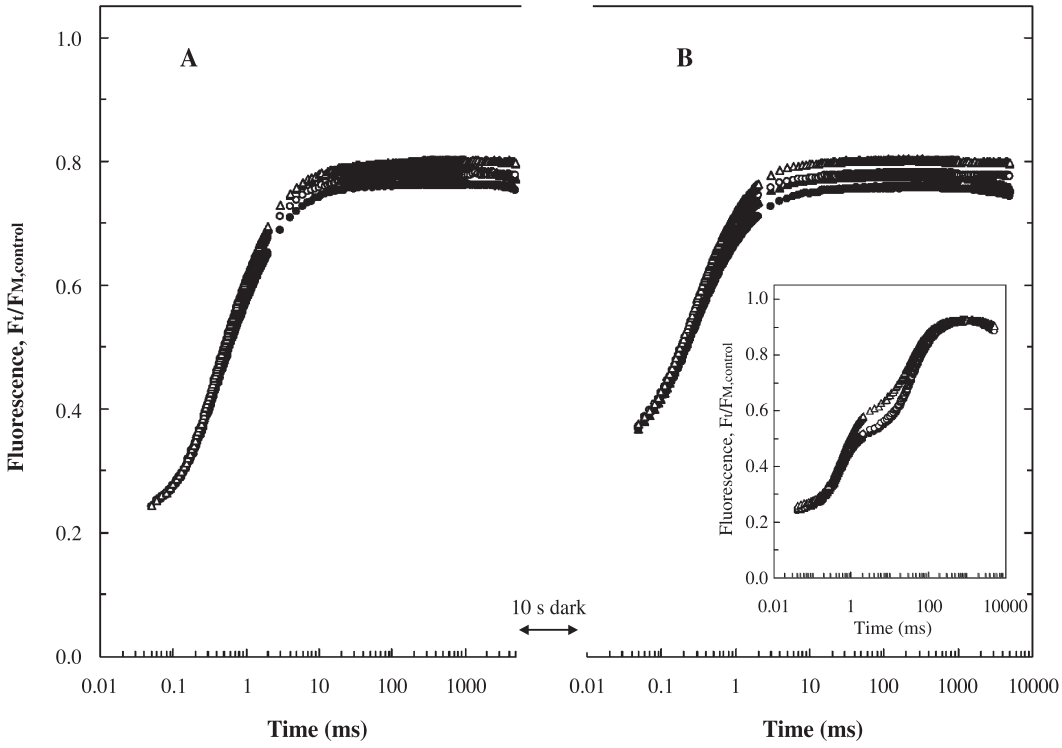

Fig. 4. Chl *a* fluorescence rise kinetics of DCMU-treated osmotically broken spinach chloroplasts incubated, in the presence of mercury (100 μM $HgCl_2$), for 60 min at room temperature in the dark under aerobic (●, ▲) or anaerobic (○, △) conditions, in the absence (●, ○) or presence (▲, △) of 250 μM NADPH. (A) First pulse transients, induced immediately after the dark incubation; (B) second pulse transients, induced after a 10-s dark interval following the completion of the first pulse transients. The insert shows, for comparison, the first pulse transients obtained from non-DCMU-treated samples incubated with mercury. For other details, see legend of Fig. 1.

obtained from the transients exhibited by DCMU-treated samples, dark-incubated in the presence or absence of NAD(P)H, both under aerobic and anaerobic conditions, as indicated in the abscissa. For each of the four treatments, the results refer to samples without pre-illumination (black columns; from Fig. 2A) and after pre-illumination with red (grey columns; from Fig. 2B) or far-red light (white columns; from Fig. 3A). The maximum fractional quenching appears at the F_M level of the sample solely treated with DCMU and following pre-illumination with far-red light. Fig. 5 includes

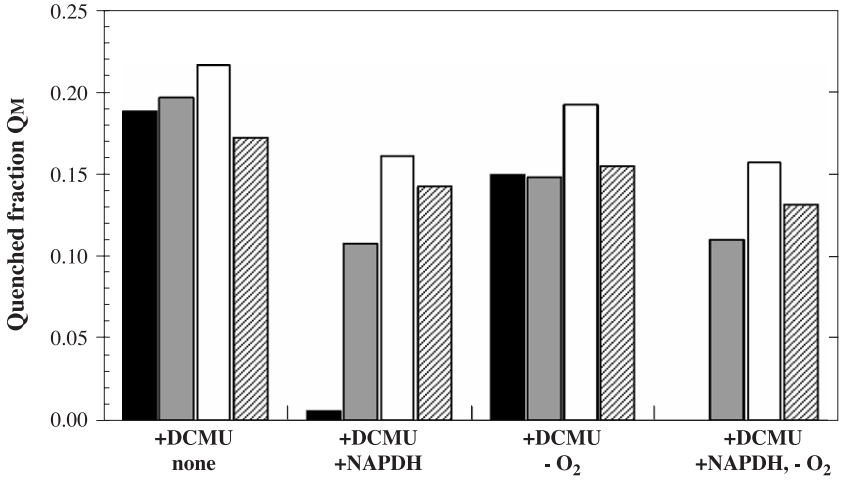

Fig. 5. Fractional quenching Q_M (at the F_M level) defined as $Q_M=(F_{M,uq}-F_M)/F_{M,uq}$ and calculated from the transients of osmotically broken spinach chloroplasts subjected to the treatments indicated in the abscissa and obtained as follows: (a) immediately after the 60-min dark incubation (black columns); (b) as in (a) but following a 5-s red light pre-illumination and a subsequent 10-s dark interval (grey columns); (c) as in (a) but following a 5-s far-red light pre-illumination and a subsequent 10-s dark interval (white columns); (d) as in (a) but in the presence of mercury (100 μM $HgCl_2$) in the incubation medium (hatched columns). For the calculation of Q_M in (a), (b) and (c), the common F_M value ($F_{M,control}$) obtained from all dark-adapted non-DCMU-treated samples in the absence of mercury was taken as $F_{M,uq}$ (subscript "uq" standing for "unquenched"); for (d) the common F_M of all dark-adapted and non-DCMU-treated samples in the presence of mercury was used instead.

also, for each of the four basic treatments (indicated in the abscissa), the fractional quenching at the F_M level obtained in the presence of $HgCl_2$ (hatched columns; from Fig. 4A). However, caution was taken concerning the reference level. The corresponding Q_M values were calculated using as $F_{M,uq}$ the common F_M value recorded in the non-DCMU-treated samples in the presence of mercury (see insert of Fig. 4), so that the F_M lowering caused by mercury, additionally to its inhibitory role on NAD(P)H-induced PQ reduction, would not be taken in account.

Collectively, our results provide strong evidence for the role of oxidised PQ as a fluorescence quencher. The next step was to investigate whether the quenching is exclusively exerted on the excited state of antenna chlorophylls. If this would be the case, the relation between Q_M, the fractional quenching at F_M, and the fractional quenching at F_0, accordingly defined as $Q_0=(F_{0,uq}-F_0)/F_{0,uq}$, is theoretically derived as shown below.

We denote the first-order de-excitation rate constants for fluorescence, photochemistry and antenna dissipation (without additional quenching) as k_F, k_P and k_N, respectively, and the second-order de-excitation rate constant for quenching as k_Q. We denote by $[PQ_q]$ the concentration of oxidised PQ that acts as a quencher, since there is no evidence that the whole of the PQ-pool plays this role. J_{abs} refers to the absorbed light intensity and g is a proportionality factor defined by the geometry of the setup.

$F_{0,uq}$, F_0 and, concomitantly, Q_0 are then given by the following formulae:

$$F_{0,uq} = gJ_{abs}\frac{k_F}{k_P + k_N} \tag{1}$$

$$F_0 = gJ_{abs}\frac{k_F}{k_P + k_N + k_Q[PQ_q]} \tag{2}$$

$$Q_0 \equiv \frac{F_{0,uq} - F_0}{F_{0,uq}} = \frac{k_Q[PQ_q]}{k_P + k_N + k_Q[PQ_q]} \tag{3}$$

Accordingly, $F_{M,uq}$, F_M and Q_M are written as

$$F_{M,uq} = gJ_{abs}\frac{k_F}{k_N} \tag{4}$$

$$F_M = gJ_{abs}\frac{k_F}{k_N + k_Q[PQ_q]} \tag{5}$$

$$Q_M \equiv \frac{F_{M,uq} - F_M}{F_{M,uq}} = \frac{k_Q[PQ_q]}{k_N + k_Q[PQ_q]} \tag{6}$$

Hence,

$$\frac{Q_0}{Q_M} = \frac{k_N + k_Q[PQ_q]}{k_P + k_N + k_Q[PQ_q]} = \frac{F_0}{F_M} \tag{7}$$

Table 1
Experimentally determined values of Q_0/Q_M and F_0/F_M

Treatment	Q_0/Q_M	F_0/F_M
+DCMU	0.249	0.247
+DCMU, +NAD(P)H	0.213	0.211
+DCMU, $-O_2$	0.244	0.239
+DCMU, +NAD(P)H, $-O_2$	0/0 (indefinable)	0.211

This theoretically derived relation can be experimentally tested, however, with the prerequisite that the measured F_0 corresponds to the fluorescence emitted when all RCs are open. The finding that DCMU alone did not affect the minimum fluorescence yield, $F_{50\mu s}$, excludes the possibility that an incomplete reopening of the RCs due to the presence of DCMU would be responsible, fully or even partly, for the differences concerning the $F_{50\mu s}$ values observed among the transients of Fig. 2A. However, for a quantitative comparison of the Q_0/Q_M and F_0/F_M ratios, a precise determination of F_0 is needed. With the fluorimeter used in our study, due to limitations related with the response time of the detector (about 30 μs), the first reliable measurement is usually considered to be at 50 μs. Though the approximation $F_0 \cong F_{50\mu s}$ brings a negligible error in routine tests, in our study, dealing with transients with widely different rise times, the error cannot be ignored.

We therefore introduced a simple method, by which the linear extrapolation of the data from 50 to 150 μs down to 10 μs was used as the F_0 value. The same data range was also used previously by Vredenberg [43] who, however, applied an exponential extrapolation. Our choice was based on the observation that all recorded transients of DCMU-treated samples, having a sigmoidal shape due to energetic connectivity between photosynthetic units, appeared linear in the initial narrow range from 50 to 150 μs. Hence, for the 11 data points recorded from 50 to 150 μs, a linear regression was considered as more suitable and, when applied, gave indeed, in all cases, R^2 values above 0.998.

The Q_0/Q_M and F_0/F_M ratios were calculated for the cases where F_0 was expected to be affected solely by PQ quenching. Table 1 shows that, for each such case, the experimentally determined value of the Q_0/Q_M ratio is almost identical to that of the F_0/F_M ratio, as theoretically predicted for the case that the quenching by oxidised PQ is exerted exclusively on the excited state of antenna chlorophylls.

4. Discussion

In the present work, we observed that the maximum fluorescence level F_M was lowered by about 20% when the chloroplasts were treated with DCMU (Fig. 1). The actinic light used in our study allowed maximal reduction of the PQ-pool in the untreated chloroplasts (control samples), as revealed by the finding that the F_M-level ($F_{M,control}$) was as high as in the case where the PQ-pool had been already non-

photochemically reduced prior to illumination (insert of Fig. 2 and [37]). Therefore, the $F_{M,control}$ value represents the true unquenched F_M.

The extent of the F_M lowering we observed is in full agreement with the results obtained by Vernotte et al. [14], who attributed the phenomenon to a non-photochemical quenching of Chl *a* fluorescence by oxidised PQ. However, several other publications have reported that the DCMU effect on F_M can be much smaller or even not appear at all [16,17,19], which has put the hypothesis of Vernotte et al. [14] in doubt. The question of non-photochemical quenching by PQ has been recently readdressed, both in the absence and presence of DCMU, by incorporating either exogenous plastoquinone [18,19] or artificial substituted quinones [19,22] into thylakoid membranes, thus enlarging the pool of oxidised (plasto)quinones.

In our study, aiming to reinvestigate the possible role of PQ as a non-photochemical quencher in DCMU-treated samples, we employed an approach based on alterations of the redox state of the native PQ-pool. This approach, similar in principle to that used by Vernotte et al. [14] in their original work on fluorescence quenching by PQ, has the advantage that it does not modify the composition of the native photosynthetic electron transport chain.

Full reduction of PQ by NAD(P)H in the dark was found to fully abolish the F_M lowering that appears in DCMU-treated samples (Fig. 2A). This finding is similar to the results obtained when the strong reductant dithionite was used [14]. However, unlike dithionite, NAD(P)H is a reductant naturally present in chloroplasts that reduces specifically PQ [35–37].

Mercury, which has been reported to inhibit the NAD(P)H-induced reduction of PQ [37], was found to inhibit also the restoration of F_M by NAD(P)H (Fig. 4). This provides strong evidence that the elimination of fluorescence quenching by NAD(P)H is exclusively related to the NAD(P)H-induced PQ reduction and not to alteration of the redox state of other components of the photosynthetic electron transport chain.

Partial reduction of PQ caused by anaerobiosis (insert of Fig. 2 and [37]), probably due to the presence of native reductants in the chloroplasts suspension, was found to result in a minor increase of $F_{M,DCMU}$ towards the $F_{M,control}$ level (Fig. 2A).

Since the rate of NAD(P)H-induced reduction of PQ is much lower than the rate of PQH_2 reoxidation by PS I activity, we could follow the effect of the latter on F_M and we observed the reappearance of quenching. The same observation was made by Vernotte et al. [14] when DCMU was added after PQ had been reduced, however, only if the reduction was induced photochemically but not when it resulted from the addition of dithionite. Moreover, the transients obtained with our experimental protocol revealed that F_M quenching started to develop again after about 30 ms of illumination (Fig. 2A), when the PS I-driven reoxidation of PQH_2 formed in the dark is initiated [20,42]. The finding that the F_M level of the second pulse transient (Fig. 2B) was much lower than that of the first pulse transient (Fig. 2A), being as low as at the end of the first pulse (F_{5s}), indicates that the decline observed in the first pulse did not arise because of an additional light-dependent process, which would be expected to relax in the dark. Pre-illumination with far-red light resulted also in the restoration of F_M quenching, even more pronounced than that observed after red light pre-illumination (Figs. 3A and 5), in accordance with the expected enhancement of PS I activity.

Collectively (Fig. 5), our results demonstrate that the lowering of F_M, appearing in the presence of DCMU, is essentially linked with the PQ redox state, with the extent of quenching being smaller when the plastoquinone pool is in a more reduced state.

So far, we discussed only the effect of the PQ redox state on F_M, where all RCs are close, i.e., when Q_A has been converted to Q_A^-. If indeed, as originally proposed by Vernotte et al. [14], oxidised PQ quenches the excited state of antenna chlorophylls, then F_0 must be also affected, as well as all other F_t values of the fluorescence transient between F_0 and F_M. In cases where non-DCMU-treated samples are used, the PQ-pool, being oxidised in the dark, gets progressively reduced during illumination, resulting in a gradual removal of both photochemical and non-photochemical quenching by oxidised PQ. However, in our study, for any fluorescence transient obtained from DCMU-treated dark adapted samples, all F_t values between F_0 and F_M were affected by the same PQ-pool redox state, as it had been established in the dark prior to illumination, since reoxidation of PQH_2 by PS I activity is not occurring in the short time range of the fast fluorescence rise. This allowed us to investigate whether F_0 was indeed quenched and to which extent.

DCMU alone was found to have no effect on F_0 (Fig. 1). This finding is not in agreement with other reports, where F_0 was found to increase in the presence of DCMU [19,21,40]. Possible explanations for this discrepancy may be related with differences in the experimental protocol: for our experiments the chloroplasts were prepared under dim light and kept in full darkness; DCMU was dissolved in water; the chloroplast suspension was incubated with DCMU for 1 h in full darkness. We would like to emphasise that, following this protocol, the same result was also obtained in other experiments with chloroplasts isolated from pea (*Pisum sativum* L.) leaves (P. Haldimann, unpublished data). DCMU was also reported to hardly affect the F_0 level in intact pea leaves [20].

Moreover, as shown in the experiments conducted in the presence of mercury, F_0 was identical for all four treatments (Fig. 4A), indicating that neither anaerobiosis nor NAD(P)H had a direct effect on the redox state of Q_A. This points out an additional advantage of using NAD(P)H as a reducing agent, since dithionite, even when added after DCMU, converts a fraction of Q_A to Q_A^-, as revealed by a pronounced increase of F_0 (see, e.g., Ref. [44]).

It can therefore be concluded that, in the transients depicted in Fig. 2A, the treatments applied affected F_0 and F_M only via the induced changes of the PQ redox state. This obviously does not hold true for F_0 when the transients were recorded after pre-illumination.

Introducing a method to improve the precision of F_0 determination, we calculated the fractional quenching Q_0 and Q_M at the open and closed states of the RCs, respectively. This was restricted to the cases where F_0 was expected to be affected solely by PQ quenching (Table 1). The finding that Q_0/Q_M ratio was equal to the corresponding F_0/F_M ratio for all these cases (Table 1) is in full accordance with theoretical predictions (formulated in Eqs. (1)–(7)) for the case that PQ quenching is solely exerted on the excited state of antenna chlorophylls.

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