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PICOSECOND TIME-RESOLVED STUDY OF $MgCl_2$-INDUCED CHLOROPHYLL FLUORESCENCE YIELD CHANGES FROM CHLOROPLASTS

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Summary

The $MgCl_2$-induced chlorophyll fluorescence yield changes in broken chloroplasts, suspended in a cation-free medium, treated with 3,-(3′,4′-dichlorophenyl)-1,1-dimethylurea and pre-illuminated, has been investigated on a picosecond time scale. Chloroplasts in the low fluorescing state showed a fluorescence decay law of the form $\exp -At^{1/2}$, where A was found to be 0.052 $ps^{-1/2}$, and may be attributed to the rate of spillover from Photosystem II to Photosystem I. Addition of 10 mM $MgCl_2$ produced a 50% increase in the steady-state fluorescence quantum yield and caused a marked decrease in the decay rate. The fluorescence decay law was found to be predominantly exponential with a 1/e lifetime of 1.6 ns. These results support the hypothesis that cation-induced changes in the fluorescence yield of chlorophyll are related to the variations in the rate of energy transfer from Photosystem II to Photosystem I, rather than to changes in the partitioning of absorbed quanta between the two systems.

Introduction

Cation concentrations have been shown to control the level of chlorophyll *a* fluorescence from higher plant chloroplasts whose bounding envelope has been broken by hypotonic shock (see ref. 1 for a recent review). Two situations can be defined: a low fluorescing state observed with low concentrations of monovalent cations (5—10 mM), and a high fluorescing state seen either in the presence of divalent cations (>1 mM) or high concentrations (100

Abbreviations: DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; MES, 2-(*N*-morpholino)ethanesulphonic acid.

mM) of monovalent cations [2,3]. The high fluorescing state is also seen with chloroplasts, prepared in a divalent-cation-containing medium, which are then resuspended in a cation-free medium [4,5]. At room temperature these fluorescence yield changes are associated with the chlorophyll molecules which serve Photosystem II (PSII) and seem to reflect the changes in the ditribution of absorbed energy between the two Photosystems [1]. Such a process would help to optimise the co-operation of PSII and Photosystem I (PSI) acting in series, probably by the transfer of singlet excitons between the respective light-harvesting pigment complexes. The exact mechanism involved remains to be determined, although some authors have argued that there may be changes in the partitioning of the incident quanta between the chlorophylls of PSII and PSI [6], while others have favoured a change in the degree of spillover from PSII to PSI (refs. 3,7,8, and ref. 1). It has been suggested that simultaneous measurement of the changes in the fluorescence intensity and lifetime of chlorophyll *a* can be used to distinguish between these two possibilities [7]. Briantais et al. [7] and, more recently, Moya et al. [8] found a nearly linear relationship between the changes in the fluorescence yield and lifetime, which indicated that PSII to PSI spillover was operative, although there was some evidence for a slight change in the quantal partitioning, in agreement with the conclusions of Butler and Kitajima [9]. However, their analysis was based upon the assumption that the fluorescence decay of the PSII chlorophyll *a* antenna system is governed by an $\exp -kt$ decay law, which we have shown not always to be the case [10,11]. We have re-investigated the fluorescence yield and lifetime relationship utilising picosecond time-resolved fluorescence spectroscopy. Changes in the fluorescence yield were induced by the addition of Mg^{2+} to illuminated chloroplasts treated with 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea (DCMU).

Materials and Methods

Chloroplasts were isolated from the leaves of young pea seedlings (*Pisum sativum*, variety Feltham First, 8—9 days old) by homogenisation for 5×3 s bursts with a Polytron blender in 0.33 M sorbitol and 20 mM 2-(*N*-morpholino)ethanesulphonic acid (MES), pH 6.5. After filtering through a muslin/cotton-wool pad, the chloroplasts were centrifuged rapidly at $2500 \times g$ for 30 s and washed once in a cation-free medium (0.33 M sorbitol and Tris base (*N*-tris-hydroxymethylaminomethane), pH 7.5) before resuspending in the same cation-free medium to a concentrated suspension.

Prior to the experiment, the suspension was diluted with a sorbitol-free cation-free medium to give a chlorophyll concentration of 0.5—1.0 mg · ml^{-1}, and DCMU was added to a final concentration of 10 μM. Any salt additions were made by microsyringe before placing the sample into a 1 mm pathlength cuvette. The transmission of the samples at 530 nm was approximately 50%.

The steady state fluorescence yield was measured using a CW He : Ne laser (wavelength 633 nm, intensity at the sample 12.5 W · m^{-2}) to excite the sample and an EMI 9558 B photomultiplier, protected by a Schott RG665 cut off filter, to detect the fluorescence intensity which was displayed on a storage oscilloscope. The fluorescence decay kinetics were investigated with the pico-

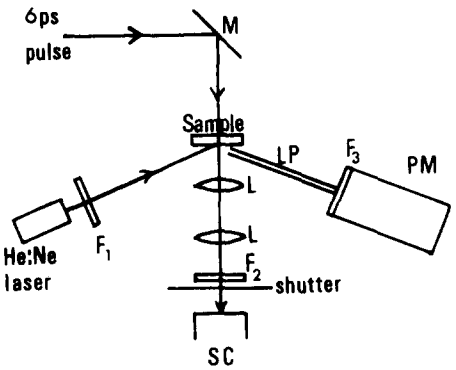

Fig. 1. The optical arrangement used to simultaneously record the steady-state fluorescence yield and the picosecond decay kinetics. M, 100% reflecting mirror; L, collection optics; F_1, neutral absorbance filter; F_2 and F_3, Schott RG665 cut off filters; SC, streak camera (Imacon 600); LP, fibre-optic light pipe; PM, EMI 9558 B photo-multiplier.

second laser : streak camera system described elsewhere [10—12], using a single 6ps (FWHM), 530 nm excitation pulse. The monitoring wavelength range was selected with a Schott RG665 cut off filter, and a streak camera speed of 500 ps · mm^{-1} was used to provide the best compromise between photon sensitivity and time resolution (88 ps). To reduce the effects of exciton-exciton annihilation, the excitation intensity was limited to a maximum value of 10^{14} photons · cm^{-2}, in general the average intensity was of the order of $5 \cdot 10^{13}$ photons · cm^{-2} (±15%). A diagram of the optical arrangement used in these experiments is shown in Fig. 1; a shutter was inserted in front of the streak camera so that the photocathode was not subjected to prolonged irradiation by the steady-state fluorescence emission.

Results

The fluorescence decay kinetics were recorded after 1—2 min illumination of the chloroplasts with the CW He : Ne laser so that a steady-state fluorescence level was obtained corresponding to the condition when all the PSII traps were closed. When the chloroplasts were isolated in low cation medium this steady-state fluorescence yield increased by approximately 50% upon the addition of 10 mM $MgCl_2$. A marked difference in the fluorescence decay kinetics was observed for the two fluorescence levels when measured at the same laser pulse intensity (±15%). Fig. 2 shows the fluorescence decay curves of chlorophyll in the absence (curve A) and the presence (curve B) of $MgCl_2$, recorded at an excitation intensity of approximately $5 \cdot 10^{13}$ photons · cm^{-2}. Both decay curves show a degree of non-exponential behaviour, as may be seen from the semi-logarithmic plots of the data in Fig. 3, although the addition of $MgCl_2$ appears to produce a decay which is predominantly governed by an exp $-kt$ decay law. The value of k was calculated to be $6.25 \cdot 10^{-4} \cdot ps^{-1}$, equivalent to a fluorescence lifetime of 1.6 ns, which is close to the 1.8 ns lifetime previously reported for *Chlorella pyrenoidosa* treated with DCMU and preilluminated [11]. Fluorescence decay curves obtained from the samples with no salt added were adequately described by an empirical decay law of the form exp $-At^{1/2}$

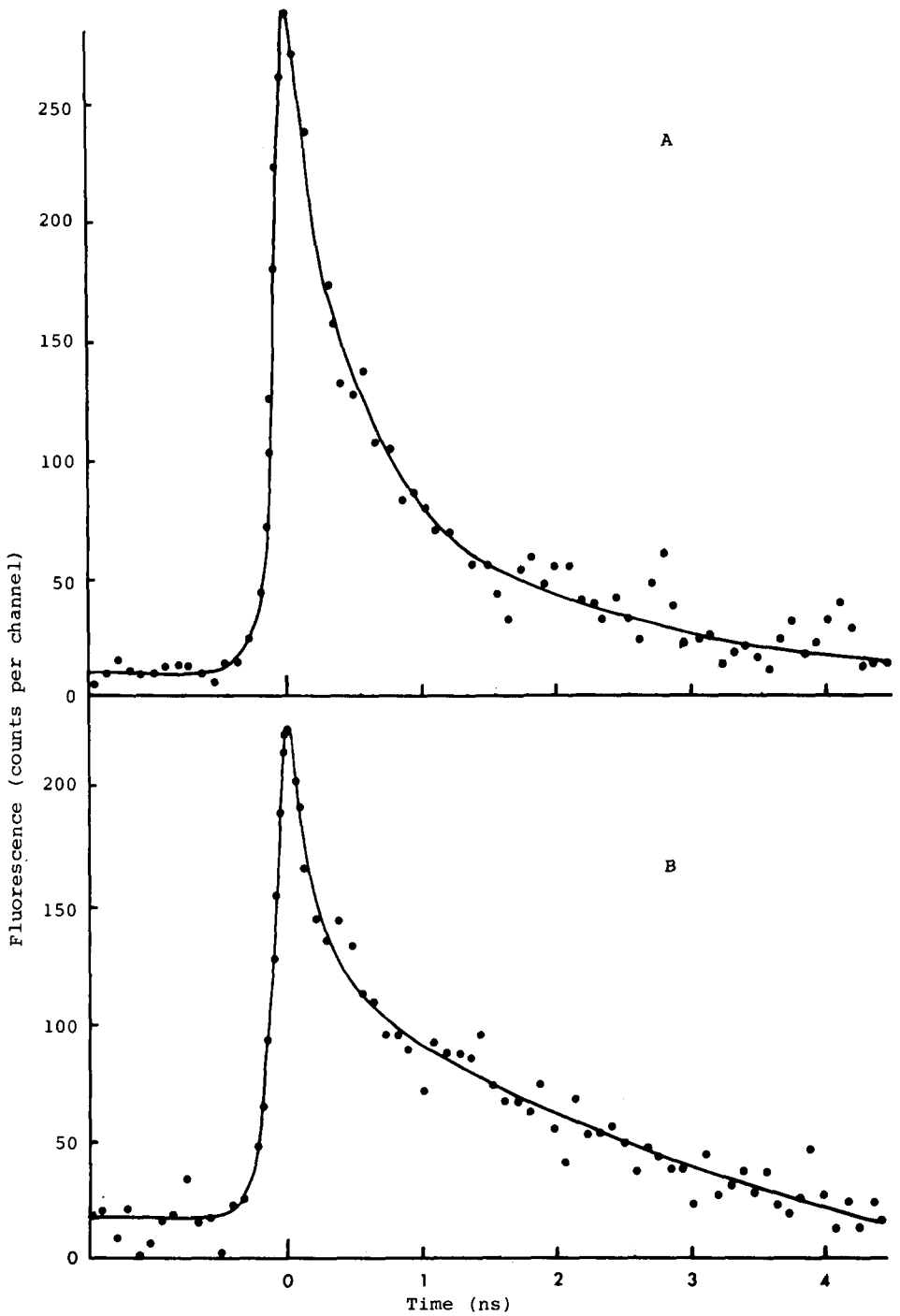

Fig. 2. The fluorescence decay of chlorophyll in broken chloroplasts treated with DCMU and pre-illuminated. A, Without cations and B, with 10 mM $MgCl_2$ added.

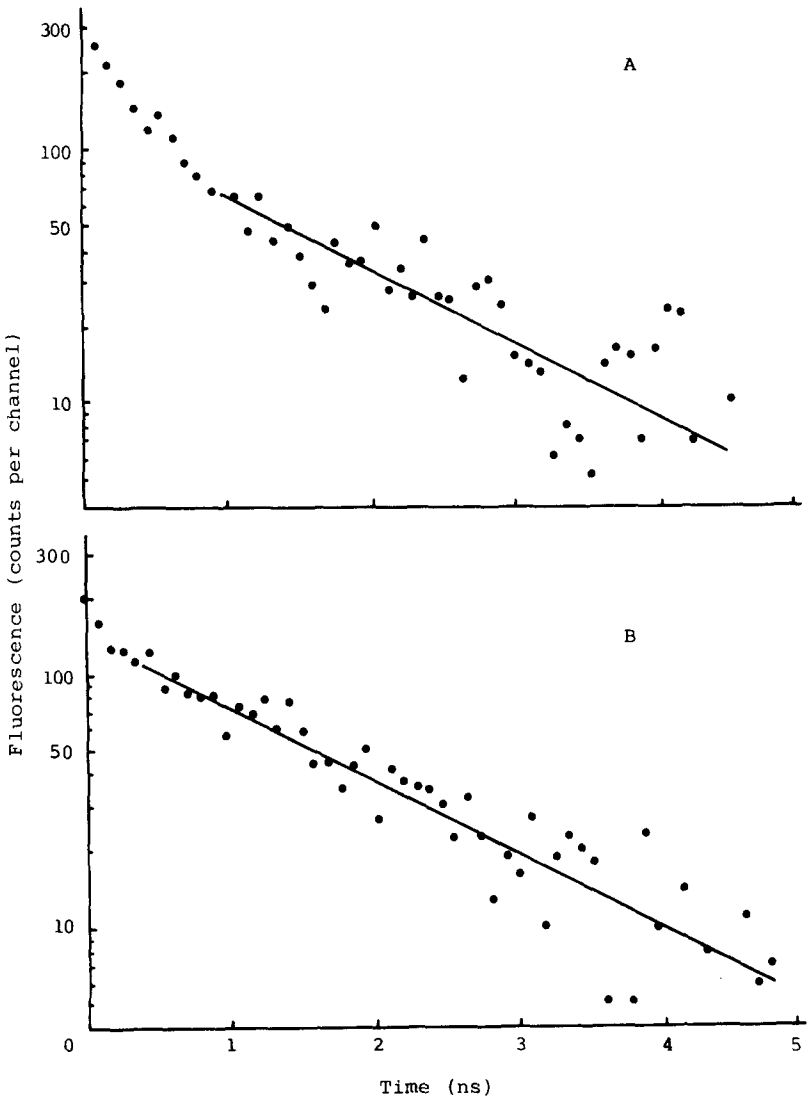

Fig. 3. Analysis of the fluorescence decay curves shown in Fig. 2. The fluorescence intensity is plotted on a logarithmic scale as a function of time, and A and B correspond to the traces shown in Fig. 2.

over the time spanned by the traces, as shown by Fig. 4 (the deviation from linearity at short times is a result of the streak camera time resolution function). From the curve shown in Fig. 4, the value of the rate constant A was calculated to be 0.052 $ps^{-1/2}$. Since the decay curve obtained from the samples with $MgCl_2$ added appears to be intermediate between an exp—kt and an exp—$At^{1/2}$ decay law, it was not possible to obtain a rate constant which would describe the complete curve. However, by extrapolating the exponential component of the decay back to zero time, the quantum yield of the emission could be calculated from the fluorescence lifetime of 1.6 ns and the relative intensity of this component; in the case of the curve in Fig. 2B, the initial component does not contribute significantly to the overall fluorescence yield. Using this semi-quantita-

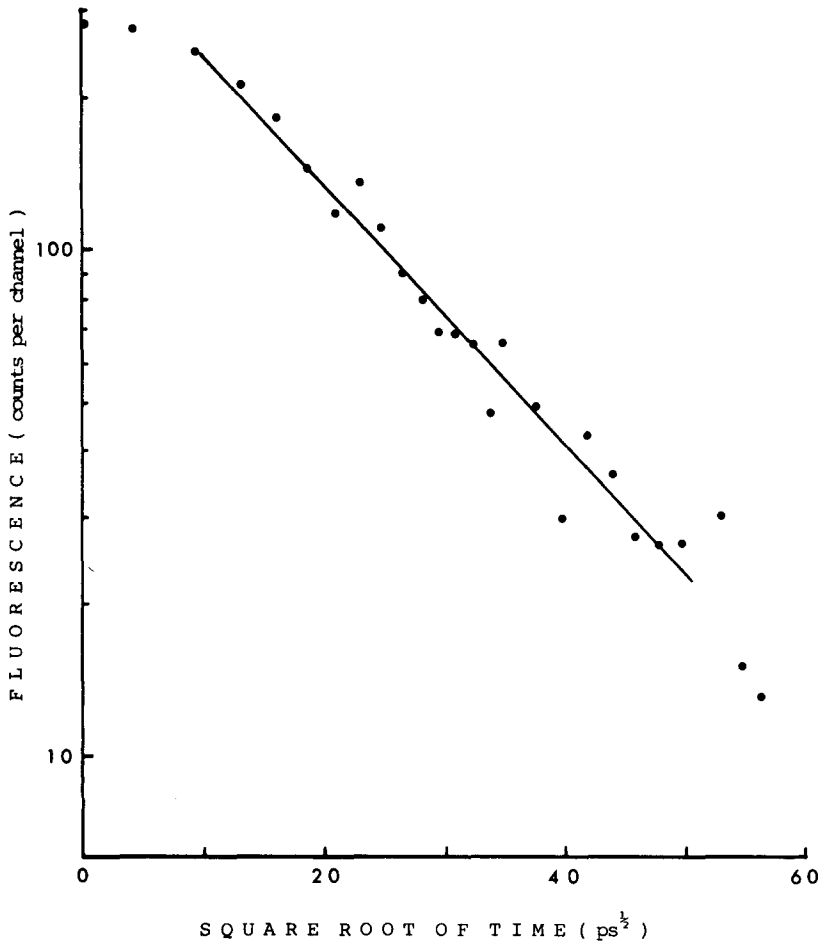

Fig. 4. Analysis of the fluorescence decay curve shown in Fig. 2A. The fluorescence intensity is plotted on a logarithmic scale as a function of the square root of time. These chloroplasts, suspended in a cation-free medium, show a fluorescence decay rate of 0.052 $ps^{-1/2}$.

tive approach, the quantum yield was estimated to be 6.5%. The quantum yield of the emission depicted in Fig. 1A was calculated from the value of the rate constant, A, to be 4.3%, using the method described previously [11]. Addition of $MgCl_2$ to the sample therefore produces a calculated increase of 51% in the fluorescence quantum yield, in excellent agreement with the observed steady-state increase of approximately 50%.

In other experiments, chloroplasts were isolated in a high fluorescing state; addition of 10 mM KCl caused a 37% decrease in fluorescence quantum yield of chlorophyll, and subsequent addition of 10 mM $MgCl_2$ brought about an increase of 34% above the initial level of fluorescence. The fluorescence decay kinetics followed the pattern already illustrated in Fig. 2: without salt addition the decay was similar to that observed with $MgCl_2$ added, although the exponential component was less obvious; with KCl added, the low fluorescing state again followed an exp $-At^{1/2}$ decay law.

Discussion

Recent reports have clearly demonstrated that the effect of cations of the fluorescence quantum yield of chlorophyll in chloroplasts is mediated via the diffuse electrical layer surrounding the negatively-charged thylakoid membrane, rather than by chemical binding to negative sites [13,14]. These cation-induced changes have been shown to require the presence of the light harvesting chlorophyll *a/b* protein complex of Thornber [15,16,19]. The nature of the interactions between PSI, PSII and light-harvesting chlorophyll · protein complexes brought about by these electrical changes, which affect the fluorescence emission from PSII, has been investigated by the phase shift technique [7,8]. This work has led to the conclusion that it is the rate of energy spillover from PSII to PSI which is changed by salt addition rather than the partitioning of absorbed quanta between the two photosystems [7]. In their analysis these authors assumed that the fluorescence decay kinetics of chlorophyll were exponential under all conditions, which is shown not to be the case in Fig. 3. However, the linear relationship between fluorescence lifetime and yield observed by Briantais et al. [7] and Moya et al. [8] will also hold for a decay law of the form $\exp -At^{1/2}$, since the 1/e lifetime is given by A^{-2} and the quantum yield is proportional to $2A^{-2}$. Clearly, changes in the decay law between the condition when all the traps are open (F_0 level) and when they are closed (F_m level) (see refs. 11 and 17) may give rise to the slight non-linearity in the plot of fluorescence lifetime against quantum yield as observed using the phase shift technique. However, this would be strongly dependent upon the time resolution of the detection system.

The fluorescence decay measurements reported in this communication only indicate changes in the decay kinetics, since the initial fluorescence intensities have not been related to the intensity of the excitation pulse. In contrast the steady-state measurements may include both changes in the decay kinetics and in the quantal partitioning [9,18]. Consequently, the agreement found between the steady-state and kinetic determinations of the change in the fluorescence quantum yield, between samples with and without $MgCl_2$ added, indicate that the variation of quantal partitioning is not a major factor in governing the quantum yield of PSII chlorophylls; this conclusion is in agreement with previous reports [7—9].

Spillover of singlet excitons from PSII to PSI appears to be the main cause of the $\exp -At^{1/2}$ decay law observed in Fig. 4. In the light of our previous investigations of energy transfer within the photosynthetic unit it is not surprising that spillover results in a chlorophyll fluorescence decay law similar to that caused by energy migration to an open reaction centre [10,11,17]. In this instance, PSI is the quenching species and the rate of spillover is determined by the rate of energy migration within the PSII chlorophyll antennae, since the lifetime of excitons in PSI is extremely short (100 ps or less) [10]. With the PSII reaction centres closed, the fluorescence decay rate resulting from spillover to PSI ($A = 0.052\ ps^{-1/2}$) is very similar to that due to quenching by an open PSII reaction centre ($A = 0.047\ ps^{-1/2}$) in the absence of spillover [10,11]. This suggests that spillover to PSI takes place via a specific site in the photosynthetic unit, otherwise a much faster decay rate would have

been expected. It should be noted that previous ps fluorescence work using *Chlorella* [11] had been carried out on cells which had been either dark-adapted or preilluminated for a short period of time to close the reaction centres. Under these conditions the intact organism would have been in State 1 which corresponds to the minimum spillover (see ref. 1). Only after prolonged illumination with light less than 680 nm does an organism like *Chlorella* change to a low fluorescing, maximum spillover state (State 2) comparable with the low salt conditions used in this work (see ref. 1). Apparently in the intact organism the State 1—State 2 shift is carefully controlled by light-induced changes in ionic levels in the intact chloroplast compartments as discussed by Barber [1] and serves as a mechanism for maximising electron flow under light limiting conditions. Moreover, it is not clear if the $\exp -At^{1/2}$ decay can be equated to the corresponding term in the Förster equation ($I_t = I_0 \exp(-kt - At^{1/2})$) since it is not yet clear that Förster energy transfer is applicable to light harvesting processes of the photosynthetic apparatus. Even if the Förster equation was applicable the rate parameter, A, is sufficiently large to dominate the decay over the time interval recorded by our instrument. At times when the kt term would become important the emission intensity would be too low for our detector.

It is interesting to note that the maximum change, which can be calculated for the quantum yield of PSII chlorophyll fluorescence on the addition of $MgCl_2$ to DCMU-treated broken chloroplasts in the low fluorescing state, is from 4.3% ($\exp -At^{1/2}$ decay law with $A = 0.052$ $ps^{-1/2}$) to 9.3% (a purely exponential decay with a lifetime of 1.6 ns). Thus, a maximum change of about 2.2-fold would be expected, and this can indeed often be observed [1].

The possible interference from exciton-exciton annihilation must also be considered in the interpretation of these results. Fluorescence quenching by this process at the low excitation intensities used should be less than 20%, if it is assumed that exciton annihilation in *Chlorella* treated with DCMU [11] is comparable to that in the chloroplasts. The close agreement between the steady-state and kinetic determinations of the fluorescence yield change indicate, however, that exciton annihilation is not a significant factor at the intensities used in these measurements. The reason for the greater sensitivity of intact *Chlorella*, compared to isolated chloroplasts, to the intensity of the laser pulse is not clear but may simply reflect a difference in the extent of the light harvesting chlorophylls in the two systems. It is well established that the chlorophyll *a*/chlorophyll *b* pigment-protein content of photosynthetic tissue can vary considerably depending on growth conditions [19]. Deviations from a pure exponential decay law observed in Fig. 2B ($MgCl_2$ added) are probably the result of residual spillover rather than the effects of exciton annihilation.

It is apparent that the rate of spillover from PSII to PSI must be dependent upon the state of the PSII reaction centre, because the same rate constant found for DCMU-treated chloroplasts ($A = 0.052$ $ps^{-1/2}$) would not be compatible with the observation that spillover is only a minor quenching pathway in the dark-adapted state (see also refs. 9 and 18). The way in which the state of the PSII trap can effect the rate of spillover poses an intriguing problem which warrants further investigation.

Finally, it should be emphasised that throughout this paper we have assumed

that changing the ionic level of the solution bathing the chloroplasts effects the degree of spillover between PSII and PSI. Although there is considerable evidence for this (see refs. 1 and 9) it has not definitely been proved. The salt-induced changes in chlorophyll fluorescence could be due to other quenching processes and this possibility should always be kept in mind.

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