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EVIDENCE FOR THE ROLE OF THE LIGHT-HARVESTING CHLOROPHYLL *a/b* PROTEIN COMPLEX IN PHOTOSYSTEM II HETEROGENEITY

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Analyses of chlorophyll fluorescence induction kinetics from DCMU-poisoned thylakoids were used to examine the contribution of the light-harvesting chlorophyll *a/b* protein complex (LHCP) to Photosystem II (PS II) heterogeneity. Thylakoids excited with 450 nm radiation exhibited fluorescence induction kinetics characteristic of major contributions from both PS II_α and PS II_β centres. On excitation at 550 nm the major contribution was from PS II_β centres, that from PS II_α centres was only minimal. Mg^{2+} depletion had negligible effect on the induction kinetics of thylakoids excited with 550 nm radiation, however, as expected, with 450 nm excitation a loss of the PS II_α component was observed. Thylakoids from a chlorophyll-*b*-less barley mutant exhibited similar induction kinetics with 450 and 550 nm excitation, which were characteristic of PS II_β centres being the major contributors; the PS II_α contribution was minimal. The fluorescence induction kinetics of wheat thylakoids at two different developmental stages, which exhibited different amounts of thylakoid appression but similar chlorophyll *a/b* ratios and thus similar PS II:LHCP ratios, showed no appreciable differences in the relative contributions of PS II_α and PS II_β centres. Mg^{2+} depletion had similar effects on the two thylakoid preparations. These data lead to the conclusion that it is the PS II:LHCP ratio, and probably not thylakoid appression, that is the major determinant of the relative contributions of PS II_α and PS II_β to the fluorescence induction kinetics. PS II_α characteristics are produced by LHCP association with PS II, whereas PS II_β characteristic can be generated by either disconnecting LHCP from PS II or by preferentially exciting PS II relative to LHCP.

Introduction

The rise in chlorophyll fluorescence on illumination of DCMU-poisoned chloroplasts is thought to reflect the kinetics of the photochemical reduction of Q, the primary acceptor of PS II [1]. First-order analyses of the growth of the area over this fluorescence-induction curve have revealed two distinct phases: a rapid sigmoidal phase followed by a slow exponential phase [2,3]. These two phases have been attributed to two different forms of PS II, termed PS II_α and PS II_β, which have different kinetics of photoinduced Q reduction [2,3]. Studies of Mg^{2+}-induced changes in the induction curve [4,5] and with mutants lacking chlorophyll *b* [6,7] have located PS II_α in the appressed regions of thylakoid membranes with PS II_β being found in the nonappressed regions. Furthermore, it has been proposed that PS II_α and PS II_β are structurally distinct [9]. PS II_α is though to have a larger absorptive cross-section, a lower

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Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; DCMU, 3-(3′,4′-dichlorophenyl)-1,1′-dimethylurea.

chlorophyll *a/b* ratio and different spectral absorption characteristics due to more light-harvesting chlorophyll *a/b* protein complex (LHCP) being associated with it than is the case for PS II_β [8,9]. Alternatively, it has been argued that the heterogeneity of the induction curve is due to incomplete DCMU-blocking of some PS II centres [10] or arises as a consequence of connectivity between PS II units [11,12]. Although no general agreement has yet been reached for the nature of this heterogeneity, it is widely accepted that first-order analysis of the induction curve provides a useful probe of PS II organization and function.

We have previously reported that the characteristics of the fluorescence induction kinetics are dependent upon the wavelength of excitation; curves for excitation at 450 nm are more exponential than those observed with 550 nm excitation [13]. Such excitation wavelength-dependent modifications have been attributed to preferential excitation of different pigment matrices associated with the PS II_α and PS II_β centres at the different excitation wavelengths [13]. In this paper the role of LHCP in this phenomenon is examined from analyses of Mg^{2+}-induced changes in the fluorescence induction curves of thylakoids excited with 450 and 550 nm radiation. Wheat thylakoids at different developmental stages which exhibit similar chlorophyll *a/b* ratios but differing degrees of membrane appression are examined together with thylakoids from a barley mutant lacking chlorophyll *b*.

Materials and Methods

Mature leaf tissue was obtained from plants of wheat (*Triticum aestivum* cv. Maris Dove), barley (*Hordeum vulgare* vc. Clermont) and chlorophyll-*b*-less barley (*Hordeum vulgare* chlorina *f*-2 mutant) grown at 20°C and a constant relative humidity of 70% under a 16 h photoperiod of a photosynthetically active photon-flux density of 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ in a Fitotron 600H environmental chamber (Fisons, U.K.). Wheat leaf tissue containing chloroplasts at different developmental stages was grown as previously described [14].

Thylakoids were prepared at 4°C by homogenization of leaf tissue in isolation medium (300 mM sorbitol/5 mM $MgCl_2$/10 mM NaCl/50 mM Hepes at pH 7.6) followed by filtration through ten layers of muslin and five layers of 25 μm nylon mesh and sedimentation at $3000 \times g$ for 90 s. The pellet was then resuspended in medium containing 5 mM $MgCl_2$/10 μM nigericin/10 mM KCl/20 μM DCMU/50 mM Hepes at pH 7.6. For Mg^{2+} depletion studies 5 mM $MgCl_2$ was substituted with 1 mM EDTA in the resuspension medium.

After dark-adaptation at 20°C for 5 min, diluted thylakoid suspensions, containing a chlorophyll concentration of 10 $\mu g \cdot cm^{-3}$, were irradiated with 75 or 100 $\mu mol \cdot m^{-2} \cdot s$ of 450 or 550 nm light (maximum half-band width of 10 nm) generated by a xenon arc source with a high radiance monochromator (Applied Photophysics Ltd., U.K.) and delivered by a liquid light guide. Fluorescence emission was monitored at 90°C to the excitation source through a 685 nm interference filter (Ealing-Beck, U.K.) with a Hamamatsu R446 photomultiplier tube. Data was digitised using DL901 transient recorders (Datalab Laboratories Ltd., U.K.) and processed by a microcomputer. First-order plots of the area growth above the fluorescence induction curves were produced using the maximal fluorescence values obtained from the samples; note that the maximal fluorescence values are not shown on all of the figures, e.g., Fig. 1B, in order to facilitate comparison of differing kinetics of the samples. Maximal fluorescence levels were determined by continuously monitoring the fluorescence signal for 5 s from the onset of irradiation of the sample.

Electron microscopy on the chloroplasts was carried out as previously described [14].

Results

The normalised fluorescence induction curves from thylakoids excited with 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ of 450 and 550 nm irradiation in the presence of 5 mM Mg^{2+} are shown, together with the semilogarithmic plots of the area growth over these curves, in Fig. 1. The first-order plots clearly demonstrate that induction curves generated with 450 nm excitation are more biphasic than those generated with 550 nm irradiation. The relative contributions of PS II_α and PS II_β to the induction curve can be quantitated using the parameter β_{max}; the intercept obtained on the *y*-axis by extrapolation to

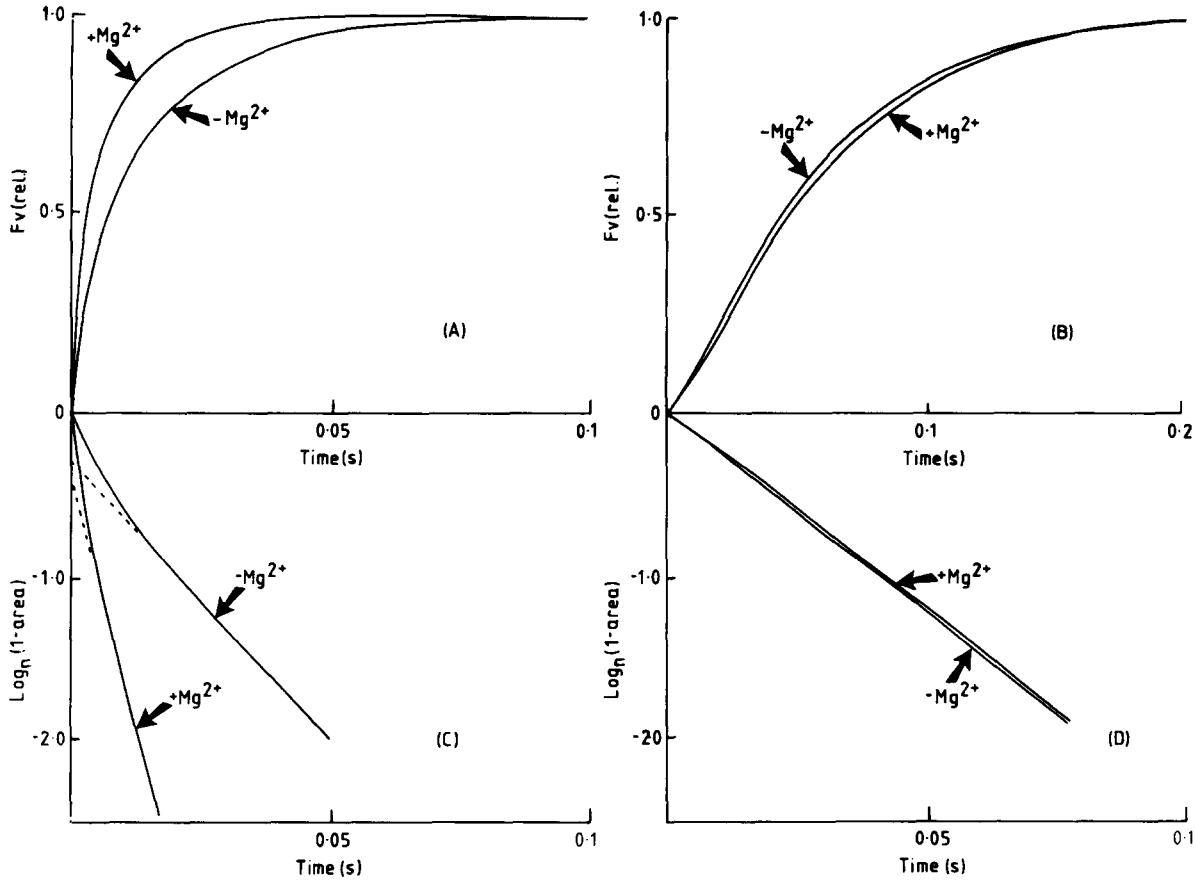

Fig. 1. Kinetics of 685 nm fluorescence emission from DCMU-poisoned wheat thylakoids excited with a photon flux density of 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ of 450 nm (A) and 550 nm (B) radiation in the presence and absence of 5 mM Mg^{2+}. First-order analyses of the kinetics of the area growth over the fluorescence induction curves were made after normalization of the induction curves on the maximal fluorescence level and are shown in (C) and (D).

zero of the slow β-phase of the semilogarithmic plot of the area growth determines β_{max} [2,10]. A decrease in β_{max} implies an increased contribution of PS II_α to the induction curve. For 450 nm excitation β_{max} was approx. 0.6 whilst at 550 nm it was always found to be greater than 0.8 suggesting that the PS II_α phase makes only a minor contribution to the fluorescence kinetics when thylakoids are excited with 550 nm radiation compared to the situation with 450 nm excitation. Changes in photon flux density between 20–120 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at the two excitation wavelengths had little effect on the shape of the induction curve and β_{max} (data not presented), thus demonstrating that the excitation wavelength dependent changes are not simply attributable to the number of photons absorbed by the photosynthetic apparatus.

The possible role of LHCP in these excitation wavelength dependent phenomena was examined by analysing fluorescence induction curves from thylakoids isolated from a chlorophyll-*b*-less barley mutant and comparing them with those from the barley wild type (Fig. 2). The fluorescence induction curves and β_{max} values generated by 450 and 550 nm excitation of the wild-type barley thylakoids were essentially the same as those reported above for wheat thylakoids. However, the fluorescence kinetics of the mutant thylakoids with 450 nm excitation were considerably different to those of the wild type (Fig. 2A); a marked increase

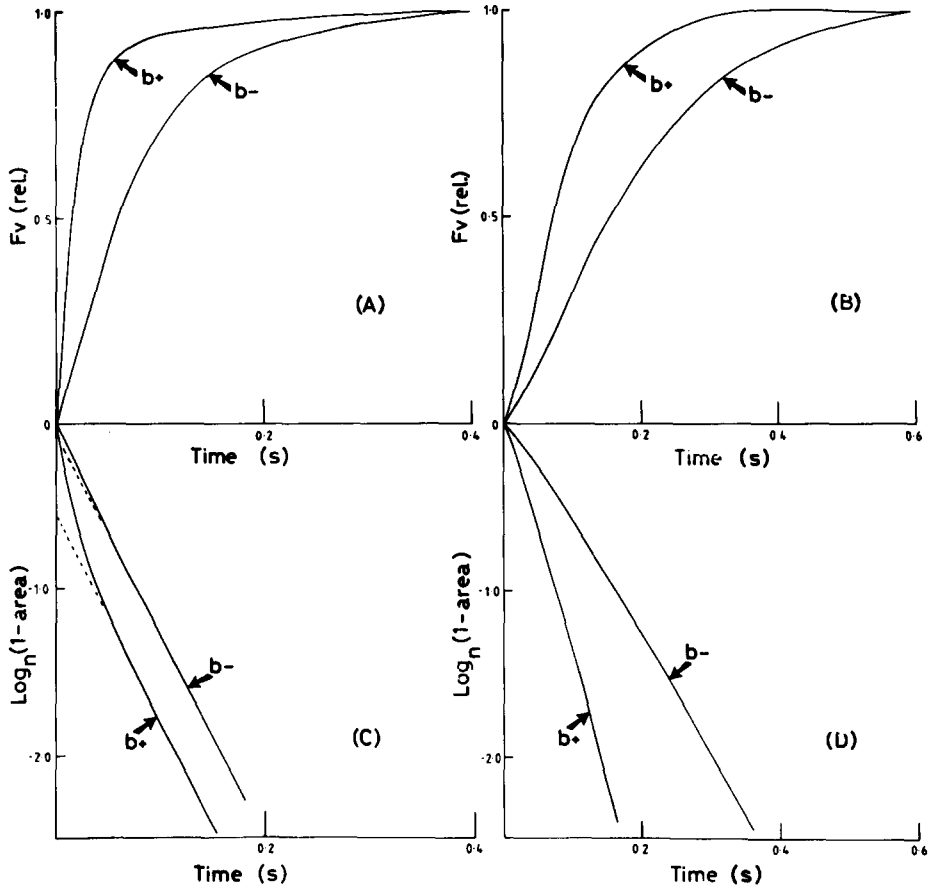

Fig. 2. Kinetics of 685 nm fluorescence emission from DCMU-poisoned thylakoids from wild-type (b+) chlorophyll-*b*-less (b−) chloroplasts when excited with 450 (A) and 550 nm (B) radiation (75 μmol·m^{-2}·s) in the presence of 5 mM Mg^{2+}. First-order analyses of the kinetics of the area growth over the fluorescence induction curves were made after normalization of the induction curves on the maximal fluorescence level and are shown in (C) and (D).

in β_{max} was observed in the mutant, implicating a loss of a significant proportion of the PS II_α component of the induction curve. No difference in the fluorescence kinetics generated by 550 nm radiation with respect to PS II_α and PS II_β components could be observed between thylakoids from the mutant and wild-type (Fig. 2); this becomes even more apparent when the data are presented on a relative time-scale such that the induction curves of the two samples are superimposed (data not shown). These data argue that LHCP contributes significantly only to induction kinetics generated with 450 nm excitation and not to those at 550 nm. Since the curves generated by 550 nm radiation consist mainly of a slow β-phase, it can be argued that LHCP does not contribute significantly to the photochemical activity of PS II_β centres, but only to PS II_α centres.

Depletion of Mg^{2+} from isolated thylakoids is known to produce changes in the structural organization of components within the membranes with consequent modifications in the distribution of excitation energy within the chlorophyll matrices [15–17]. Of particular importance here is the Mg^{2+}-induced modification of the α-phase, but not the β-phase, of the induction curve [4,5]. This effect can be observed for wheat thylakoids excited with 450 nm radiation (Fig. 1). When the thylakoids were excited with 550 nm radiation Mg^{2+}-depletion had a negligible effect on the

kinetics (Fig. 1). These observations are consistent with the hypothesis that there is only a minimal contribution of PS II_α centres to the induction curve generated by 550 nm radiation; PS II_β centres, which are insensitive to Mg^{2+} depletion [4,5], make the major contribution to these kinetics. The presence of a Mg^{2+} effect with 450 nm excitation, but not with 550 nm excitation, is consistent with a significant contribution of LHCP to the kinetics generated with 450 nm radiation but not with 550 nm radiation. Mg^{2+} has been shown to modify the degree of energetic coupling between LHCP and PS II [5,10,18,19] and these data presented here argue that such effects implicate a coupling of LHCP with PS II_α centres but not with PS II_β centres.

It is not readily apparent from these Mg^{2+} data whether the Mg^{2+}-induced effects are due primarily to changes in membrane appression or to intrinsic modifications to PS II-LHCP organization, which are not related to changes in membrane appression. This question can be addressed by examining the fluorescence kinetics from thylakoids at different stages of development. Chloroplasts along the length of the leaves of monocotyledons exhibit a progressive developmental sequence [14]. In the light-grown first leaf of 8-day-old wheat plants, chloroplasts isolated from cm segments taken from the base and tip on the leaf had a similar chlorophyll *a*/*b* ratio of approx. 3.4. Previous studies [20] have shown that differences in chloroplast ultrastructure exist between

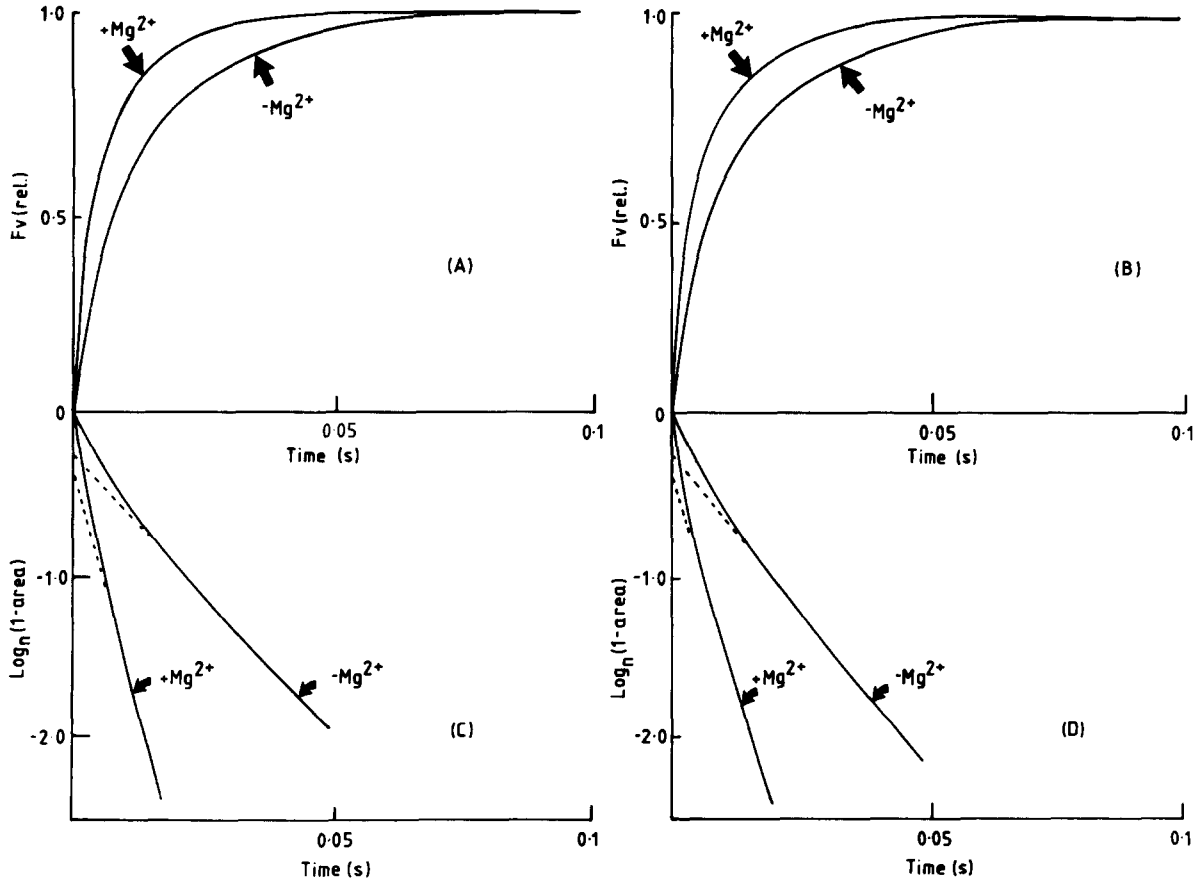

Fig. 3. Kinetics of 685 nm fluorescence emission from DCMU-poisoned thylakoids isolated from leaf tissue taken from 0–1 cm (A) and 10–11 cm (B) from the base of 8-day-old wheat leaves. Thylakoids were excited with 350 nm radiation (100 $\mu mol \cdot m^{-1} \cdot s^{-1}$) in the presence and absence of 5 mM Mg^{2+}. First-order analyses of the kinetics of the area growth over the fluorescence induction curves were made after normalization of the induction curves on the maximal fluorescence level and are shown in (C) and (D).

these two developmental stages, as illustrated by leaf tip chloroplasts having an average of 6.4 thylakoids per granum, whilst in chloroplasts from the basal cm of the leaf this ratio is 3.8. Although the absolute amount of thylakoid membrane appression in these chloroplasts is difficult to determine, it would appear that there is an increase in the degree of thylakoid appression from the basal to the tip region of the leaf [20]. The fluorescence kinetics and the first-order analyses of the two thylakoid preparations from the base and tip of the wheat leaf when excited with 450 nm radiation in the presence and absence of Mg^{2+} are shown in Fig. 3. In the presence of Mg^{2+} both thylakoid preparations exhibit a β_{max} of approx. 0.6 indicating that the relative contributions of PS II_α and PS II_β to the kinetics are similar at both stages of development. On removal of Mg^{2+} from the thylakoids there was a significant and similar reduction in the contribution of the PS II_α phase for thylakoids at both stages of development. These data demonstrate that Mg^{2+} depletion has the same effect on the fluorescence kinetics of both thylakoid developmental stages despite the difference in thylakoid appression and thus suggest that such Mg^{2+}-induced effects are more likely to be attributable to changes in the LHCP-PS II association than to changes in membrane appression. It would also seem likely, since the β_{max} values of the two stages of development are similar, that the relative contributions of PS II_α and PS II_β to the fluorescence kinetics are determined by the ratio of LHCP to PS II rather than by degree of membrane appression.

Discussion

The data presented demonstrate that the fast α-phase of the fluorescence induction, generally observed on irradiating thylakoids with broad band light, can be almost totally removed by exciting with narrow band green light of 550 nm. At this excitation wavelength the slow phase is the dominant component of the PS II fluorescence kinetics. The loss of the α phase on excitation at 550 nm, although analogous to the effect of Mg^{2+} depletion (see Fig. 2) cannot be attributed to the structural reorganization of PS II complexes within the membrane as has been suggested for the Mg^{2+}-induced changes [4,5]. The greatly reduced α-phase in the kinetics of thylakoids of the barley chlorophyll-*b*-less mutant, which lack LHCP [21,22], gives rise to the suggestion that the presence of LHCP may be required for PS II_α characteristics. Alternatively, it might be argued that the reduced thylakoid appression in the mutant [23–25] would produce a considerably increased proportion of PS II in the nonappressed membranes and therefore an increased contribution of the β-phase to the fluorescence kinetics. This question can be examined by considering the data obtained from wheat thylakoids at different stages of development, which have similar chlorophyll *a*/*b* ratios and therefore presumably similar proportions of LHCP to PS II but different amounts of thylakoid appression. The relative contributions of the α- and β-phases to the fluorescence kinetics of these developing membranes are very similar. If membrane appression was important in determining the appearance of PS II_α then it would be predicted that there should be a large difference in the PS II_α contribution to the fluorescence kinetics between these two developmental stages. The data from developing wheat thylakoids, together with that from the chlorophyll-*b*-less barley mutant, argues that LHCP plays a major role in determining the α-phase but does not contribute significantly to the β-phase of the fluorescence kinetics. On the basis of this hypothesis, the loss of the phase on excitation with 550 nm radiation suggests that this wavelength preferentially excites chlorophyll-*a*-matrices closely associated with PS II traps, i.e., the PS II core, whereas 450 nm radiation is much more strongly absorbed by LHCP and enables the LHCP to contribute significantly to PS II trap closure. This argument is consistent with the fact that 450 nm radiation is more strongly absorbed by chlorophyll *b* than by chlorophyll *a*, as indicated by the absorption spectra of the isolated chlorophyll species [26] and isolated chlorophyll-protein complexes [27,28]. The Mg^{2+}-induced depletion of the α-phase with excitation at 450 nm can be explained by Mg^{2+} producing separation of LHCP from PS II, as has been often previously reported [5,10,18,19]. We discount the possibility that depletion of the α-phase may be attributable to increased LHCP, or LHCP-PS II, interactions with PS I, since thylakoids isolated from the base

of the 8-day old wheat leaf have been found previously to exhibit considerably less energetic interaction between LHCP-PS II and PS I, i.e., less excitation-energy transfer, than thylakoids isolated from the tip of the leaf [14] yet the contributions of the α- and β-phases to the PS II fluorescence kinetics (Fig. 3) are similar in both of these thylakoid preparations.

The proposed role of LHCP and excitation wavelength in determining the contribution of the α- and β-phases to the fluorescence kinetics from thylakoids in the presence and absence of 5 mM Mg^{2+} is diagrammatically represented in Fig. 4. The data presented in this paper are consistent with the hypothesis that either preferential excitation of PS II relative to excitation of the chlorophyll *b* of LHCP or the physical separation of LHCP from PS II will result in the loss of the

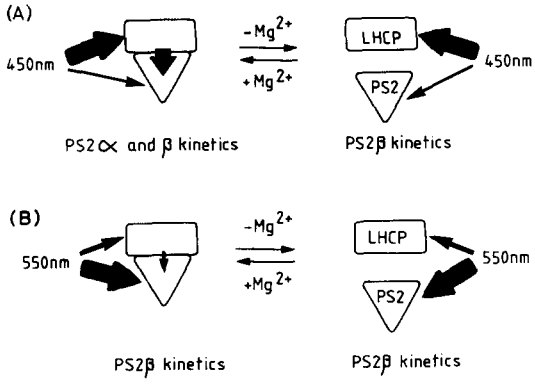

Fig. 4. Illustration of how the contribution of PS II_β kinetics to the total PS II fluorescence kinetics can be increased either by removal of Mg^{2+} or by excitation with 550 nm radiation. (A) Excitation with 450 nm radiation, which preferentially excites chlorophyll *b*, generates both PS II_α and PS II_β kinetics, since excitation energy from both LHCP and PS II core chlorophylls will be transferred to PS II reaction centres. Removal of Mg^{2+} dissociates LHCP from PS II thus preventing efficient energy transfer from LHCP to PS II; excitation energy absorbed directly by the PS II core will new be mainly responsible for PS II trap closure and result in PS II_β kinetics. (B) Excitation with 550 nm radiation, which preferentially excites chlorophyll *a* and results in PS II trap closure primarily by excitation energy absorbed directly by PS II core chlorophylls, produces predominantly PS II_β kinetics. Dissociation of LHCP from PS II on removal of Mg^{2+} will have very little effect on the PS II fluorescence kinetics, since the contribution to PS II photochemistry by energy transfer from LHCP to PS II is not large even in the presence of Mg^{2+} compared to that by direct excitation energy absorption by PS II.

α-phase of the fluorescence induction kinetics. The α-phase of the kinetics will only become prominent when LHCP is closely associated with PS II and it is being efficiently excited in comparison with the PS II core. This is consistent with arguments that PS II_α and PS II_β are not discrete, physical entities which cannot be interconverted, but are simply reflections of the proportion of LHCP, either physically or energetically, connected to PS II [5,10,18,19].

There are a number of reports in the literature which may seen incongruous with the above suggestions and should be commented upon. Hodges and Barber [10] have shown that the β-phase of the fluorescence induction kinetics is lost in the presence of dithionite, whilst the α-phase is unaffected. In the context of our model this may be explained if dithionite cannot reduce the PS II primary acceptors when LHCP is attached to PS II. This could result from the PS II traps being in a more hydrophobic environment within the membrane when PS II is attached to LHCP, and is compatible with the suggestion that LHCP-PS II units are preferentially located in appressed regions of the thylakoids while disconnected PS II units are in the nonappressed regions [4–6,9]. Such an argument would also explain why PS II_α and PS II_β centres have different midpoint redox potentials for Q reduction [29,30]. It has been argued that the sigmoidicity of the fluorescence induction curve is associated with PS II_α; however, we observe a marked sigmoidicity in the absence of a significant α-component when thylakoids are excited with 550 nm radiation (see Fig. 1). This sigmoidicity can be simply attributed to PS-II–PS-II interactions. It is well established that energy transfer between PS II units will result in a sigmoidal fluorescence induction [31–35], and since the organization of the PS II units in the thylakoid membranes excited with 450 and 550 nm radiation must be the same at the inset of irradiation, as in the experiment reported in Fig. 1, it is logical to expect energy transfer between the PS II units to occur with both 450 and 550 nm radiation. The occurrence of any energy transfer between PS II units excited with 550 nm radiation would result in a sigmoidicity of the kinetics which are representative of the slow β-phase.

In conclusion, it would appear that the ability

to modify the PS II fluorescence induction kinetics with respect to the relative contributions of PS II_α and PS II_β by simply changing the excitation wavelength offers a useful probe with which to examine the interaction between LHCP and PS II within the thylakoid membranes under different conditions. Currently, we are using this technique to investigate the changes in LHCP-PS II interactions that are induced by LHCP phosphorylation.

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