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MEMBRANE PHOSPHORYLATION LEADS TO THE PARTIAL DETACHMENT OF THE CHLOROPHYLL *a*/*b* PROTEIN FROM PHOTOSYSTEM II

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The hypothesis that the chlorophyll fluorescence decline due to membrane phosphorylation is caused principally by the detachment and removal of LHCP from the LHCP-PS II matrix is examined. It is demonstrated that when membranes are phosphorylated in the dark (a) the fluorescence decline is greater when excited by light enriched in wavelengths absorbed mainly by LHCP (475 nm) than when excited by light absorbed to a large extent also by the PS II complex (435 nm), (b) titration with different artificial quenchers of chlorophyll fluorescence is unchanged after the phosphorylation-induced fluorescence decline, and (c) the F_v/F_m ratio does not change after the phosphorylation-induced fluorescence decline. These data indicate that it is indeed principally LHCP that interacts with the quencher (PS I presumably). This interaction involves a small fraction of the total PS II-coupled LHCP, which becomes functionally detached from the LHCP-PS II matrix.

Introduction

The phosphorylation of membrane polypeptides by ATP and a thylakoid-bound kinase [1,2] causes a decrease in the chlorophyll fluorescence emission of approx. 15–20% (see review by Haworth et al. [3]). This probably involves the phosphorylation of LHCP. Activation of the kinase requires reduction of the plastoquinone pool [4–6]. There is a growing conviction that the major change leading to the fluorescence decline is a decrease in the optical cross-section of PS II. Thus it is suggested that some LHCP may become detached from PS II and subsequently become associated with and transfer its excitons to PS I [3,7–10], though strictly speaking there is no evidence yet to directly involve LHCP in this process. There also seem to be differences in data among the different groups proposing this idea. Thus Arntzen and colleagues [3,10] have found that in the presence of saturating concentrations of magnesium ions most of the quenching is to the F_v fluorescence with no significant decrease in F_0 fluorescence. On the contrary, Horton and Black [7] have found that both F_v and F_0 decline, leading to an unchanged F_v/F_m ratio. Arntzen and coworkers [3,8] have suggested that a loosely coupled (to PS II) or 'free' LHCP moves from the grana regions into the stroma regions to be quenched by PS I. On the

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Abbreviations: LHCP, light-harvesting chlorophyll *a*/*b* protein; PS II, the Photosystem II core complex; PS I, Photosystem I; F_m, maximum fluorescence (all traps closed); F_0, non-variable fluorescence (all traps open); F_v, variable fluorescence ($F_m - F_0$); DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; DBMIB, dibromothymoquinone; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

other hand, the possibility has been entertained that the quenching involves a classical spill-over type interaction of the LHCP-PS II complex with PS I [12]. Such a mechanism should lead to a decreased F_v/F_m ratio and, as mentioned above, there is disagreement on this point.

In the present work we have tested the idea of LHCP detachment from PS II and its subsequent quenching (by PS I presumably) utilising several different approaches. (a) Such a mechanism predicts a greater fluorescence quenching when fluorescence is excited by light absorbed preferentially by LHCP rather than when it is absorbed more equally by PS II and LHCP. This information is important, as to date there are no data which directly indicate LHCP detachment, but rather the suggested decrease in PS II cross-section following phosphorylation is assumed to be due to LHCP detachment. (b) This mechanism also predicts that the fluorescence emission after phosphorylation is largely from the nonquenched and still coupled LHCP-PS II. Such fluorescing units are expected to have rate constants for the various processes that compete for LHCP-PS II excitons little changed with respect to the nonphosphorylated control (a small increase in the rate constant for energy transfer from LHCP to PS II may be expected due to the decreased size of the LHCP bed [13]. Such a possibility has been investigated by titration of the F_m fluorescence with quenchers of chlorophyll fluorescence [14;30]. (c) The mechanism predicts little or no change in the F_v/F_m ratio provided the coupling between PS I and the LHCP detached from LHCP-PS II is tight. In the light of the conflicting data on this point, we have reinvestigated it taking care to eliminate possible artifacts induced by the preillumination treatment commonly used to reduce the plastoquinone pool, a condition required for membrane phosphorylation [4–6]. To this end, the plastoquinone pool was reduced chemically, in the dark. While this manuscript was in preparation, we learned that a similar approach was also used by Telfer et al. [9]. Our experimental findings confirm the idea that spillover changes are not significantly involved in the phosphorylation-induced fluorescence decline, which is best explained as the detachment and subsequent quenching by PS I of a small amount of LHCP from PS II. This LHCP, in the presence of Mg ions and before phosphorylation, seems tightly coupled to PS II [30].

Material and Methods

Chloroplasts were prepared from young, freshly harvested spinach leaves by a brief homogenisation in an extraction buffer containing 30 mM Tricine (pH 8)/10 mM $MgCl_2$/10 mM NaCl/0.4 M sucrose. The extract was centrifuged at $1500 \times g$ for 5 min, and the pellet was resuspended in the same buffer minus sucrose and centrifuged as before. The chloroplasts were then resuspended in the sucrose-containing medium and stored in ice for 1–2 h before starting the experiment. The reaction medium for all experiments contained 0.1 M sucrose/10 mM NaCl/10 mM NaF/10 mM $MgCl_2$/30 mM Tricine (pH 8). Chloroplast membranes were phosphorylated in the dark by incubation for 8 min in the above reaction medium, which in addition contained 1 mM NADP/5 mM glucose-6-phosphate/5 μM ferredoxin/glucose-6-phosphate dehydrogenase in large excess (1 μl of the Boehringer enzyme from yeast (grade II suspension) per ml), and 0.5 mM ATP. Control membranes were similarly treated but without ATP.

Bennett [15] has shown that the NADPH-ferredoxin system for reducing plastoquinone in the dark activates protein kinase and leads to LHCP phosphorylation. We have noticed that the fluorescence decline which results from this treatment depends on (a) the presence of ATP and (b) the reduction of the plastoquinone pool (from fluorescence induction measurements performed in the presence of reduced DBMIB we estimate that 80–90% of the plastoquinone pool is reduced by NADPH-ferredoxin in these conditions). Removal of ferredoxin greatly reduces both the ATP-induced fluorescence decline and the reduction of plastoquinone. However, we would mention that the fluorescence decline induced in this way is substantially irreversible upon removal of the NaF and the reducing system by dilution.

Fluorescence induction was measured according to Jennings et al. [16]. Titration of fluorescence with DBMIB and *m*-dinitrobenzene was performed as described by Jennings et al. [17,30].

Results

Analysis of the room temperature excitation spectra of the chlorina barley mutant which lacks LHCP [18] and its parental wild-type indicates that at 475 nm the absorption cross section of LHCP is about 3.5–4 times greater than that of PS II at this wavelength, with respect to the situation at 435 nm (unpublished data). It is therefore to be expected that if membrane phosphorylation leads to LHCP detachment from the PS II-LHCP matrix and to its subsequent association with and quenching by PS I [3,7–10], the quenching will be greater at 475 nm than at 435 nm. On the contrary, no such difference at these two wavelengths would argue against such an idea. It can be seen from Table I that when fluorescence was excited at 475 nm the fluorescence quenching was considerably greater (18.9%) than when it was excited at 435 nm (15.8%). Analysis of variance of the quenching at these two wavelengths indicates that they are significantly different at $P \leq 0.01$.

We have checked that the phosphorylation treatment did not lead to changes in the amount of light absorbed at the two wavelengths by measuring the chloroplast absorbance in a spectrophotometer and correcting for scattering flattening according to Latimer and Eubanks [19]. No absorption differences have been detected upon phosphorylation of the membranes. We therefore conclude that the fluorescence quenching data indicate that LHCP excitons are quenched to a greater extent than PS II excitons upon membrane phosphorylation.

TABLE I

THE EFFECT OF MEMBRANE PHOSPHORYLATION ON F_m

Fluorescence was excited at either 435 or 475 nm (half-band width, 8 nm) and measured at 681 nm. DCMU (25 μM) and chlorophyll (4 μg/ml) were present. Analysis of variance of the quenching data indicates that quenching at the two wavelengths is significantly different at the 99% level.

Excitation wavelength (nm)	Phosphorylated	Non-phosphorylated	Quenching (%)
435	56.4	67.0	15.8
475	71.4	88.1	18.9

We have demonstrated elsewhere [14] that titration of the chlorophyll fluorescence with artificial quenchers is useful in distinguishing between homogeneous and heterogeneous endogenous fluorescence quenching. Homogeneous quenching signifies that the endogenous quenching process involves the entire fluorescing bed and is formally equivalent to the rate constant of the endogenous quencher competing with all other rate constants for the PS II-LHCP excitons. On the other hand, heterogeneous quenching means that only a part of the fluorescing bed is quenched by the endogenous quenching process. In this case, the quenching rate constant competes for the PS II-LHCP excitons only in those complexes which are quenched. We have therefore titrated the fluorescence yield of thylakoids before and after phosphorylation with artificial quenchers. The data (Fig. 1) are represented as the $F_{n-1} - F_n/F_{n-1}$ versus $F_i - F_n/F_i$ plot (double quenching plot; see Refs. 14, 17 and 30). In the case of homogeneous quenching, the ratio of the intercepts on the $F_{n-1} - F_n/F_{n-1}$ axis should equal the ratios of the F_i values. For heterogeneous quenching the ratio of the intercepts is less than the ratio of the F_i values. From Fig. 1 it can be seen that membrane phosphorylation leads to a markedly heterogeneous type of quenching with no evident change in the double quenching plot. Thus we conclude that the phosphorylation-induced quenching mechanism

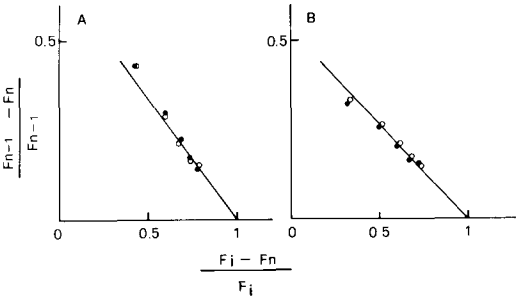

Fig. 1. Titration of the fluorescence (F_m) of phosphorylated and non-phosphorylated chloroplasts with successive additions of (A) DBMIB (0.6 μM) and (B) *m*-dinitrobenzene (125 μM). DCMU (25 μM) was always present. The excitation wavelength was 475 nm and the emission wavelength was 682 nm. F_i, initial fluorescence before addition of the quencher. F_n, fluorescence after *n* additions of quencher. The fluorescence decline due to phosphorylation was 18% (A) and 14% (B). (○), non-phosphorylated; (●), phosphorylated.

TABLE II

FLUORESCENCE INDUCTION PARAMETERS FOR PHOSPHORYLATED AND NON-PHOSPHORYLATED MEMBRANES

The excitation light was provided by a Corning 4-96 filter and was measured at 681 nm. DCMU (25 μM) was present.

	Phosphorylated	Non-phosphorylated
F_0	12.9	14.9
F_m	55.9	64.5
F_v/F_m	0.77	0.77

does not effectively compete with the various rate constants that determine the fluorescence emission after phosphorylation. Such a situation is most readily envisaged in terms of phosphorylation leading to detachment of a part of the fluorescing bed from the PS II-LHCP matrix, and the subsequent, virtually complete quenching of the fluorescence of these detached units.

According to Duysens and Sweers [20], Duysens [21] and Butler and Kitajima [22], the conversion of the F_0 fluorescence level to the F_m level during fluorescence induction is considered to be due to the conversion of a strong photochemical quencher (the oxidised PS II reaction centre) into a weakly quenching form (the reduced PS II reaction centre). Thus the interaction of a quencher with PS II is expected to quench the F_m fluorescence more than the F_0 level, leading to a decrease in the F_v/F_m ratio. However, in the case of the above-mentioned hypothesis, that the phosphorylation-induced quenching involves the detachment and subsequent, virtually complete quenching of a part of the PS II-LHCP matrix, such a change in the F_v/F_m ratio is not predicted. Thus we have measured the influence of thylakoid phosphorylation on the F_v/F_m ratio. The data (Table II) show that this remains unchanged.

Discussion

We demonstrate here that chloroplast membrane phosphorylation leads to a significantly greater fluorescence quenching when the excitation light is absorbed predominantly by LHCP than when it is absorbed to a relatively greater extent by PS II. Therefore, titration with fluorescence quenchers indicates that phosphorylation probably leads to the detachment and subsequent quenching of a part of the antenna of PS II from the PS II-LHCP matrix, and this conclusion is supported by the lack of change in the F_v/F_m ratio upon phosphorylation. It seems therefore likely that it is, in fact, mostly LHCP which becomes detached from the PS II-LHCP matrix and quenched (probably by PS I). In terms of this concept, a decline in the fluorescence of 15–20% is expected to indicate the detachment and quenching of about 15–20% of the total PS II-LHCP matrix. Since about 70–80% of the chlorophyll of this matrix is expected to be associated with LHCP [23], we estimate that in these experiments up to around 25% of the total LHCP may become detached.

It should be pointed out that removal of magnesium ions from chloroplasts also leads to a greater quenching when fluorescence is excited with 475 nm than with 435 nm light [24,25]. However, studies with artificial quenchers [14,30] have shown that this quenching process is completely homogeneous, thus differing from the markedly heterogeneous quenching due to phosphorylation as reported here. This points to a fundamentally different mechanism for the two phenomena (Mg ion removal and thylakoid phosphorylation). The present suggestion that phosphorylation leads to the detachment and quenching of some LHCP from the PS II-LHCP matrix, whereas lowering the Mg ion concentration from 2.5 to 0.5 mM is not expected to lead to such an effect [14,30], probably explains this difference.

Our data which indicate that membrane phosphorylation does not change the F_v/F_m ratio in the presence of 10 mM $MgCl_2$ agree with those of Horton and Black [7,26], Krause and Behrend [27], and Telfer et al. [9], but differ from those of Haworth et al. [11], Kyle et al. [8], and Saito et al. [28]. It is difficult to understand why these differences in data exist. We have been careful here to eliminate, by carrying out this process in the dark, any possible artifacts that involve the light pretreatment usually given to accomplish phosphorylation. The possibility that the developmental state of the chloroplasts may lead to different results of this kind is suggested by Black et al. [29]. They showed that the phosphorylation of mature membranes did not lead to F_v/F_m changes when

the Mg-ion concentration was saturating, whereas with immature chloroplasts F_v/F_m decreases were observed.

Thus our data are best interpreted in terms of a model in which membrane phosphorylation induces the movement of some LHCP away from the LHCP-PS II bed towards the fluorescence quencher (PS I). We have shown elsewhere [14], on the basis of equal F_m/F_0 ratios using either 475 or 435 nm excitation light, that in the presence of Mg ions LHCP is strongly coupled to PS II. Thus we are unable to ageee with Kyle et al. [8] and Haworth et al. [11] that the LHCP that becomes quenched after phosphorylation belongs to a 'free' or a loosely coupled LHCP population, at least not in mature spinach chloroplasts with a high F_v/F_m ratio.

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