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AN INVESTIGATION OF THE MECHANISTIC ASPECTS OF EXCITATION ENERGY REDISTRIBUTION FOLLOWING THYLAKOID MEMBRANE PROTEIN PHOSPHORYLATION

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Accompanying thylakoid membrane protein phosphorylation is a redistribution of energy between PS II and PS I; mechanistic aspects of this redistribution have been investigated in both a mature and a developing chloroplast system. Data are presented which suggest that the mechanism of these changes is dependent upon the developmental status and/or morphological characteristics of the chloroplast.

Introduction

Higher plants and algae are able to adapt to changes in the spectral quality and intensity of light to control the relative rates of photon delivery to the two photosystems. Adaptation to PS-II-enriched light is called State 2 and to PS-I-enriched light State 1. Experiments using isolated chloroplasts have indicated that reversible phosphorylation of thylakoid proteins is the likely in vivo mechnism for the state transitions (see Refs. 1–4 for reviews). Protein phosphorylation causes a decrease in the yield of chlorophyll fluorescence when assayed at room temperature [5,6] and fluorescence meaurements at −196°C indicated an increase in rate of excitation of PS I at the expense of PS II [5–7]. Although several proteins are phosphorylated, the evidence suggests that it is the phosphorylation of LHCP which controls excitation distribution [8,9]. Controversy exists concerning the mechanism by which phosphorylation causes changes in the relative rates of photon delivery to PS II and PS I [1,10]. Two mechanisms are possible; firstly, the absorption cross section of PS I (α) may increase after phosphorylation and secondly, there may be an increase in transfer of energy from PS II to PS I (spillover). Information concerning the mechanism of the redistribution of energy has been gained from examination of the room temperature fluorescence induction curves recorded for phosphorylated and nonphosphorylated thylakoids [5,11,12]. A decrease in absorption cross section of PS II would decrease the fluorescence yield at F_o to the same extent as at F_m, while a preferential quenching of F_v would be used as evidence for an increase in spillover. There have been reports of both types of fluorescence change; thus, Horton and Black [5,14] and Telfer et al. [12] showed that the F_v/F_m ratio was unchanged by phosphorylation, whereas Bennett et al. [6] and Kyle et al. [11] demonstrated a large

Abbreviations: LHCP, light-harvesting chlorophyll-protein complex; PS I, II; Photosystem I, II; F_m, fluorescence yield when all PS II traps are closed; F_0, fluorescence yield when all PS I traps are open; F_v, $F_m - F_0$; DCMU, 3-(3′,4′-dichlorodiphenyl)-1,1-dimethylurea; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

decrease in this parameter. Analysis of fluorescence transients at −196°C suggested that phosphorylation increased both α and spillover [13].

In subsequent work it was shown that phosphorylation can bring about a large decrease in the F_v/F_m ratio if assays were carried out in 1 mM $MgCl_2$ rather than 5–10 mM [14]. This apparent increase in spillover following phosphorylation at low Mg^{2+} is the likely result of partial unstacking of the thylakoids, since it had previously been shown that phosphorylation increased the Mg^{2+} requirement for the high fluorescent state associated with membrane appression [15]. These findings can be explained by the forces responsible for thylakoid stacking [16,17]. Destabilisation of membrane appression requires an increase in space charge density which may be brought about by a decrease in level of the screening cations or by an increase in membrane surface charge [18]; at low Mg^{2+} a larger destabilising effect might result from phosphorylation. This idea led to the suggestion that the effect of protein phosphorylation on excitation distribution will depend also upon the composition and morphology of the thylakoid membrane, since these factors will also determine the surface charge properties [1,10,14]. For example, during chloroplast development a progressive increase in stacking occurs, and it might be predicted that phosphorylation would produce different effects on spillover and absorption cross section when thylakoids from mature peas and those from peas grown under an intermittent-light regime are compared.

Materials and Methods

Peas (*Pisum sativum*, var. Kelvedon Wonder) were grown for 8–10 days in complete darkness and then transferred to an intermittent-light regime of 2 mins light + 118 mins dark for a period of 48 h. Plants grown under these conditions have been shown to incorporate preferentially chlorophyll *a* into the thylakoid membrane. Chloroplasts isolated from these leaves are photosynthetically competent but have very little LHCP [19]. The plants were subsequently exposed to continuous light for various periods which results in an increase in chlorophyll *b* relative to chlorophyll *a* and an increase in LHCP and degree of stacking [20]. Mature thylakoids were obtained from plants grown under a 16 h photoperiod for 12–16 days.

Thylakoids were isolated as described in Ref. 11 and illuminated with white light (40 $W \cdot m^{-2}$) for 10 min in reaction medium containing 0.33 M sorbitol/10 mM NaCl/5 mM $MgCl_2$/10 mM NaF/50 mM Hepes (pH 7.6)/1 μM nigericin/1 μM valinomycin plus or minus 0.2 mM ATP at a chlorophyll concentration of 20 or 50 $\mu g \cdot cm^{-3}$. The extent of rise in chlorophyll fluorescence induced by addition of Mg^{2+} after various 'greening' times was measured under low intensity actinic light in the presence of 50 μM DCMU. Phosphorylated and nonphosphorylated thylakoids were depleted of Mg^{2+} as described previously [15] and the F_m measured before and after addition of 5 mM $MgCl_2$. Chlorophyll fluorescence induction transients at room temperature were measured as before [5] in the presence of 5 mM $MgCl_2$.

Fluorescence induction curves (20 μg chlorophyll per cm^3) at −196°C were measured at an angle of 30°C from the broad blue band actinic light via a bifurcated fibre optic. Emission at 695 and 740 nm was detected by Hamamatsu red-sensitive side-window photomultiplier tubes and defined by RG665 cut-off and Ealing narrow bandpass interference filters. Data was recorded onto a Datalab DL4000 transient recorder and energy-distribution parameters determined according to the models of Butler [21,22].

Results

Fig. 1 depicts the relationship between both the chlorophyll-*a*-to-chlorophyll-*b* ratio and the influence of Mg^{2+} on membrane stacking (as measured by the increase in PS II chlorophyll fluorescence) of thylakoids from the intermittent-light plants relative to 'greening' time. As the ratio of chlorophyll-*a*-to-chlorophyll-*b* decreases the degree of stacking of cation-depleted membranes induced by addition of Mg^{2+} ions increases. This would indicate a progressive increase in concentration of LHCP and its insertion into the membrame as LHCP is the major chlorophyl-*b*-containing light-harvesting protein complex of the thylakoid membrane, of which a surface-exposed side chain is required for stacking and grana formation (for

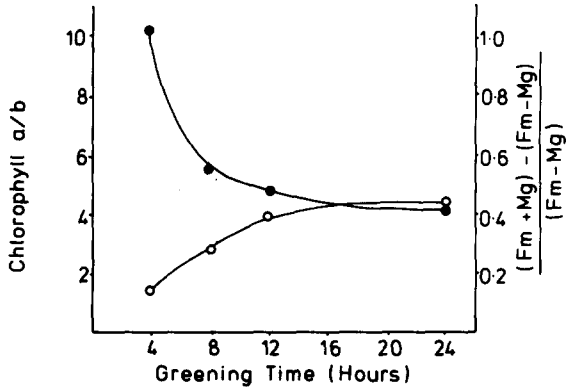

Fig. 1. Chlorophyll a/b ratio (●) and the Mg^{2+}-induced increase in the maximum level of fluorescence (○) in thylakoids isolated from peas grown under intermittent light and exposed to various lengths of continuous light.

reviews see Refs. 3, 17 and 23). LHCP thus has more than one role in the membrane, being involved in both the harvesting of light energy and associated phenomena and also in the control of the structural profile of the membrane. Hence, this method of plant growth provides a system with which to explore the effects of phosphorylation of LHCP on thylakoids at different stages of development.

Incubation of intermittent-light pea thylakoids with ATP resulted in no fluorescence decrease and it took 4 h of continuous illumination before the plants reached a developmental state capable of showing the 'normal' 15–20% decrease in yield associated with protein phosphorylation (Table I).

TABLE I

ATP-INDUCED FLUORESCENCE DECREASE IN INTERMITTENT-LIGHT THYLAKOIDS

Thylakoids were isolated from intermittent-light peas exposed to different periods of greening in continuous light and their fluorescence yield determined after illumination in the presence and absence of ATP.

Greening time (hours)	$F_{-ATP} - F_{+ATP}/F_{-ATP}$ (%)
0	0
0.5	3.5
1.0	11.7
2.0	13.4
4.0	21.0

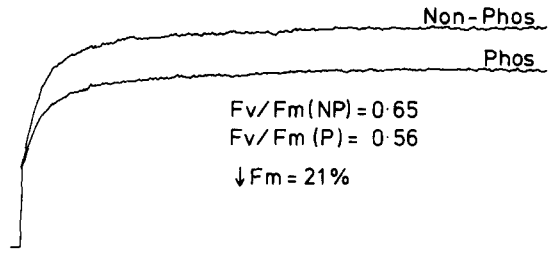

Fig. 2. Chlorophyll fluorescence induction curves (+50 μM DCMU) of thylakoids isolated from intermittent-light peas greened for 4 h and illuminated in the presence (P) and absence (NP) of ATP.

At 4 h the chlorophyll a/b ratio was still greater than 5. Fig. 2 shows chlorophyll-fluorescence induction curves of phosphorylated and nonphosphorylated thylakoids isolated from intermittent-light peas which have been subjected to 4 h continuous light. It is evident that phosphorylation induced a large decrease in the F_v/F_m ratio, from 0.65 in nonphosphorylated thylakoids to 0.56 in the sample illuminated with ATP. It should be added that increasing the level of Mg^{2+} to 20 mM did not alter the effect of phosphorylation on the F_v/F_m ratio showing that the effect is not due to any increase in the Mg^{2+} requirement of the intermittent-light thylakoids. After longer exposure of these plants to continuous light the effect of phosphorylation on F_v diminished (relative to the decrease in F_m) until after 24 h no significant change in F_v/F_m was seen (Fig. 3). Fig. 4 shows induction curves of phosphorylated and non-phosphorylated mature thylakoids. The mechanism of fluorescence

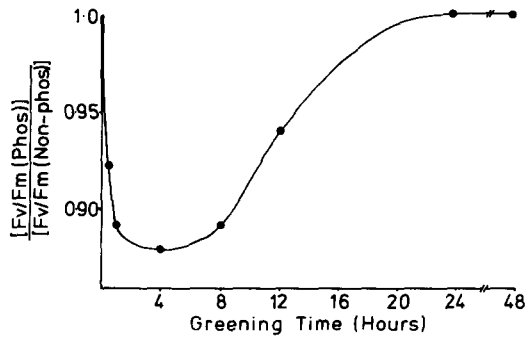

Fig. 3. Decrease in F_v/F_m (after phosphorylation) of intermittent-light pea thylakoids relative to greening time, normalized to F_v/F_m of nonphosphorylation thylakoids.

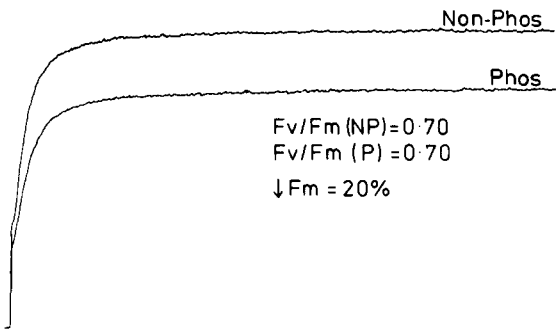

Fig. 4. Chlorophyll fluorescence induction curves (+50 μM DCMU) of thylakoids isolated from mature peas and illuminated in the presence (P) and absence (NP) of ATP.

decrease in this case is clearly different; no decrease in the F_v/F_m ratio resulted from phosphorylation indicating that the major change in fluorescence yield was due to a reduction in the absorption cross section of PS II.

The results from room-temperature induction curves suggested that phosphorylation of partially greened intermittent-light thylakoids caused an increase in spillover. However, a variety of processes may bring about preferential quenching of F_v including alteration of intrinsic reaction-centre parameters [24]. Therefore, data obtained from fluorescence induction curves at 695 nm (PS-II emission) and 740 nm (PS-I emission) at −196°C were used to determine various excitation distribution parameters according to the bipartite model of Butler and Kitajima [21,22]. We have calculated the values of α and β (absorption cross-sections of PS I and PS II, respectively, $\alpha + \beta = 1$) in addition to the yields of energy transferred to PS I subsequent to its absorption by the PS-II antennae. Butler's model considers two types of energy-transfer process; energy transfer from the antenna chlorophyll of PS II to PS I (Φ_T(II-I)) and energy transfer from the reaction-centre chlorophyll of PS II to PS I (Φ_t(II-I)). It is this latter type which can account for the specific quenching of the variable fluorescence of PS II. Values of these parameters are presented for mature thylakoids and for intermittent light thylakoids which have been greened for 16 h. (Thylakoids from plants greened for 4 h could not be used for this experiment as the technique involves the use of membranes with and without added Mg^{2+}, the changes in 4 h plants being to small to obtain suitable data. However, these 16 h membranes still showed a decrease in the room temperature F_v/F_m ratio of 0.06 compared with only 0.01 in mature thylakoids). As shown in Table II a substantial increase in α occurs after phosphorylation of both mature and

TABLE II

FLUORESCENCE PARAMETERS DETERMINED FROM FLUORESCENCE CURVES RECORDED AT −196°C USING PHOSPHORYLATED (P) AND NONPHOSPHORYLATED (NP) THYLAKOIDS PREPARED FROM PARTIALLY GREENED (16 h) INTERMITTENT-LIGHT AND MATURE PEAS

Parameter	Intermittent-light peas		Mature peas	
	Nonphosphorylated	Phosphorylated	Nonphosphorylated	Phosphorylated
Φ_T(II-I)(0)	0.043	0.150	0.036	0.064
Φ_T(II-I)(M)	0.127	0.380	0.098	0.169
Φ_t(II-I)	0.050	0.138	0.095	0.112
α	0.21	0.35	0.21	0.31
β	0.79	0.65	0.79	0.69
Φ_t(II-I)P/Φ_t(II-I)NP	2.76		1.18	
αP/αNP	1.67		1.48	
$\frac{\alpha + \Phi_T(\text{II-I})(\text{M})\text{P}}{\alpha + \Phi_T(\text{II-I})(\text{M})\text{ NP}}$	2.17		1.56	

α, β, absorption cross-sections of PS I and PS II, respectively; Φ_T(II → I) (0), Φ_T(II → I)(M), energy transfers to PS I at F_0 and at F_m, respectively; Φ_t(II → I), energy transfer from reaction center chlorophyll of PS II to PS I.

intermittent-light thylakoids, increasing by 48% and 67%, respectively. In addition to this change there is also an increase in the yield of energy transfer from PS II to PS I in each case. Both of these factors together ($\alpha + \Phi_T$(II-I)) contribute a very significant increase in the excitation rate of PS I, this being more than doubled in intermittent-light peas and increased by 56% in mature peas (see ratios in Table II). Further analyses give values of Φ_t(II-I), the yield of transfer from the reaction-centre chlorophyll of PS II to PS I. In the case of intermittent-light thylakoids, which exhibit a pronounced quenching of variable fluorescence of PS II, this parameter is increase by almost 3-fold whereas in mature thylakoids this increases by only 18%. The mechanism of energy redistribution due to protein phosphorylation in mature thylakoids is mainly one which increases α with some contribution from energy transfer. In contrast, the intermittent-light thylakoids exhibit a substantial increase in both α and energy transfer, notably in Φ_t(II-I).

Discussion

The results presented in this paper clearly demonstrate that the mechanism of the change in excitation distribution caused by protein phosphorylation depends upon the composition of the thylakoid membrane. In particular the content of LHCP and the consequent extent of membrane appression seems to be a crucial consideration. In intermittent-light thylakoids no ATP-induced fluorescence change occurs and it seems that approx. 4 h of greening are required before the maximum extent of quenching can be demonstrated. Thus, LHCP is required for the observation of ATP-induced fluorescence quenching as shown by earlier work [25,26]. At this stage of development a polypeptide with an M_r of 9000 is the major phosphoprotein (rather than LHCP) but it has been clearly shown that the fluorescence change is due only to LHCP phosphorylation [9]. Thus the difference in quenching mechanism cannot be attributed to the different phosphoprotein composition.

In addition to the requirement for LHCP a further prerequisite for observation of an ATP-induced state transition is the lateral segregation of PS II and PS I into the appressed and unappressed membrane domains respectively [3,18,23]. It seems that very little LHCP is required for the creation of these domains, since from 4 h to maturity (covering a change in chlorophyll a/b ratio from more than 5 to less than 3) an approximately constant proportion of ATP-induced fluorescence quenching is seen. Despite the similarity of the magnitude of the ATP-induced quenching it is clear that in thylakoids partially depleted of LHCP, phosphorylation causes a large spillover change, whereas at maturity the spillover change in negligible. This conclusion was based on analysis of fluorescence-inducton curves recorded at both +20 and −196°C. A likely explanation for this difference in quenching mechanism is that phosphorylation of immature thylakoids results in more extensive unstacking than that occurring in mature thylakoids. The greater destabilising effect of phosphorylation in the immature thylakoids is explainable in terms of the magnitude of the forces promoting and inhibiting membrane appression [17,18]. These forces are likely to be different in membranes with different protein composition. In mature thylakoids, phosphorylation of LHCP is normally insufficient to destabilize membrane appression except at low Mg^{2+} concentration [12,14]. However, if a large amount of phosphorylation is seen then, even in mature thylakoids, increased unstacking and spillover may occur. Such effects have been reported by Kyle et al. [11] and have been seen on occasions in our laboratory when phosphorylation induced an exceptionally large (more than 30%) decrease in fluorescence yield [14].

The present results suggest that the mechanisms of energy redistribution brought about by protein phosphorylation will show considerable variation between species and within a single species depending on developmental state and growth conditions. This variation may not be without significance, since a state change dependent upon spillover rather changes in absorption cross-section will have implications in terms of electron-transfer interaction between PS I and PS II.

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