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Spinach-thylakoid phosphorylation: studies on the kinetics of changes in photosystem antenna size, spill-over and phosphorylation of light-harvesting chlorophyll *a*/*b* protein

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The kinetics of LHCP phosphorylation and associated changes in photosystem cross-section and energy 'spill-over' from PS II to PS I have been examined in isolated spinach chloroplasts. During an initial phosphorylation period of 3–6 min, in the presence of saturating concentrations of Mg^{2+}, the increase in PS I and decrease in PS II cross-section are largely completed, as judged by both measurements of the steady-state redox state of Q and fluorescence yield changes. This corresponds to a period of rapid ^{32}P incorporation into the low-molecular weight LHCP polypeptide. Subsequent to this initial 3–6-min period there is substantial further phosphorylation of both LHCP polypeptides, which is not accompanied by significant changes in photosystem cross-section, even after the chloroplasts had been unstacked with extensive mixing of PS I and PS II by Mg-removal. It is suggested that there exists a specific 'mobile' population of LHCP molecules which is rapidly phosphorylated and which may be enriched in the low-molecular-weight polypeptide. In addition, measurements of the kinetics of the 'spill-over' changes upon either Mg^{2+} addition or removal indicate that the continued phosphorylation of LHCP is able to increase the 'spill-over' process under favourable ionic conditions.

Introduction

In recent years the phosphorylation of membrane thylakoid proteins has been extensively studied in terms of the regulation of the energy distribution between the Photosystems II and I. In this respect attention has been particularly directed towards the phosphorylation of the LHCP polypeptides [1,2]. Phosphorylation of LHCP by a thylakoid-bound protein kinase, activated by the reduction of the plastoquinone pool [2–4], is thought to lead to the detachment of a fraction of the LHCP from the PS II-LHCP matrix and to its subsequent association with PS I [5–10]. This process presumably involves the lateral translation of some of the LHCP from the grana partition zone (where the PS II-LHCP matrices are located) to the stroma-exposed membranes (enriched in PS I complexes [11]). This idea is supported by the pulse-chase experiments of Kyle et al. [12] and Larsson and Andersson [13]. Thus phosphorylation would lead to an increase in the PS I optical cross-section and a decrease in that of PS II. On

Abbreviations: LHCP, light-harvesting chlorophyll *a*/*b* protein complex; PS, Photosystem; NADP, nicotinamide-adenine dinucleotide phosphate; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Q, primary electron acceptor quinone of Photosystem II; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl] glycine; Chl, chlorophyll.

the other hand dephosphorylation of the phosphorylated LHCP by endogenous phosphatases is thought to lead to the reassociation of this protein-chlorophyll complex with the PS II matrix [14,5].

This relatively simple picture of the effects of LHCP phosphorylation on energy distribution between the two photosystems pertains to a situation in the presence of saturating concentrations of the electrostatic double layer screening cations. In the presence of subsaturating concentrations of screening cations thylakoid phosphorylation seems to bring about quite large changes in the direct transfer of PS II excitons to PS I (spill-over) in addition to the changes in cross-sections mentioned above [15–17].

Whilst there is a general consensus on the overall consequences of LHCP phosphorylation, the situation is much less clear regarding the mechanistic aspects. It was previously thought that a close correlation existed between the phosphate incorporation into LHCP and its detachment from the PS II matrix and subsequent association with PS I (usually measured as the decline in the PS II fluorescence emission). We have recently demonstrated that this is not the case in spinach chloroplasts suspended in the presence of saturating concentrations of Mg^{2+}. It was shown that after an initial period of 3–6 min, while substantial phosphate incorporation into LHCP continued, the quenching of PS II fluorescence was almost complete [18]. Thus in fully stacked thylakoids a large amount of phosphorylated LHCP appears to be unable to migrate from the PS II-LHCP matrix towards the PS I-enriched membrane zones. However, as distinct from the situation in the presence of saturating concentrations of Mg^{2+}, upon lowering the concentration of the screening cation to subsaturating levels it was possible to demonstrate considerable further quenching of PS II fluorescence after the initial 3–6 min period. The additional fluorescence quenching seemed to involve predominantly a 'spill-over' type interaction rather than a change in photosystem cross-section. In the present study we have further examined the relationship between spinach thylakoid phosphorylation during and after the initial 3–6 min period and both photosystem cross-section changes and 'spill-over' type changes.

Materials and Methods

Chloroplasts were prepared from freshly harvested spinach leaves which had been kept in the dark for 1–2 h, as described previously [9,18]. Phosphorylation of the membranes (200–400 μg Chl/ml) was performed in the dark in the presence of the NADPH-ferredoxin reducing system (added to both control- and phosphorylated-membrane samples [9,18]) in a medium containing 30 mM Tricine (pH 8.0), 0.1 M sucrose, 10 mM NaCl, 10 mM NaF, 5 mM $MgCl_2$ and was initiated by addition of 1 mM Mg-ATP. At various times aliquots were removed and the reaction terminated either by addition of a 100–200-fold excess of ice-cold phosphorylation medium or by addition of ice cold 80% acetone to precipitate the proteins for SDS-polyacrylamide gel electrophoresis.

Fluorescence measurements were carried out in a home-built apparatus at a chlorophyll concentration of 2–4 μg/ml in the presence of 25 μM DCMU at 20°C. The excitation light was filtered through a Corning 4-96 filter and fluorescence was measured at 691 nm (Balzers B-40 interference filter). Fluorescence quenching is expressed as $(F_{max}(-ATP) - F_{max}(+ATP))/F_{max}(-ATP)$ where all F_{max} values were determined at each time point. The kinetics of fluorescence changes associated with either Mg^{2+} or EDTA addition to chloroplasts were measured with continual stirring of the chloroplast suspension.

Steady-state measurements of the redox state of the primary PS II electron acceptor Q during non-cyclic electron transport from water to NADP were performed as described previously [10].

Digitonin fractionation was according to Boardman and Anderson [19] in the Tricine buffer described above at a chlorophyll concentration of 200 μg/ml.

Delipidated proteins were fractionated on 15% acrylamide slab gels by SDS-polyacrylamide gel electrophoresis and the specific incorporation into the LHCP polypeptides determined by excision of the protein bands as described previously [18]. A relative stoichiometry of the low molecular weight/high molecular weight polypeptide of 1 mol: 3 mol has been determined by quantitative SDS-polyacrylamide gel electrophoresis [20] for both the purified LHCP and isolated thylakoids [21].

Results and Discussion

Effect of phosphorylation on LHCP movement

A typical time-course of ^{32}P incorporation into the two major LHCP polypeptides and the phosphorylation-induced fluorescence quenching is shown in Fig. 1a. These data confirm our previous observation [18] that while the fluorescence quenching, in the presence of saturating concentrations of Mg^{2+}, is largely completed during the initial 3–6 min, incorporation into both the LHCP polypeptides continues long after this period. It can be seen that the ^{32}P incorporation into the two LHCP polypeptides is roughly equal during the initial few minutes. Subsequently, most incorporation is into the high-molecular-weight band. However, as the low-molecular-weight band is approx. 3-times less abundant than the high-molecular-weight band [21], the specific activity of incorporation into this polypeptide is very much higher than that into the high-molecular-weight band (Fig. 1b), during the first few minutes. Subsequently, the specific activity of incorporation into the low-molecular-weight band, after correction for the molar abundance, remains somewhat higher than that into the high-molecular-weight band, though the difference is greatly reduced.

We have previously demonstrated that thylakoid phosphorylation leads to quite large increases in the steady-state level of oxidised Q during non-cyclic electron transport to NADP [10]. This is thought to be due to both an increase in the PS I and a decrease in the PS II photochemistry following the phosphorylation-induced movement of LHCP from the PS II matrix regions to PS I. Thus the steady-state redox level of Q is a sensitive index of the phosphorylation-induced changes in optical cross-section. We have measured the time course of the effect of phosphorylation on this parameter (Fig. 2). As with the phosphorylation-induced fluorescence decline the major increase in oxidised Q levels was observed in the initial 3–6 min of phosphorylation, after which time little or no further change was detected. Thus it is concluded on the basis of both fluorescence and Q measurements that the increased phosphorylation after the initial 3–6 min period leads to little further LHCP movement from PS II to PS I.

We have also investigated the possibility that the failure of continued phosphorylation, after the initial 3–6 min period, to provoke substantial LHCP movement away from the PS II-LHCP

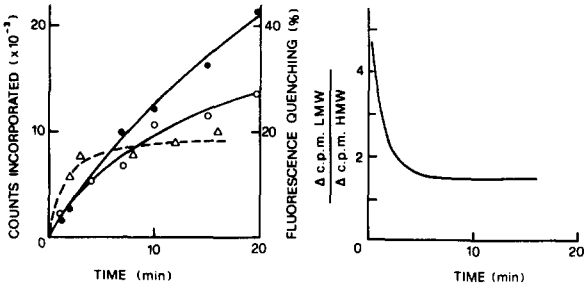

Fig. 1. Time-course of γ-^{32}P incorporation into the two LHCP polypeptides and of the fluorescence quenching due to thylakoid phosphorylation. (a) Total counts incorporated into the low-molecular-weight (○) and high-molecular-weight (●) polypeptides and the fluorescence quenching (△). (b) The ratio of the incremental ^{32}P incorporation into the low- (LMW) and high-molecular-weight (HMW) polypeptides. The ratio curve was calculated from the data in part (a) of this figure taking into account the relative abundance of the two polypeptides (see Materials and Methods).

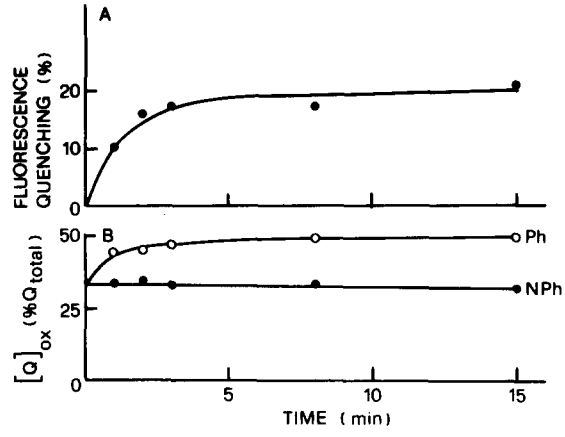

Fig. 2. The time-course of the phosphorylation-induced fluorescence quenching and the steady-state level of oxidised Q during non-cyclic electron transport from water to NADP. Fluorescence determinations (a) and Q measurements (b) were performed as described in Materials and Methods. For the Q measurements the light intensity was 6.5 $J \cdot m^{-2} \cdot s^{-1}$ (Corning 4-96 filter) and Gramicidin (1 μM), Ferredoxin (3.6 μM) and NADP (0.5 mM) were added. ph, phosphorylated thylakoids; nph, non phosphorylated thylakoids (see Materials and Methods).

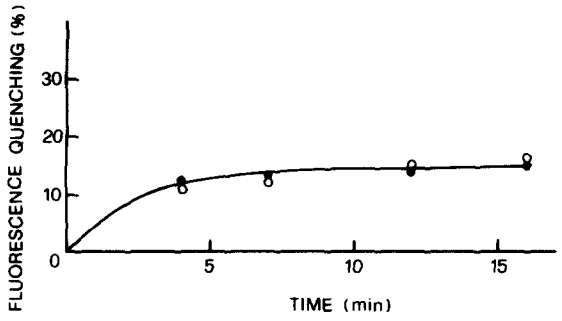

Fig. 3. The effect of thylakoid unstacking and restacking on the phosphorylation-induced quenching of the chlorophyll fluorescence. Phosphorylation was performed under the usual conditions in the presence of $MgCl_2$ (5 mM). At various times aliquots were removed and diluted 200-fold in ice-cold buffer either with (●) or without (○) $MgCl_2$ and incubated in the cold for a further 1.5 h. After this time $MgCl_2$ (5 mM) was added back to the Mg^{2+}-deprived samples which were then incubated for a further 1 h in the cold when fluorescence measurements were commenced. The fluorescence values (arbitrary units) for the different experimental treatments were: chloroplasts incubated with $MgCl_2$, 100; chloroplasts incubated without $MgCl_2$, 42; chloroplasts to which $MgCl_2$ was readded, 97.

matrices may be due to some kind of steric hinderance. Conceivably only the LHCP-located peripherally in the matrix has the potential to move out of the grana, while the LHCP complexes less peripherally located may not be able to detach from the PS II matrices following phosphorylation. It has been suggested that there may be a specifically 'mobile' population of LHCP molecules and that in fact it is only this 'mobile' LHCP which migrates upon phosphorylation [12,13]. A 'mobile' population of LHCP could correspond to those LHCP molecules which are peripherally located in the PS II matrices. We have examined this possibility by removal of Mg ions from the phosphorylated thylakoids in order to unstack the membranes and disrupt the PS II-LHCP matrices. Subsequently, Mg^{2+} was added and after incubation for a suitable period the fluorescence was measured and compared with that of phosphorylated membranes which had not been subjected to the unstacking treatment. As shown in Fig. 3 the phosphorylation-induced fluorescence decline for both unstacked and subsequently restacked chloroplasts was similar both quantitatively and kinetically to that for chloroplasts which had remained stacked in the presence of Mg^{2+}-following phosphorylation. This result would seem to rule out the possible involvement of steric hinderance factors associated with PS II matrix or grana structure. The data appear to support the idea that only a distinct population of phosphorylated LHCP can migrate and become associated with PS I in the presence of saturating concentrations of screening cations. We suggest that this population of phosphorylated LHCP molecules correspond to the 'mobile' LHCP of Kyle et al. [12] and Larsson and Andersson [13]. As changes in the photosystem cross-section occur during the initial 3–6 min period it may be that the 'mobile' LHCP is much more rapidly phosphorylated than the 'static' LHCP population. As noted above the low-molecular-weight polypeptide component is phosphorylated at a much greater rate than the high-molecular-weight polypeptide component during the initial 3–6 min period (Fig. 1). Thus the 'mobile' population may contain a high concentration of phosphorylated low-molecular-weight polypeptide. This interpretation of our kinetic data would be in agreement with the recent fractionation studies of Larsson and Andersson [13].

Effect of phosphorylation on 'spill-over' changes

We have also examined the effect of thylakoid phosphorylation on the fluorescence change kinetics associated with the 'spill-over' type interaction between PS II and PS I both upon Mg^{2+} removal (EDTA-chelation) and Mg^{2+} addition. The 'spill-over' interactions are known to be controlled by the space charge density associated with the electrostatic double layer [22–24]. Thus if thylakoid phosphorylation were to increase the membrane surface charge density, as is expected, the fluorescence change kinetics should be altered. A typical fluorescence rise upon Mg^{2+} addition to chloroplasts which had been deprived of Mg^{2+}, is represented in Fig. 4. It can be seen that thylakoid phosphorylation significantly slowed down this rise. On the other hand thylakoid phosphorylation appreciably increased the rate of the fluorescence decline upon EDTA addition to Mg^{2+}-incubated chloroplasts (Fig. 4b). We believe that these data are best explained in terms of an increased negative surface charge density in phosphorylated membranes in agreement with the suggestion of

Barber [25]. This causes a slower separation of the PS I complexes from the PS II-LHCP complexes upon Mg^{2+} addition and a more rapid intermixing of the two photosystems upon EDTA addition.

We have subjected the data from the Mg^{2+}-addition experiment to the analysis suggested by Vernotte et al. [26]. Combining the hypothesis of Forster energy transfer between the two photosystems with a coulomb-type interaction, these authors describe the kinetics of the fluorescence rise upon Mg^{2+} addition to thylakoids in terms of the kinetics of the distance R separating the PS II and PS I particles. According to this analysis a plot of R^3 vs. time gives a straight line with a slope proportional to the ratio between the coulomb-type force which cause the distance between the two photosystems to increase and the frictional coefficient of the thylakoid membrane. In Fig. 5 are shown the plots of R^3 vs. time of phosphorylated and non-phosphorylated thylakoids. It can be seen that phosphorylation decreases the slope, indicating either a decrease in the above-mentioned coulomb-type force or an increase of the frictional coefficient. The latter hypothesis of increased resistance of the membrane upon phosphorylation seems to be ruled out by the fact that with EDTA addition the rate of the fluorescence decline, and hence of PS II and PS I intermixing, is increased by phosphorylation (Fig. 4b). Thus the data are explained in terms of a decrease in the coulomb-type forces causing the two photosystems to migrate away from each other, presumably due to increased PS II–PS II separation associated principally with LHCP phosphorylation.

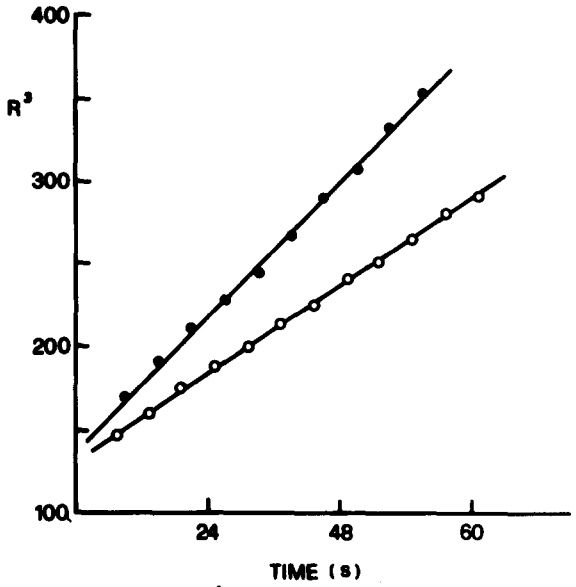

Fig. 5. Kinetics of R^3 changes with time after addition of $MgCl_2$ to phosphorylated (○) and non-phosphorylated (●) chloroplasts. R is the average distance between the PS I and PS II particles and was calculated from the fluorescence data (see Fig. 4a) using the approach of Vernotte et al. [26].

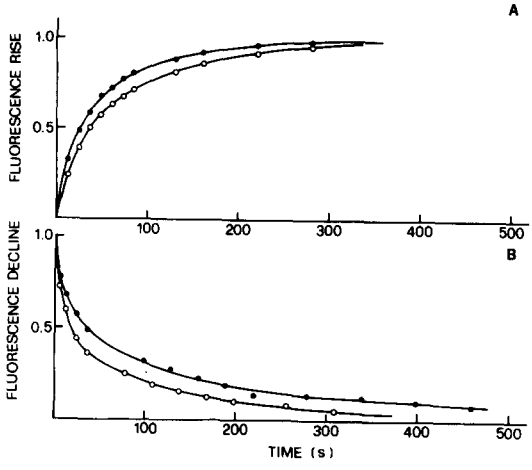

Fig. 4. The effect of thylakoid phosphorylation on the fluorescence change kinetics due to either Mg addition or Mg chelation. Chloroplasts were phosphorylated for 15 min, as described in Materials and Methods, and then (a) diluted 200-fold with the Mg^{2+}-free ice-cold phosphorylation buffer and incubated for a minimum time of 1 h when $MgCl_2$ (5 mM) was added and the fluorescence rise kinetics measured at 20°C. The data are normalized to the maximum fluorescence change. Fluorescence after Mg^{2+} addition was 2.1-times that before Mg^{2+} addition. (b), as in (a), except incubation following phosphorylation was in the presence of $MgCl_2$ (5 mM). Subsequently EDTA (6 mM) was added and the fluorescence decrease measured. The data are normalized to the maximum fluorescence decrease. Fluorescence after EDTA addition was 0.54 times that before EDTA addition.

We have demonstrated in the preceding section that LHCP phosphorylation after the initial 3–6 min period has little or no influence on LHCP movement and the associated changes in cross-section of the two photosystems. In Table I data are presented on the kinetics of 'spill-over' changes caused by Mg^{2+} and EDTA addition to thylakoids phosphorylated for either 5 or 15 min. It can be seen that phosphorylation for 15 min led to a more pronounced deceleration of the Mg^{2+}-in-

TABLE I

THE EFFECT OF THYLAKOID PHOSPHORYLATION FOR DIFFERENT TIMES ON THE HALF-TIMES OF THE FLUORESCENCE CHANGES CAUSED BY Mg OR EDTA ADDITION

For experimental conditions see legend to Fig. 4. The errors represent the standard deviation multiplied by Student's 't' for the 95% confidence level.

Treatment	Non-phosphorylation $t_{1/2}$ (s)	Phosphorylation $t_{1/2}$ (s)	Phosphorylation $t_{1/2}$ / Non-phosphorylation $t_{1/2}$
Mg addition			
phosphorylation time: 5 min	27.6	34.7	1.26 ± 0.1
phosphorylation time: 15 min	24.4	39.2	1.61 ± 0.25
EDTA addition			
phosphorylation time: 5 min	39.1	29.8	0.76 ± 0.06
phosphorylation time: 15 min	45.8	24.8	0.54 ± 0.09

duced fluorescence rise than after 5 min. In the case of EDTA addition the phosphorylation-induced acceleration of the fluorescence decline was greater after 15 min than after 5 min. This latter result implies that between 5 and 15 min a substantial increase in charge density occurred within the grana partition zone themselves. We have directly confirmed this result by examining ^{32}P incorporation into the LHCP of the heavy ($10\,000 \times g$) digitonin fraction (grana). This increased approx. 2-fold between 5 and 15 min, as did the ^{32}P incorporation into the total (unfractionated) LHCP. The light fraction has not been examined as we have been unsuccessful in obtaining total recovery of the phosphorylated protein and observe losses of between 20–40%. The reasons for the inefficient recovery are not known.

Thus in both the above-described situations, where the fluorescence changes produced by Mg^{2+} addition or removal are largely caused by 'spill-over' changes, the effect of phosphorylation was greater after 15 min than after 5 min. This observation supports our previous report [18] that in the presence of subsaturating Mg^{2+} concentrations the phosphorylation-induced fluorescence quenching continues to increase with increasing phosphorylation time after the initial 3–6 min period. We therefore conclude that the increased charge density due to membrane phosphorylation after the initial 3–6 min is effective in influencing 'spill-over' interactions between PS II and PS I, whenever the ionic condition permits such an in-

TABLE II

DISTRIBUTION OF ^{32}P INTO LHCP POLYPEPTIDES IN UNFRACTIONATED THYLAKOIDS AND THE DIGITONIN HEAVY FRACTION AFTER PHOSPHORYLATION FOR TWO DIFFERENT TIMES

Chloroplasts were phosphorylated for 5 and 15 min and incubated for 30 min on ice before addition of Digitonin to a final concentration of 0.5% and left on ice for a further 30 min with gentle shaking. After removal of aliquots for the determination of the ^{32}P into the unfractionated thylakoids the reaction was terminated by addition of 10 volumes of cold phosphorylation buffer and the heavy fraction collected by centrifugation ($10000 \times g$, 30 min). The specific incorporation into the LHCP polypeptides was determined following fractionation by SDS-polyacrylamide gel electrophoresis as described in Materials and Methods.

	Total ^{32}P in LHCP polypeptides (counts/20 min)		Counts heavy fraction / Counts unfractionated thylakoids
	unfractionated thylakoids	heavy fraction	
Phosphorylation time: 5 min	23069	10557	0.46
Phosphorylation time: 15 min	40441	18444	0.46

teraction to occur. However, as distinct from the initial phosphorylation phase when the low-molecular-weight polypeptide of LHCP is very rapidly labelled, during this second phase substantial phosphorylation of both polypeptides occurs. There is therefore no evidence to suggest that the effects of phosphorylation on 'spill-over' involve a specific LHCP population.

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