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Modification of the interaction between Photosystem II and the light-harvesting chlorophyll *a*/*b*-protein complex by protein phosphorylation in developing wheat thylakoids exhibiting different degrees of lateral heterogeneity

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The effects of phosphorylation of thylakoid polypeptides at 2 and 5 mM Mg^{2+} on the chlorophyll fluorescence induction characteristics of developing wheat thylakoids, exhibiting differing degrees of lateral heterogeneity of intrinsic protein complexes, were studied. The relative contributions of the fast and slow components of F_v, defined by β_{max}, were the same at all stages of thylakoid development. On phosphorylation at 5 mM Mg^{2+}, β_{max} increased similarly at all stages of thylakoid development. Such changes in β_{max} indicate that the magnitude of phosphorylation-induced disconnection of the light-harvesting chlorophyll *a*/*b* protein complex from Photosystem II was independent of the degree of lateral heterogeneity of the thylakoid membranes. At 2 mM Mg^{2+} phosphorylation also increased β_{max} at all developmental stages, but to a lesser extent than was observed at 5 mM Mg^{2+}. Discussion is made of how changes in the lateral heterogeneity of the pigment-protein complexes within the thylakoid that occur during membrane development and on reduction of Mg^{2+} concentration can modify the effects of protein phosphorylation on the energetic interactions between the complexes.

Introduction

The distribution of excitation energy within the photochemical apparatus of thylakoid membranes can be regulated by a process often termed the State 1–State 2 transition [1–5]. An important feature of the regulation of the state transition is the dissociation of phosphorylated LHC II from PS II [5–11]. However, it is often difficult to differentiate which changes in membrane characteristics are attributable to the removal of phosphorylated LHC II from PS II and which are due to increased interaction between the phosphorylated LHC II and PS I. One approach to overcome this problem is to induce a desegregation of LHC II-PS II complexes in the membrane prior to LHC II phosphorylation by reducing the Mg^{2+} concentration around the membranes [12–14]. This produces a decrease in the degree of thylakoid

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Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; F_m, maximal fluorescence level; F_0, minimal fluorescence level; F_v, fluorescence of variable yield ($F_v = F_m - F_0$); Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; LHC II, light-harvesting chlorophyll *a*/*b* protein complex of Photosystem II; PS, Photosystem.

appression with a resultant decrease in the segregation of LHC II-PS II from PS I [12–14]; however, reduction of Mg^{2+} also induces a dissociation of LHC II from PS II [15–18] and the possibility that interactive effects between Mg^{2+} reduction and LHC II phosphorylation may complicate and possibly distort the primary effect of LHC II phosphorylation on LHC II-PS II interaction should not be ignored. The developing wheat thylakoid offers a useful system with which to examine the effects of LHC II phosphorylation in membranes having a poor lateral heterogeneity of LHC II-PS II and PS I complexes at high Mg^{2+} concentrations. The development of the ability to perform a State 1–State 2 transition correlates with an increased degree of thylakoid appression [19–21], implying that the absence of the transition at early developmental stages is due to a poor segregation of LHC II-PS II and PS I complexes within the membranes [19,20]. Since the LHC II-to-PS II ratio does not change significantly during chloroplast development in wheat [19,20] the early stages of thylakoid development offer a situation to examine the effect of LHC II phosphorylation on the interaction between LHC II and PS II in membranes where the degree of randomization of LHC II-PS II and PS I complexes is changing naturally without resorting to a reduction in Mg^{2+} concentration. This developmental system also allows an examination of the effects of lowering Mg^{2+} concentration on LHC II-PS II interactions as the lateral segregation of LHC II-PS II and PS I complexes increases.

In this study an examination is made of the effects of LHC II phosphorylation and reduction in Mg^{2+} from 5 to 2 mM on the chlorophyll fluorescence induction characteristics of DCMU-treated wheat thylakoids at three developmental stages which exhibit different degrees of lateral heterogeneity.

Materials and Methods

Wheat leaf tissue containing chloroplasts at different developmental stages was grown as described previously [19]. Thylakoids were prepared from leaf sections taken 0–1 cm, 1–2 cm and 3–4 cm from the leaf base, as described previously [22]. Thylakoids used for fluorescence kinetic studies in the presence of DCMU were suspended in assay medium at a chlorophyll concentration of 10 $\mu g \cdot cm^{-3}$ with a final concentration of 5 mM or 2 mM $MgCl_2$. After 10 min incubation in the dark at 20°C thylakoids were phosphorylated by the addition of ATP (200 μM) and incubated for 5 min in white light at a photon flux density of 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Following addition of DCMU (20 μM) after dark adaptation for 2 min, thylakoids were then irradiated with light generated from a quartz-iodine source (Volpi) and passed through a 580 nm short pass cut-off filter (Ealing 35-5362) to give a photon flux dnesity of 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Fluorescence was detected at right angles to the actinic beam through a 685 nm interference filter (Ealing-Beck) by a photomultiplier tube (Hamamatsu R928). Data were captured and digitised using DL901 transient recorders (Datalab Laboratories Ltd.) and processed by microcomputer. A sampling rate of 20 kHz was used to resolve F_0. β_{max} values were calculated from first-order plots of the area growth over the fluorescence induction curve as previously described [22].

In order to examine thylakoid phosphoproteins isolated thylakoids were treated with [γ-^{32}P]ATP as previously described [23], except that the incubation conditions and period were the same as those described above to phosphorylate thylakoids in the presence of 5 mM Mg^{2+} prior to measurement of fluorescence induction kinetics.

Thylakoid proteins were fractionated by electrophoresis on polyacrylamide gels using a modification of the method of Laemmli [24] which utilized a linear gradient of 8–15% acrylamide stabilized by a 4–8 M gradient of urea in the resolving gel. Following electrophoresis, polypeptides were visualized by silver staining [25] and labelled phosphoproteins were visualized by autoradiography using Kodak X-omat AR film.

Results and Discussion

Chlorophyll fluorescence induction characteristics in the presence of DCMU were examined for thylakoids at the three stages of development found in tissue 0–1 cm, 1–2 cm and 3–4 cm from the base of the 4-day-old wheat leaf. Fluorescence induction curves from these thylakoids before and after phosphorylation of the thylakoid proteins in

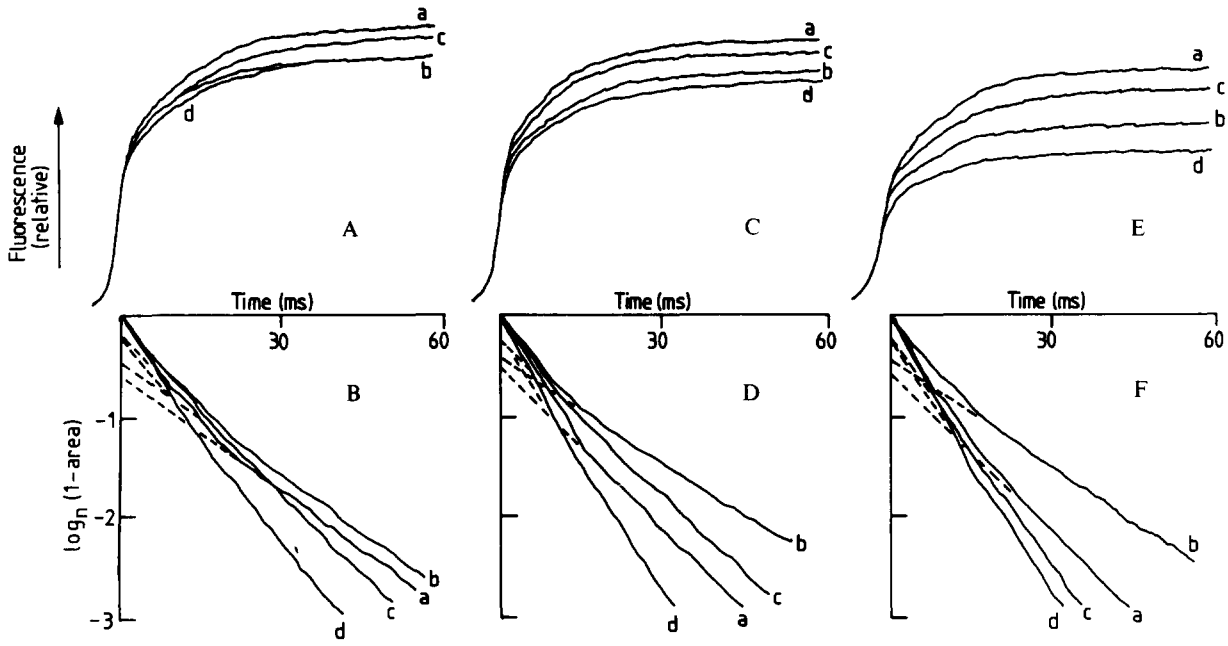

Fig. 1. Kinetics of 685 nm fluorescence emission from phosphorylated (c) and (d) and non-phosphorylated (a) and (b) DCMU-poisoned thylakoids isolated from 0–1 cm (A), 1–2 (C) and 3–4 cm (E) sections of 4-day-old wheat leaves in the presence of 5 mM Mg^{2+} (a) and (c) and 2 mM Mg^{2+} (b) and (d). First-order analyses of the kinetics of the area growth over the respective fluorescence induction curves made after normalization on the maximal fluorescence level are shown in (B), (D) and (F).

TABLE I

EFFECT OF PHOSPHORYLATION AND Mg^{2+} CONCENTRATION ON THE FLUORESCENCE INDUCTION CHARACTERISTICS OF DEVELOPING WHEAT THYLAKOIDS

Comparison of the fluorescence parameters F_0, F_m, F_v/F_m and β_{max} for non-phosphorylated and phosphorylated thylakoids isolated from different regions of 4-day-old light-grown wheat leaves and suspended with 2 or 5 mM Mg^{2+}. The decrease in the connectivity of LHC II with PS II was estimated from the β_{max} values as described in the text. Data are the means of 10 replicates and standard errors of the means are given where applicable.

Leaf section (cm from base)	Mg^{2+} (mM)	Phosphorylated	F_0	F_m	F_v/F_m	β_{max}	Decrease in LHC-PS II connectivity (%)
0–	5	–	94 (2)	168 (3)	0.440	0.55 (0.01)	0
	5	+	89 (1)	161 (4)	0.447	0.80 (0.02)	31
	2	–	91 (1)	151 (2)	0.397	0.69 (0.01)	20
	2	+	91 (2)	148 (2)	0.385	0.79 (0.01)	30
1–2	5	–	96 (2)	157 (4)	0.389	0.54 (0.01)	0
	5	+	91 (1)	148 (3)	0.385	0.77 (0.01)	30
	2	–	88 (1)	137 (2)	0.358	0.66 (0.02)	18
	2	+	85 (1)	131 (3)	0.351	0.76 (0.02)	29
3–4	5	–	79 (1)	138 (2)	0.428	0.54 (0.01)	0
	5	+	72 (1)	125 (2)	0.424	0.79 (0.01)	31
	2	–	66 (1)	105 (2)	0.371	0.67 (0.01)	19
	2	+	62 (1)	88 (2)	0.295	0.78 (0.02)	31

the presence of 2 and 5 mM Mg^{2+} are shown in Fig. 1 together with the semi-logarithmic plots of the area growth over these curves. The values of the fluorescence parameters F_0, F_m, F_v/F_m and β_{max} associated with these induction characteristics are given in Table I. At 5 mM Mg^{2+} phosphorylation of thylakoid proteins produced a significant decrease in both F_0 and F_m only for membranes at the two later stages of development. Such changes in F_0 and F_m with a constancy of F_v/F_m are consistent with previously reported effects of phosphorylation on mature thylakoids and are indicative of a phosphorylation-induced reduction in PS II antenna size [12,13,26–28]. It should be noted that the magnitude of these phosphorylation-induced fluorescence changes are considerably smaller in the developing wheat thylakoids than in mature thylakoids.

The polypeptide phosphorylation profiles of thylakoids isolated from 0–1 cm and 3–4 cm from the leaf base and phosphorylated in the presence of [γ-^{32}P]ATP and 5 mM Mg^{2+} are shown in Fig. 2. Since the apoproteins of LHC II (molecular weight approx. 27000) can be seen to be present and heavily phosphorylated at both stages of development, phosphorylation-induced changes in LHC II-PS II interactions would be expected to be observed throughout thylakoid development.

The interaction between LHC II and PS II can be examined from analyses of the first-order plots

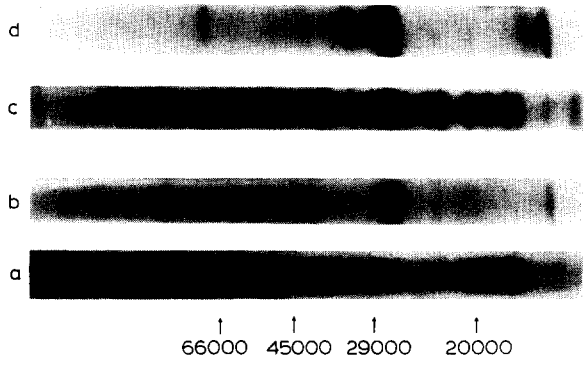

Fig. 2. Silver stained gels (a, c) with their respective autoradiograms (b, d) after electrophoretic polypeptide fractionation of phosphorylated thylakoid membranes (labelled with ^{32}P) isolated from 0–1 cm (a, b) and 3–4 cm (c, d) sections of 4-day-old wheat leaves. Numbers under the gels indicate the migration of proteins of known molecular weight.

of the growth of the area over the fluorescence induction curve [12,22]. Such first-order plots demonstrate a fast α phase and a slow β phase of the induction kinetics (see Fig. 1). Extrapolation to zero of the linear slow β phase produces an intercept on the y-axis that has been designated as the parameter β_{max}, wich defines the relative contributions of the α and β phases to the induction of variable fluorescence [12,22,31]. It has been demonstrated that the fast α kinetics are characteristic of a PS II complex in association with LHC II, whilst the slower β kinetics emanate from PS II complexes disconnected from LHC II [22]. Thus, β_{max} can be used to estimate the degree of connectivity between LHC II and PS II. Concern has been expressed recently regarding the validity of analyses of the first-order plots of growth of the area over the fluorescence induction curve for the study of PS II heterogeneity [32]. Criticism has been based on the difficulty of accurately determining F_m [32]. Such criticism is valid if the gradients of the α and β phases of the plots are being used to estimate the rate constants of the α and β kinetics. It is evident from the studies of Bell and Hipkins [32] and our own unpublished data that although errors in the estimation of F_m will modify the gradients of first-order plots of the α and β kinetics, such errors do not modify significantly the relative contributions of the α and β phases to the induction curve and thus do not modify β_{max}.

The β_{max} values of non-phosphorylated thylakoids at the three developmental stages in the presence of 5 mM Mg^{2+} were remarkably similar (Fig. 1 and Table I), implying that the degree of connectivity between LHC II and PS II was the same throughout chloroplast development. This hypothesis was substantiated by the similarity of the half-rise times for variable fluorescence from these thylakoids (Table II), which demonstrated that the efficiency of light capture and utilization for primary photochemistry by PS II was similar at the three developmental stages. Phosphorylation of thylakoids at all stages of development in the presence of 5 mM Mg^{2+} produced a similar increase in β_{max} which was indicative of a decrease of approximately 30% in the connectivity of LHC II with PS II. This observation is consistent with an estimated disconnection of 25% of LHC II

TABLE II

HALF-RISE TIMES FOR VARIABLE FLUORESCENCE INDUCTION IN DEVELOPING WHEAT THYLAKOIDS

Data are the means of 10 replicates determined from thylakoids isolated from different regions of 4-day-old wheat leaves. Standard errors of the means are given.

Leaf section (cm from base)	Half-rise time (ms)
0–1	8.0 ± 0.12
1–2	8.1 ± 0.11
3–4	8.0 ± 0.15

from PS II on phosphorylation of spinach thylakoid proteins in the presence of 10 mM Mg^{2+} [28]. Since the degree of lateral segregation of LHC II-PS II and PS I complexes in the membranes at the three developmental stages is thought to be very different [19–21], these data imply that the changes in β_{max} induced by protein phosphorylation are independent of the degree of lateral segregation of the photosystem complexes within the membranes and are simply indicative of the connectivity between LHC II and PS II.

Lowering the Mg^{2+} concentration from 5 to 2 mM will reduce the segregation LHC II-PS II and PS I complexes in thylakoids which exhibit a high degree of lateral heterogeneity [12–14,33]. Such a reduction in Mg^{2+} concentration produced decreases in both F_0 and F_m for thylakoids at all three developmental stages, the decrease in F_m being proportionally greater than in F_0, thus producing a decrease in F_v/F_m (Table I). This effect increased in magnitude with increasing development of the membranes and was consistent with the suggestion that the degree of segregation of LHC II-PS II from PS I complexes is greater in the later stages of chloroplast development [19–21]. Reduction of the Mg^{2+} level would be expected to produce greater increases in the energy transfer between PS II and PS I as thylakoid development proceeds, since the lateral segregation of the PS II and PS I complexes is greater at the later stages of development. Lowering the Mg^{2+} concentration also produced an increase in β_{max}, the magnitude of which was similar at all three developmental stages and implies a constant degree of disconnection (approx. 19%) of LHC II from PS II throughout chloroplast development. The consistent increase in β_{max} at the three stages of thylakoid development on reduction of the Mg^{2+} concentration whilst the magnitude of the reduction in F_v/F_m increased with development suggests that the changes in F_v/F_m are indicative of an increase in energy transfer (spillover) from PS II to PS I, and that this phenomenon does not modify the β_{max} value; β_{max} being modified only by changes in the connectivity between LHC II and PS II.

Phosphorylation of the thylakoid proteins in the presence of 2 mM Mg^{2+} produced different changes in the fluorescence kinetics at the three developmental stages, although in all cases β_{max} increased to a similar extent, indicating a 10% decrease in connectivity between LHC II and PS II (Table I). Thylakoids isolated from the basal cm of the leaf showed no significant decrease in F_0 or F_m (Table I). In the more mature thylakoids isolated from the leaf tip phosphorylation produced a decrease in both F_0 and F_m. The decrease in F_m was considerably greater than in F_0 and produced a larger decrease in F_v/F_m than was found at the earlier developmental (1–2 cm) stage (Table I). This preferential quenching of F_v relative to F_0 by phosphorylation at 2 mM Mg^{2+} has been reported previously for pea thylakoids [13,14] and is thought to indicate that both a detachment of LHC II from PS II and an increase in energy transfer from PS II to PS I occurs on phosphorylation [13,14]. This interpretation of the data is consistent with the greater effect of phosphorylation at 2 mM Mg^{2+} on the decrease in F_v/F_m at later stages of thylakoid development where a greater lateral heterogeneity exists than at early stages of development.

An important conclusion from this study is that irrespective of the degree of lateral segregation of LHC II-PS II and PS I complexes in the developing thylakoid membrane phosphorylation at 5 mM Mg^{2+} produces identical effects on β_{max}, which can be taken to be indicative of a 30% decrease in the connectivity between LHC II and PS II. Reduction of the Mg^{2+} level, like phosphorylation, produces a disconnection of LHC II from PS II at all developmental stages, however only approx. 20% of the LHC II is disconnected on Mg^{2+} reduction compared to approx. 30% on phosphorylation. More importantly Mg^{2+} reduction pro-

duces an increase in energy transfer from PS II to PS I presumably by inducing a randomization of LHC II-PS II and PS I complexes; this phenomenon is reduced in thylakoids at early stages of development which exhibit a poor degree of LHC II-PS II segregation from PS I. At 2 mM Mg^{2+} removal of LHC II from PS II on phosphorylation is also accompanied by an increased energy transfer from PS II to PS I that is not observed on phosphorylation at 5 mM Mg^{2+} and is considerably reduced in thylakoids which exhibit poor lateral segregation of PS II and PS I because of their developmental state. Clearly lowering the Mg^{2+} from 5 to 2 mM produces complications in studying the effects of phosphorylation on membrane functions by reducing the potential for the disconnection of LHC II from PS II on phosphorylation by approx. 66% and also by increasing the proximity between PS II and PS I complexes such that an increase in energy transfer from PS II to PS I will occur on phosphorylation that does not occur on phosphorylation of thylakoids with a poor lateral heterogeneity of PS II and PS I at 5 mM Mg^{2+}.

This study has demonstrated and emphasized the potential value of the parameter β_{max} as a probe for studying the degree of connectivity between LHC II and PS II. The remarkable constancy of β_{max} in non-phosphorylated membranes and the constancy of the phosphorylation and Mg^{2+}-induced changes in β_{max} at all stages of thylakoid development together with the fact that the absorptive cross-section of LHC II-PS II complexes in non-phosphorylated membranes remained unchanged during development despite the occurrence of large changes in the degree of lateral segregation of LHC II-PS II and PS I complexes argues strongly that β_{max} is simply dependent on the degree of association of LHC II with PS II and is not influenced by modification in the interactions of LHC II or PS II with PS I.

Although phosphorylation induces changes in the association of LHC II with PS II throughout thylakoid development, at early developmental stages, where the membranes exhibit a poor lateral segregation of photosystem complexes, phosphorylation does not simultaneously produce changes in excitation energy distribution to PS I, as is the case in mature membranes. This suggests that phosphorylation-induced changes in LHC II-PS II organization may serve a function other than the regulation of energy distribution to PS I, e.g., the protection of PS II from photoinhibition.

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