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Complex formation in plant thylakoid membranes. Competition studies on membrane protein interactions using synthetic peptide fragments

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Abstract

Thylakoid membranes of pea were used to study competition between extra-membrane fragments and their parental membrane-bound proteins. Phosphorylated and unphosphorylated fragments of light harvesting complex II (LHC II) from higher plants were used to compete with LHC II for interactions with itself and with other thylakoid protein complexes. Effects of these peptide fragments of LHC II and of control peptides were followed by 80 K chlorophyll fluorescence spectroscopy of isolated thylakoids. The phosphorylated LHC II fragment competes with membrane-bound phosphoproteins in the phosphatase reaction. The same fragment accelerates the process of dark-to-light adaptation and decreases the rate of the light-to-dark adaptation when these are followed by fluorescence spectroscopy. In contrast, the non-phosphorylated LHC II peptide does not affect the rate of adaptation but produces results consistent with inhibition of formation of a quenching complex. In this quenching complex we propose that LHC II remains inaccessible to the LHC II kinase, explaining an observed decrease in LHC II phosphorylation in the later stages of the time-course of phosphorylation. The most conspicuous protein which is steadily phosphorylated during the time-course of phosphorylation is the 9 kDa (psbH) protein. The participation of the phosphorylated form of psbH in the quenching complex, where it is inaccessible to the phosphatase, may explain its anomalously slow dephosphorylation. The significance of the proposed complex of LHC II with phospho-psbH is discussed.

Abbreviations: LHC II – light harvesting complex II; PS II – Photosystem II; PS I – Photosystem I

Introduction

Adaptation of chloroplast thylakoid membranes to changes in light intensity and light quality is accompanied by phosphorylation or dephosphorylation of several thylakoid proteins (Allen 1992). Most of these belong to Photosystem II (PS II). Membrane fractionation studies (Anderson and Andersson 1982; Kyle et al. 1984) are consistent with lateral movement of light harvesting complex II (LHC II) from PS II to PS I upon phosphorylation, but the productive binding of LHC II to PS I has not been consistently observed (Allen 1992). While most thylakoid protein kinase reactions are redox-dependent (Silverstein et al. 1993a), thylakoid protein phosphatase reactions are redox-independent and kinetically heterogeneous (Silverstein et al. 1993b). LHC II is dephosphorylated more rapidly than other proteins. The 9 kDa protein exhibits an extremely low rate of dephosphorylation (Allen and Findlay 1986; Cheng et al. 1994). Other phosphoproteins are dephosphorylated at various rates between these two extremes (Cheng et al. 1994). It has been shown by Demming et al. (1987) that changes in chlorophyll fluorescence are not in a simple relation to LHC II phosphorylation. The adaptation of thylakoid membranes by protein phosphorylation to changes in light conditions may then be a more complex process

than simple movement of LHC II proteins between PS II- and PS I-enriched domains.

Here we present results of low-temperature fluorescence spectroscopy which are consistent with formation of a quenching complex in which LHC II participates in its unphosphorylated form. Phosphorylation of LHC II reaches its maximum in an early stage after which LHC II becomes dephosphorylated and protected against further phosphorylation. This dephosphorylation of LHC II is accompanied by increasing phosphorylation of the 9 kDa phosphoprotein whereas the majority of other thylakoid phosphoproteins maintain a constant degree of phosphorylation. We therefore suggest that the quenching complex is formed between 9 kDa phosphoprotein in its phosphorylated form and LHC II in its unphosphorylated form.

Materials and methods

Peptide synthesis

Peptides were synthesized by a solid phase, t-Boc strategy (Barany and Merrifield 1980). The peptides RKSATTKKVASSGSP and SRPLSDQEKRKQISVRGLAGVENV were supplied by the Dr. Ivo Blaha group in the Institute of Organic Chemistry and Biochemistry, Prague. The phosphorylated peptide RKSA[T(PO_3)]TKKVASSGSP was synthesized by the same methods. In place of phosphorylated threonine, Boc-Thr[OPO($OPh)_2$] was incorporated and a deprotection step carried out as described by (Grehn et al. 1987). This peptide was supplied by Dr. Henry Franzén from the Biomedical Unit at Lund University.

Thylakoid preparation

Thylakoid membranes were isolated from pea (Pisum sativum). Peas were grown at 20 °C in a 12-hour light and 12-hour dark cycle. Leaves were harvested from 14–16-day-old seedlings towards the end of the dark phase of the cycle. Thylakoids were prepared by the method described in (Harrison and Allen 1991). Thylakoids were stored on ice in darkness for at least 60 minutes before the measurements. Results were independent of the age of thylakoid preparation up to 6 hours after isolation.

Dark-to-light adaptation

Thylakoid membranes were suspended to give the final concentration of 5 μg Chl ml^{-1} in the suspending medium given by (Harrison and Allen 1991) to which ATP and peptides were added to give final concentration of 3 mM ATP and 2 mM peptides. A peptide-free thylakoid suspension was used for the control experiment. Samples were illuminated with a fluorescent strip lamp giving a light intensity of 5 μmol $m^{-2} \cdot s^{-1}$ while working with the phosphorylated peptide and 8.5 μmol $m^{-2} \cdot s^{-1}$ while working with the non-phosphorylated peptide. Samples were pipetted at different time intervals from the suspension into the fluorescence cuvette and frozen immediately in darkness to the temperature of liquid nitrogen. Fluorescence spectroscopy was carried out using a Perkin-Elmer LS-5 luminescence spectrometer.

Light-to-dark adaptation

Fully light-adapted thylakoids after 2.5 hours of illumination in a suspension of concentration 10 μg Chl · ml^{-1} with the light intensity 8.5 μmol $m^{-2} \cdot s^{-1}$ were suspended in an equal volume of solution containing 4 mM peptide in darkness. The samples for fluorescence measurements were taken in the same way as for the dark-to-light adaptation.

Phosphorylation measurements

For measurement of the amount of ^{32}P incorporated into membrane proteins during the phosphorylation, we prepared a suspension containing [γ-^{32}P] ATP in concentration of 0.1 mM at a specific activity 2.4 μCi · ml^{-1}. The concentration of thylakoid membranes was 0.2 mg Chl · ml^{-1}. Samples were taken at time intervals from 15 seconds to 2 hours. At each time interval a sample (100 μl) of suspension was withdrawn and precipitated in Eppendorf tubes containing 0.9 ml of acetone pre-cooled to −20 °C. Tubes were placed on ice for 30 minutes and centrifuged at 13,000 rpm for 2.5 minutes. The pellets were redissolved in 2% SDS sample buffer at 70 °C for one hour, after which SDS-PAGE (Laemmli 1970) and autoradiography were performed. Measurement of the ^{32}P-labelling of the protein bands was performed by phosphorimaging with a Molecular Dynamics PhosphorImager SI. Background labelling was subtracted for each lane.

Results

Isolation of thylakoids

Thylakoids were isolated from pea leaves harvested before the light was switched on in the growth chamber. Isolation of chloroplasts and their lysis were performed in low light. The isolated thylakoids were incubated in darkness for several hours. We have found no difference in behavior between thylakoids incubated for 1 and 6 hours and it may be concluded that in the freshly isolated thylakoids the complete oxidation of the plastoquinone pool does not take more than 1 hour.

Fluorescence measurements

The light-do-dark and dark-to-light adaptation was followed by measurement of emission spectra at 80 K of samples withdrawn at the times indicated and frozen in liquid nitrogen. The light intensity was chosen in order to keep the depletion of PS II fluorescence in the presence of peptides in the time scale which may be followed by the experimental arrangement used. Differences in the range of up to 15% of the total fluorescence intensity were observed both for the initial values and for the kinetics of adaptation between various preparations of thylakoids.

As a result of dark-to-light adaptation, the PS II emission band of isolated thylakoid membranes becomes depleted in the chlorophyll b (Chl b) as may be seen from the loss of the 473 nm excitation component in excitation spectra of fluorescence emission 685 and 695 nm (Fig. 1). No subsequent increase in the Chl b component was observed in the excitation spectra of PS I at 735 nm (Fig. 1C) and no rise of a separate emission peak for the free antenna systems (AS) was observed (Fig. 2). The reversibility of the light-dependent depletion of Chl b absorption was also checked. The fully light-adapted thylakoids (2.5 hours after the beginning of the adaptation) were put into darkness and the fluorescence changes were followed. In about 30 minutes the fluorescence of PS II increased by about 25% but no further increase was observed in the next 18 hours (Fig. 3, control). In all the procedures, the control experiment was performed under the same conditions in absence of ATP. A decrease of up to 5% in the relative fluorescence intensity of fluorescence emission at 686 to 735 nm was observed after 2.5 hours of illumination in the absence of ATP. Incubation in darkness had no influence on the fluorescence spectrum.

Fig. 1. Superposition of fluorescence excitation spectra of dark adapted (solid line) and fully light adapted thylakoids (dashed line) at 685 (Fig. 1A), 695 (Fig. 1B) and 735 (Fig. 1C). Spectra were obtained using slit width 2.5 nm on both excitation and emission monochromators. Spectra were normalised with respect to the maximum at 435nm. The depletion of chlorophyll b component in the spectrum at 685 and 695 nm may be clearly seen from the decrease of the intensity at 473 nm. No such effect was observed in the excitation spectrum at 735 nm. The symmetrical maximum seen at 460 nm in Fig. 1A and at 466 nm in Fig. 1B are most likely second order interference artefacts arising from the use of unblocked grating monochromators.

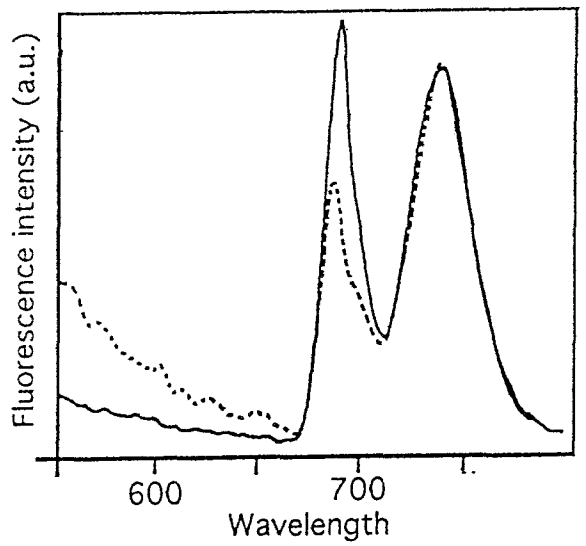

Fig. 2. Fluorescence emission spectra of dark-adapted (solid line) and light-adapted thylakoids (dashed line). Spectra were obtained using 480 nm excitation with a slit width 2.5 nm on both excitation and emission monochromators. A blue green filter blocking all light of wavelengths above 530 nm was installed in the excitation pathway in order to remove the second order interference artefacts arising from the use of unblocked grating monochromators. Spectra were normalised with respect to the maximum at 735 nm. A decrease in intensity of the fluorescence emission at both 685 and 695 nm is observed whereas no new emission peak appears.

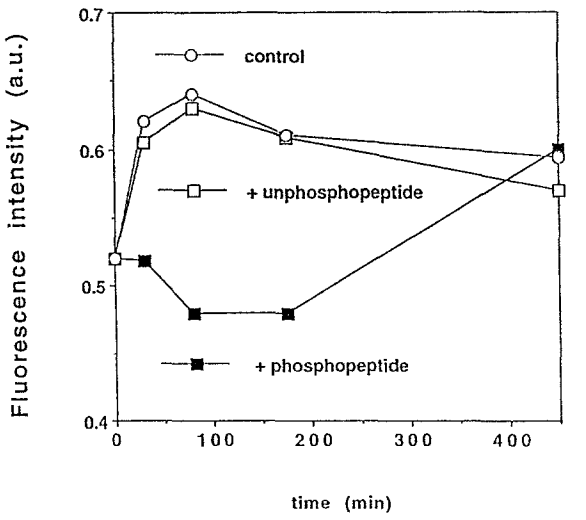

Fig. 3. Time course of dark adaptation of fully light adapted thylakoids. Value on the y axis represents the ratio of fluorescence intensity at 685 nm to 735 nm (F_{685}/F_{735}). The slowdown of the adaptation in presence of the phosphorylated peptide is apparent. The original F_{685}/F_{735} in the dark adapted thylakoids before the light adaptation was 1.2.

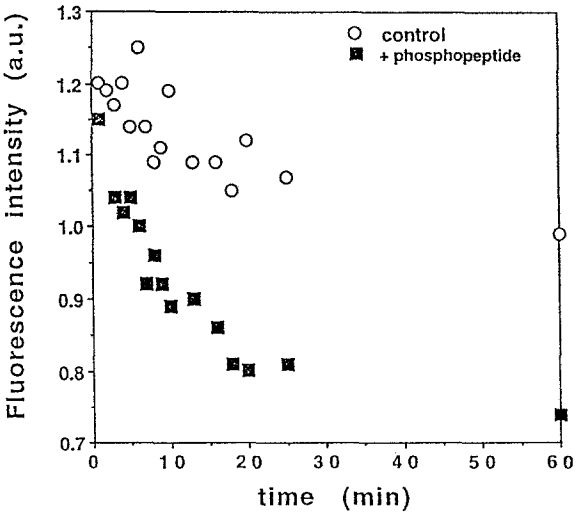

Fig. 4. Time course of light-adaptation of dark adapted thylakoids. Value on the y axis represents the ratio of fluorescence intensity at 685 nm to 735 nm (F_{685}/F_{735}). Significant acceleration of adaptation in the presence of phosphorylated peptide is observed.

In the next series of experiments we followed the influence of the phosphorylated peptide fragment of LHC II on the rate of the dark-to-light adaptation. If the main consequence of adaptation is disconnection of LHC II from PS II by its phosphorylation and subsequent binding of phosphorylated LHC II to antenna system of PS I, we would expect competition of the phosphopeptide in this process. The addition of the phosphorylated peptide fragment of LHC II leads to acceleration of the adaptation (Fig. 4) which may be explained by its competition with membrane phosphoproteins in the phosphatase reaction (Cheng et al. 1994). This explanation is supported by the fact that addition of the phosphorylated peptide markedly decreased the rate of dark adaptation of fully light-adapted thylakoids (Fig. 3). The phosphorylated peptide itself had no effect on either the emission or the excitation spectra of thylakoids at various stages of adaptation (data not shown).

In the presence of high concentrations of non-phosphorylated peptide the depletion of PS II in Chl b was not significantly accelerated (data not shown). However, major changes were observed in the emission spectrum, where a new peak at 697 nm appears (Fig. 5). A shift of the emission maximum of PS I from 735 nm to 731 nm also occurred. The new peak at 697 nm and the blue shift of PS I were not observed in the presence of phosphorylated peptide or at advanced stages of light adaptation of thylakoid membranes (not

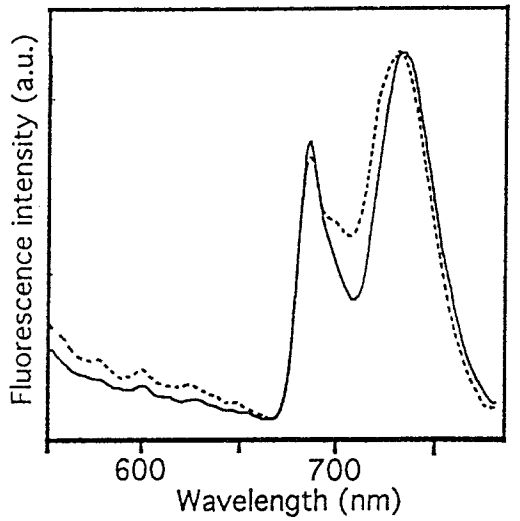

Fig. 5. Fluorescence emission spectra of light adapted thylakoids after 60 min of adaptation in presence of the non-phosphorylated peptide (dashed line) and without peptide (solid line). Spectra were obtained using 480 nm excitation with a slit width 2.5 nm both on excitation and emission monochromator. The blue green filter blocking all light on wavelengths above 530 nm was installed as in Fig. 2. Spectra were normalised with respect to the maximum at 735 nm or 731 nm. Note that a new peak appears with an apparent maximum at 697 nm.

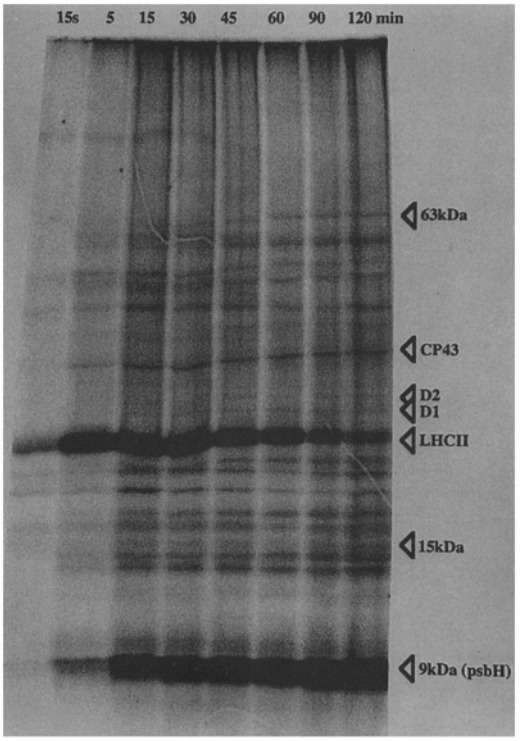

Fig. 6. Autoradiographic scan of the SDS page of thylakoids at various stages of light adaptation. Samples were taken at the time intervals shown. Arrows show positions of bands used for quantification.

shown). No detectable differences were observed in the excitation spectra of the light-adapted thylakoids in the presence or absence of the non-phosphorylated peptide (not shown). The light-to-dark adaptation was unaffected by addition of non-phosphorylated peptide (Fig. 3).

In order to check the possibility of non-specific effects caused only by the influence of high concentration of peptide (for example non-specific absorption, effects on ion transport, effects on phase separation) we measured the dark-to-light adaptation also in the presence of 2 mM and 4 mM peptide SRPLSDQEKRKQISVRGLAGVENV. This peptide represents a fragment of the phosphorylation site of glycogen phosphorylase (Chan et al. 1982). In this case neither changes in the rate of adaptation nor a change in the spectrum was observed (data not shown).

Measurement of incorporation of ^{32}P

The time course of incorporation of ^{32}P into various phosphoproteins in the thylakoid membrane was followed for 2 hours (Fig. 6). The phosphorylation of LHC II and of the 9 kDa phosphoproteins exceeds that of the other phosphoproteins by several times at various stages of the adaptation (Fig. 7). Phosphorylation of LHC II reaches its maximum in about 15 minutes whereas 9 kDa phosphoprotein is increasingly phosphorylated for at least another 1.5 hours. Detailed analysis of the times course of phosphorylation and dephosphorylation of proteins from thylakoid membranes illustrates the complexity of the phenomena. Some proteins are phosphorylated by a steadily active kinase, some proteins are phosphorylated by light-dependent kinase, some proteins are phosphorylated by both kinases and some proteins are phosphorylated only in darkness (Figs. 6 and 7), and also (L. Cheng et al., unpublished). Heterogeneity has been found also in the time course of the light-dependent phosphorylation (Fig. 6). There are proteins whose phosphorylation achieves a maximum value and then declines (e.g. LHC II), proteins the phosphorylation level of which is constant, and proteins which are increasingly phosphorylated during the time-course (e.g. 9 kDa). As a control we measured the level of phosphorylation of several other membrane phosphoproteins whose maximal level of ^{32}P incorporation was achieved within 15

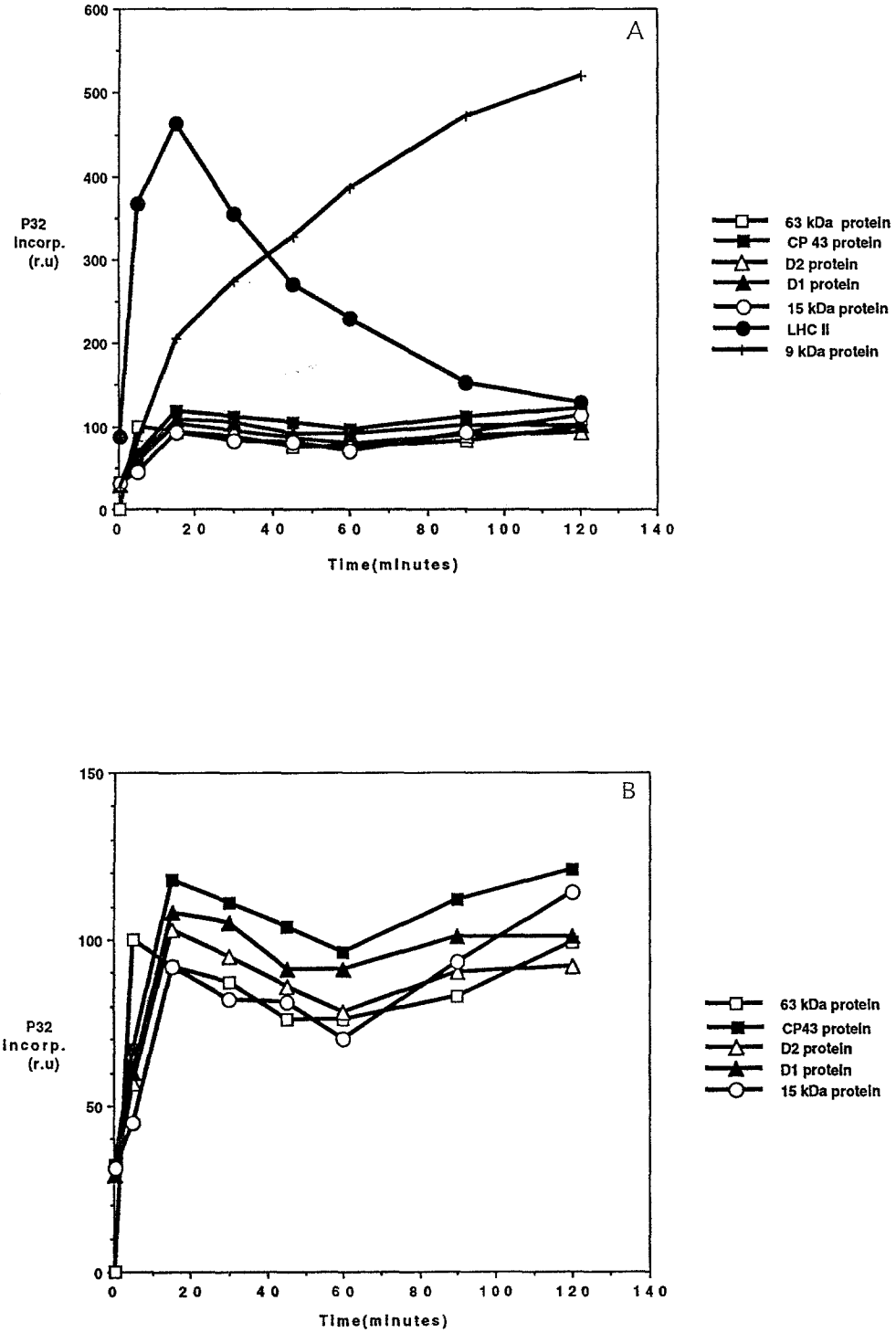

Fig. 7. Time course of ^{32}P incorporation into the thylakoid membrane proteins. The phosphorylation of LHC II at 15 minutes largely exceeds that of other phosphoproteins. The majority of phosphoproteins maintain a constant level of phosphorylation (Fig. 7B), whereas the phosphorylation of LHC II decreases with time and the phosphorylation of the 9 kDa protein increases.

minutes and did not increase further with time. In order to eliminate the possibility that we observed a kinetic difference in the phosphatase reaction when the kinase reaction becomes limited by depletion of ATP, we performed two control experiments. In the first, a fresh mixture of ATP was added to the system after 15 minutes of adaptation. In the second, after 15 minutes of adaptation membranes were spun down, a new batch of dark adapted thylakoids was added, and their phosphorylation was followed. In all cases the phosphorylation of membranes followed the same time course as seen in Fig. 7A (data not shown).

Conclusions

The dark-to-light adaptation of thylakoid membranes consists mainly of depletion of PS II in chlorophyll b (Chl b)-containing antenna systems. Such a mechanism has been proposed by (Kyle et al. 1984). The fact that this depletion is not accompanied by an increase in the corresponding antenna component of PS I or by the appearance of a new fluorescence emission peak for the free antenna system shows that there is an efficient quenching mechanism for the Chl b-containing component of the antenna systems. Such a quenching mechanism does not completely exclude the possibility of migration of minor part of the antenna to the PS I. The fact that this mechanism does not lead to immediate quenching of all the PS II fluorescence suggests that the antenna systems are no longer productively connected to PS II. In the purple bacterium *Rhodospillium rubrum*, where the energy difference between the chlorophylls of the antenna systems and the photosystem is much higher than in the case of plant PS II, about 25% of the energy transferred to the photosystem is detrapped by the antenna systems (Timpman et al. 1993). In the case of plants, the detrapping rate should be even higher. Formation of a tight complex between the reaction centre and antenna system is necessary for productive energy transfer to PS II. As the random contacts between the proteins in the membrane are very frequent (Grasberger et al. 1986), the free antenna systems must quench very efficiently in order to prevent energy transfer to PS II.

Analogues of the N-terminus of LHC II are known to be substrates for the LHC II kinase (Bennett et al. 1987; Michel and Bennett 1989) and phosphatase (Sun et al. 1993). Recent results from our group (Cheng et al. 1994) show that the phosphorylated form of the LHC II N-terminal fragment may compete efficiently with the phosphoprotein phosphatase reaction. This result is quite consistent with the influence of the phosphorylated peptide on the rate of adaptation of isolated thylakoids described here.

By analogy, the non-phosphorylated peptide should be expected to compete with the membrane-bound phosphoproteins in the reaction of the kinase. A decrease in the rate of the kinase reaction, however, has been observed only at concentrations of the peptide between 0.5 and 1.34 mM, whereas at lower peptide concentrations the kinase was apparently activated and at higher peptide concentrations the phosphorylation of both the peptide and the membrane proteins declined (L. Cheng et al., unpublished). The influence of the unphosphorylated peptide on the rate of thylakoid protein phosphorylation may be interpreted as an unspecific phenomena related to changes in the surface charge and ionic strength in the solution. This interpretation is supported by the influence of a peptide that is not recognized by the membrane bound kinase on the rate of phosphorylation of membrane proteins (White et al. 1990). In our experiments, nevertheless, we observed the appearance of the new emission peak at 697 nm only in the presence of the non-phosphorylated LHC II peptide. This spectral change suggests that the non-phosphorylated LHC II fragment specifically blocks efficient quenching of energy captured by free antenna complexes. The 77 K emission spectrum of isolated, aggregated LHC II shows two maxima at 680 and 697 nm and a broad shoulder towards longer wavelengths (Moya and Tapie 1984). A similar spectrum was observed for isolated LHC II in vesicles (Mullet and Arntzen 1980). Diluted LHC II has a sharp maximum at 680 nm. Superposition of the spectrum of the aggregated antenna on the spectrum of light-adapted thylakoids gives a spectrum equivalent to the spectrum found in our experiments (Fig. 5). In the presence of the non-phosphorylated peptide most probably the fluorescence of free aggregated LHC II is observed whereas the depletion of PS II in LHC II is not affected: the quenching mechanism has been blocked.

The specificity of influence of peptides on adaptation is clearly seen from the difference of the results in the presence of the phosphorylated, the non-phosphorylated and the glycogen-phosphorylase peptides. In a diluted solution in which ATP forms one of the negatively-charged ions, the concentration of ATP in the proximity of the membrane surface is to a large extent dependent on the charge on the surface (Cevc 1990). The membrane surface charge itself would be changed by non-specific binding of positively charged

peptides (Sackmann 1994). The experiments involving phosphorylated peptide represent a special case because the phosphorylated peptide is dephosphorylated by a membrane bound phosphatase (Cheng et al. 1995) and therefore binds to a class of binding sites on the membrane inaccessible to unphosphorylated peptides. The influence of the phosphorylated peptide on the concentration of ions in the membrane-solution interface may therefore be different from that of the other peptides (Cevc 1990). One should concentrate on qualitative changes in the spectrum rather than on the changes in the rate of adaptation.

On the basis of our results we propose a two-step mechanism for light adaptation. As a first step LHC II is liberated from its binding site at PS II by partial phosphorylation. Free, partially phosphorylated LHC II immediately forms the quenching complex with other proteins. These proteins are either constantly present in the membrane or also released from their original position by phosphorylation. Alternatively, based on our knowledge of rapid dephosphorylation of LHC II, the quenching complex may be built around the completely dephosphorylated LHC II.

The mechanism proposed here predicts that phosphorylation of LHC II will reach a maximum and then decline or stay constant at constant illumination. Among the proteins of the thylakoid membrane, LHC II reaches its maximum phosphorylation in approximately 15 minutes, and subsequently its phosphorylation declines (Figs. 6 and 7). The majority of other phosphoproteins, including known phosphoproteins CP43, D1 and D2, reach their maximum of phosphorylation within 15 minutes and then stay steadily phosphorylated (Fig. 7). In contrast, the 9 kDa phosphoprotein becomes increasingly phosphorylated for 2 hours. The decline of LHC II phosphorylation excludes the simple mechanism according to which LHC II fluorescence is quenched solely by LHC II migration outside the PS II region of the membrane, since this predicts a steady level of phosphorylation of LHC II in the final stage of adaptation. More likely a quenching complex is built in which one of the other phosphoproteins of thylakoid membranes participates. A necessary requirement for this mechanism is that either the second component of the quenching complex has higher affinity for the liberated antenna than PS II or the PS II binding site for the LHC II is modified, or both. In each case, if a single introduced phosphate group per unit of liberated LHC II is expected, the total number of phosphate groups introduced either to the second component of the quenching complex or to the modified LHC II binding protein should be equal to the total amount of phosphate groups introduced to LHC II. The total number of phosphate groups introduced to LHC II is difficult to examine as the level of its phosphorylation at any time is a result of both the kinase and the phosphatase reactions, the nature of which is in many important aspects unknown. Nevertheless, it is clear from the phosphorylation pattern seen on Figs. 6 and 7, that only the number of phosphate groups introduced to the 9kDa phosphoprotein may possibly equal the number of phosphate groups introduced to LHC II in the maximum of its phosphorylation. Thus the 9 kDa protein may be expected to form the second main component of the quenching mechanism.

The 9 kDa protein has been isolated from oxygen-evolving PS II particles (Farchaus and Dilley 1986) and it may be considered that phosphorylation of the 9 kDa phosphoprotein may be responsible for alteration of the PS II binding site for LHC II. A weak sequence similarity of the 9 kDa phosphoprotein with LHC II has been reported (Hind et al. 1986; Allen and Findlay 1986). The difficulties in isolation of 9 kDa from the light harvesting proteins when both are specifically extracted from oxygen-evolving PS II particles (Farchaus and Dilley 1986) may indicate that phospho-9 kDa protein forms a complex with LHC II and itself forms the component responsible for LHC II quenching. Analogously to protection of LHC II in its unphosphorylated form from the kinase reaction by its binding to the quenching complex, the anomalously slow dephosphorylation of the 9 kDa phosphoprotein may be a consequence of its protection from the phosphatase reaction in the quenching complex. The psbH protein may itself be a pigment binding protein (Allen and Findlay 1986) on the pathway of excitation energy transfer from LHC II to PS II reaction centre (Allen and Holmes 1986). In possible contrast to mechanisms based on formation of a single, tight complex are the results of membrane fractionation experiments (Stefánsson et al. 1994) which show that the stromal fraction in thylakoid membranes phosphorylated for 15 minutes at conditions similar to those used in this article becomes enriched in LHC II but not in PS II proteins, including the 9 kDa. No data are, nevertheless, available at present about the changes in protein composition at later stage of phosphorylation. The results described here suggest that phosphorylation of psbH initiates non-photochemical radiationless excitation decay, thereby deflecting excitation energy from the path to PS II photochemistry. Whether the site of

this decay lies in LHC II or in phospho-psbH remains to be determined.

Acknowledgements

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