Biochimica et Biophysica Acta, 722 (1983) 234–241
Elsevier Biomedical Press

BBA 41227

CHANGES IN THYLAKOID POLYPEPTIDE PHOSPHORYLATION DURING MEMBRANE BIOGENESIS IN *CHLAMYDOMONAS REINHARDII* y-1

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(Received June 28th, 1982)

Key words: Thylakoid membrane; Protein phosphorylation; Chloroplast biogenesis; (C. reinhardii)

Thylakoid polypeptide phosphorylation has been studied in vivo and in vitro during plastid differentiation in *Chlamydomonas reinhardii* y-1. Pulse labeling cells at different stages of greening with [^{32}P]orthophosphate revealed differences in the pattern of protein phosphorylation. In the early phase of greening the 44–47 kDa reaction center II polypeptides were labeled but the 22–24 kDa polypeptides of the light-harvesting chlorophyll *a*/*b*-protein complex (LHC) were not. Later in the greening, coinciding with the formation of the antenna of Photosystem I and membrane stacking, the converse was found. Furthermore, the 22–24 kDa polypeptides of grana lamellae were less labeled than the same polypeptides found in the corresponding stroma lamellae. Polypeptides in the molecular mass range of 32–34 kDa were phosphorylated at all stages following the onset of greening. Dark-grown cells did not incorporate ^{32}P in vivo or in vitro into the polypeptides present in the residual thylakoids. Similarly, cells greened in the presence of chloramphenicol, in which the synthesis of reaction centers is inhibited, showed no light-stimulated phosphorylation in vitro. However, the residual 32–34 kDa and 44–47 kDa polypeptides found in thylakoids of these cells were phosphorylated in vivo, whereas the LHC polypeptides synthesized in the presence of chloramphenicol were not. Phosphorylation of the LHC polypeptides (22–24 kDa) in these cells occurred if new reaction center polypeptides and all antennae components were formed, following removal of the inhibitor and further incubation of the cells in the light. Phosphorylation of LHC polypeptides was not resumed if active reaction centers were formed in the absence of complete restoration of all antenna components (incubation in the dark or light with addition of cycloheximide). It is concluded that phosphorylation is correlated with the thylakoid polypeptide content and organization.

Introduction

The light-harvesting chlorophyll *a*/*b*-protein complex (LHC) constitutes the main antenna of PS II [1]. Following the original observation by Bennett [2] that the apoprotein of the LHC is phosphorylated, there has been a considerable amount of interest in the possible functional significance of this phenomenon in photosynthesis. A light-dependent protein kinase [3,4] and light-independent phosphoprotein phosphatase [5] have been identified in isolated thylakoids from chloroplasts of higher plants. These activities phosphorylated and dephosphorylated the LHC in vitro [2,5]. Further in vitro studies have revealed that a correlation exists between phosphorylation of the LHC

Abbreviations: LHC, light-harvesting chlorophyll *a*/*b*-protein complex; CP, chlorophyll-protein complex; LDS, lithium dodecyl sulphate; PS, photosystem.

and uncoupler-insensitive fluorescence quenching, analogous to a State 1–State 2 transition in vivo [6–8]. More recently, measurement of cytochrome *f* photoreduction in thylakoids prephosphorylated in vitro has led to an alternative interpretation of the physiological role of thylakoid polypeptide phosphorylation, namely, in modulating the interaction between LHC and reaction center II [9].

We have previously shown that chloroplasts of *Chlamydomonas reinhardii* contain several phosphoproteins in addition to the LHC, which appear to be associated with PS II [10]. Phosphorylation was found to be light independent in vivo, and the pattern of phosphorylation was drastically altered by the absence of the nonphosphorylated 44–47 kDa reaction center II polypeptides which form the apoproteins of CP III and CP IV [10,11]. It was of interest, therefore, to extend the study of the phosphorylation phenomenon to developing thylakoids in order to see whether the pattern of phosphorylation might be correlated with the changes in membrane organization which occur during plastid differentiation. A mutant of *C. reinhardii*, designated y-1, was used in this study. Chlorophyll synthesis in this strain is obligatorily dependent upon light due to a single nuclear point mutation [12]. Consequently, heterotrophic growth in the dark results in an almost complete loss of chloroplast photosynthetic membranes [13]. The assembly of the photosynthetic membranes during regreening can be resolved as a multistep process, involving 70 S and 80 S ribosome translation products [12,14]. The normal developmental process can be specifically altered by addition of the 70 S translation inhibitor chloramphenicol, resulting in cells containing thylakoids which do not have photochemical activity. These cells can be subsequently repaired in the light, dark or in the light in the presence of cycloheximide, giving rise to different photosynthetic phenotypes [12,14]. Cells repaired under the first condition exhibit essentially normal photosynthetic behavior; however, while photosynthetic activity is restored in cells repaired under the other two conditions, they have an abnormally high light requirement to saturate photochemistry [14]. In the present study, phosphorylation of thylakoid membrane proteins has been investigated in vivo and in vitro during normal and perturbed development.

Materials and Methods

Cultivation of cells and greening protocol

C. reinhardii y-1 cells were propagated in the dark at 25°C for four to five generations in a semicontinuous cell-culturing apparatus with acetate as a carbon source [13]. The general protocol for the greening and repair experiments has been detailed elsewhere [13,14]. Cells were greened under nondividing conditions at 25°C ($2 \cdot 10^7$ cells/ml) in growth medium under cool fluorescent light ($1.6 \cdot 10^4$ erg $\cdot$ cm$^{-2} \cdot$ s^{-1}). Chloramphenicol, when required, was added directly to the cell suspension (200 μg/ml). Cycloheximide was added from a stock solution, to give a final concentration of 2 μg/ml.

Phosphorylation of thylakoid membrane proteins in vivo and in vitro

At specific times in the greening process or after repair under various conditions, following 6 h of greening in chloramphenicol, samples were taken and phosphorylated in vivo or in vitro as described previously [10]. Cells were pelleted, washed and resuspended in Tris-buffered medium (20 mM Tris-HCl, pH 7.4), containing 10 μM orthophosphate. [^{32}P]Orthophosphate (Nuclear Research Center, Beer Sheva) was added to give a final specific radioactivity of 0.5 Ci/mmol, and cells were incubated for 30 min, with continuous agitation, in the same light regime used for the greening. Labeling was carried out in the absence of any translation inhibitors. Cells were disrupted by sonication in ice (Branson sonifier setting 5, three 15-s periods with intervals of 15 s), and thylakoids were prepared by centrifugation of the homogenate in a discontinuous sucrose gradient (15, 30, 60%, w/v) in Tris-HCl (50 mM, pH 7.2) at 80 000 $\times$ *g* for 90 min. Thylakoids for the in vitro assay were prepared by the same centrifugation procedure but the cells were broken by French pressure cell treatment (4000 lb/inch^2). Isolated thylakoids were incubated for 15 min at 25°C in the light, in the presence of [γ-^{32}P]ATP (0.15 Ci/mmol) (Amersham International Ltd.). The reaction was terminated by centrifugation in an Eppendorf microcentrifuge and the membranes were subsequently resuspended in electrophoresis sample mixture [10].

Thylakoid polypeptides were fractionated by LDS-polyacrylamide gel electrophoresis according to the methodology of Chua [15]. Gels were stained with Coomassie brilliant blue R, destained, dried and exposed to X-ray film (Agfa-Gevaert Curix RP2) with an enhancement screen (Agfa-Gevaert MR400). The concentration of protein was determined according to the method of Lowry et al. [16], using bovine serum albumin as standard, and chlorophyll concentration was measured by the procedure of Arnon [17].

Measurements of fluorescence emission at room temperature and at liquid nitrogen temperature, and photosynthetic oxygen evolution

Fluorescence induction measurements were performed at room temperature in an apparatus previously described [18]. Excitation was provided by a Tungsten halogen light source passing through a 496 nm filter (Corning, transmitting between 380 and 600 nm), to give a photon flux of $4.4 \cdot 10^3$ erg $\cdot$ cm$^{-2} \cdot$ s^{-1} incident on the sample cuvette. The photomultiplier was protected by a 685 nm interference filter (Baird Atomic half-bandwidth 40 nm). Diuron (0.01 mM) was added after 30 s of preincubation in the dark. Fluorescence emission spectra of samples in liquid nitrogen were recorded in an Aminco-Chance dual-wavelength recording spectrophotometer adapted for this kind of measurement, as described by Gershoni and Ohad [19]. Oxygen evolution in saturating light was measured polarographically using a Clark-type oxygen electrode.

Preparation of grana and stroma lamellae

Subfractionation of thylakoids into grana and stroma lamellae was accomplished by digitonin treatment [20]. Large thylakoid vesicles were prepared by the procedure of De Petrocellis et al. [21], with the modifications previously described [10]. Benzamidine (2 mM) and a phosphatase inhibitor, sodium molybdate (20 mM), were included in the buffer used in the subfractionation.

Results

The process of greening of the y-1 mutant in the presence or absence of chloramphenicol, with subseuqnet repair in the light or dark or light plus cycloheximide, has been extensively studied [12,14]. Table I details the photosynthetic parameters which were measured in the present study in order to characterize the various stages of development at which the cells were pulse labeled with [^{32}P]orthophosphate. The fluorescence index $(F_m - F_s)/F_o$ can be used as a measure of the presence of rate-limiting steps, beyond PS II, in photosynthetic electron transfer [14]. It has been shown previously that in photosynthetic cells which evolve oxygen in saturating light but have defective antennae, the value of this index was less than 0.3 [14]. As may also be seen in Table I, cells grown in the dark for four to five generations contain only a small amount of chlorophyll, and a lag phase of about 2 h is observed in chlorophyll synthesis. The onset of oxygen evolution in saturating light precedes the rise of the fluorescence index above zero and approaches a plateau after 8 h of greening. At this stage of the greening, the fluorescence emission spectrum at liquid nitrogen temperature is essentially the same as for light-grown cells (Fig. 1). As previously shown, the appearance of a significant peak at 714 nm corresponding to the antenna

TABLE I

CHANGES IN THE PHOTOSYNTHETIC CHARACTERISTICS OF y-1 CELLS DURING THE GREENING PROCESS

CAP, chloramphenicol; CHI, cycloheximide.

Time of greening (h)	Chloro phyll concen-tration (μg/10^7 cells	O_2 evolution (μmol/h per 10^7 cells)	$(F_m - F_s)/F_o$
0	0.8	0	0
2	1.8	1.1	0
4	3.0	4.4	0.2
8	9.0	5.3	1.1
6 plus CAP	4.2	0	0
6 plus CAP ↓ 8 light	8.0	1.97	0.6
6 plus CAP ↓ 8 dark	4.2	0.86	0.23
6 plus CAP ↓ 8 CHI light	4.3	1.34	0.2

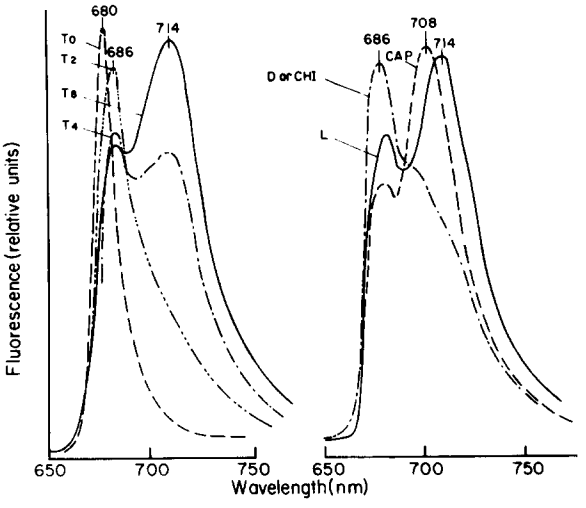

Fig. 1. Uncorrected low-temperature fluorescence emission spectra of y-1 cells during greening (left panel), and following greening for 6 h in chloramphenicol (CAP) with subsequent repair in the light (L), dark (D), or light plus cycloheximide (CHI) (right panel).

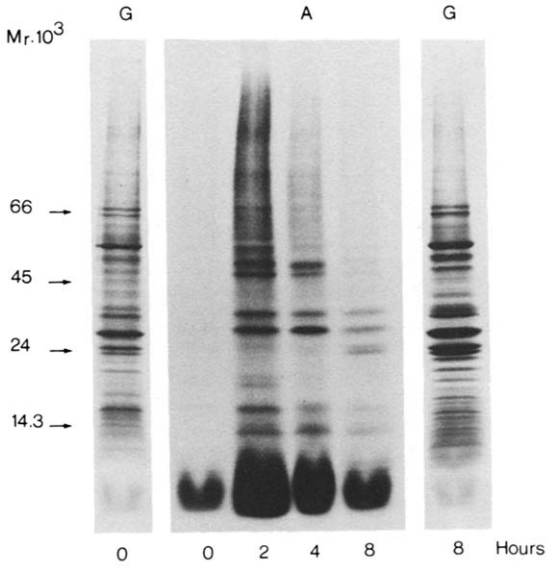

Fig. 2. Changes in the pattern of thylakoid polypeptide phosphorylation during thylakoid biogenesis in *C. reinhardii* y-1. Samples of cells were taken from a greening culture at various times (0, 2, 4 and 8 h) and were pulse labeled with [^{32}P]orthophosphate. G, gel; A, autoradiograph.

of PS I constitutes a late event in the greening process [22,23]. Chlorophyll synthesis is not inhibited in cells greened in chloramphenicol, and a fluorescence emission peak at 686 nm, corresponding to the LHC, is observed at liquid nitrogen temperature. In addition, there is a major peak at 708 nm which is thought to arise from a disorganized PS I antenna [14,24] (Fig. 1). Essentially, chlorophyll is not synthesized in cells repaired in the dark or in the light plus cycloheximide, although oxygen evolution is restored (Table I). However, a significant change in the low-temperature fluorescence emission spectrum takes place where the 708 nm maximum shifts to a longer wavelenght.

In vivo phosphorylation of thylakoid polypeptides during greening

Dark grown cells were pulse labeled with [^{32}P]orthophosphate in the dark. There was no incorporation of label into the polypeptides of the residual thylakoids found in these cells (Fig. 2). The same result was obtained if dark-grown cells were pulse labeled in the light (data not shown).

Within a few hours, however, following exposure to continuous light, high levels of phosphorylation were observed. The pattern of incorporation differed markedly from that observed in fully greened cells (Fig. 2). Specifically, the reaction center II polypeptides in the molecular mass range 44–47 kDa were labeled, and the lower molecular mass components of the LHC (22–24 kDa) were not. Late in the greening process all the components of the LHC were found to be phosphorylated, and at this stage the CP III and CP IV apoproteins were no longer significantly labeled. Polypeptides in the 32–34 kDa region were phosphorylated in cells at all stages following the onset of greening.

A late event in plastid development is the morphological differentiation of the thylakoid membrane into grana and stroma [13]. Fully greened y-1 cells were pulse labeled with [^{32}P]orthophosphate, and thylakoids were prepared and subfractionated into grana and stroma lamellae. The grana (10 000 × *g* pellet) fraction was enriched in PS II components (reaction center II, LHC and 32–34 kDa polypeptides). The P-700 apoprotein and α- and β-subunits of the coupling factor were more abundant in the stroma fraction (160 000 × *g* pellet), the 50 000 × *g* pellet comprising membrane fragments of intermediary composition (Fig. 3).

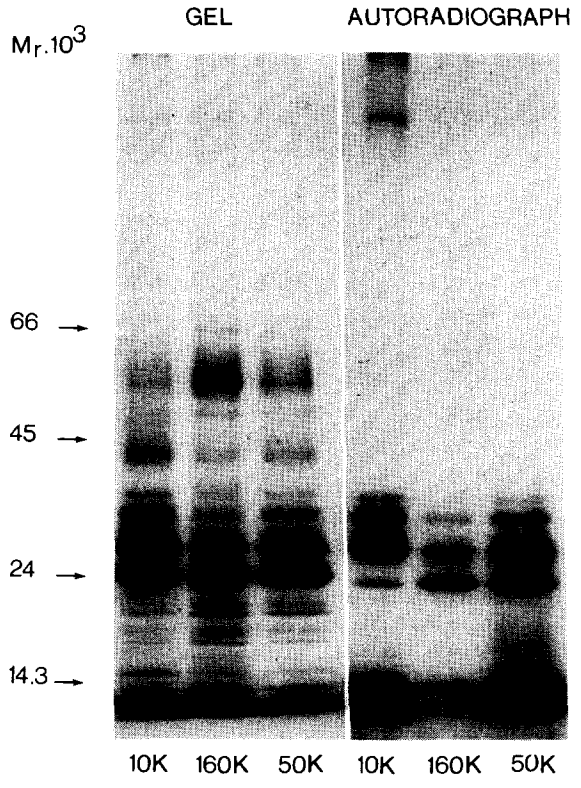

Fig. 3. A comparison of the phosphorylation of grana and stroma lamellae. Light-grown y-1 cells were pulse labeled with [^{32}P]orthophosphate and subfractions were prepared from the purified thylakoids. The chlorophyll *a/b* ratio of the three subfractions were as follows: 10K, 1.6; 50K, 2.0; and 160K, 2.4.

As might be expected, therefore, the grana were more phosphorylated than the stroma. However, it is interesting to note that there was a considerable difference in the incorporation of phosphate into the 22–24 kDa polypeptides of the LHC between the subfractions. In the grana lamellae the incorporation of phosphate into these polypeptides was considerably lower than the amount of protein present, as visualized by Coomassie brilliant blue staining.

In vitro phosphorylation of thylakoid polypeptides during greening

In agreement with the data obtained in vivo, no light-stimulated phosphorylation of the LHC or 32–34 kDa polypeptides in thylakoids isolated from dark-grown cells was observed, compared with membranes prepared from cells late in the greening process (Fig. 4). The phosphorylation of a minor polypeptide with an estimated molecular mass of 40 kDa also occurred in the dark (not shown). It is interesting, however, that the labeling of this band in vitro, which might correspond to the γ-subunit of the coupling factor [25], decreased during the greening. Thylakoids obtained from cells early in the greening process, despite being photochemically active (Table I), showed very little light-stimulated phosphorylation in vitro of the LHC and 32–34 kDa polypeptides (Fig. 4). Furthermore, in complete contrast to the results obtained in vivo, no phosphorylation of the reaction center II polypeptides (44–45 kDa) was observed in vitro. Similarly, cells greened in the presence of chloramphenicol and then repaired in the dark exhibited very low activity in vitro as compared with cells repaired in the light (Fig. 5).

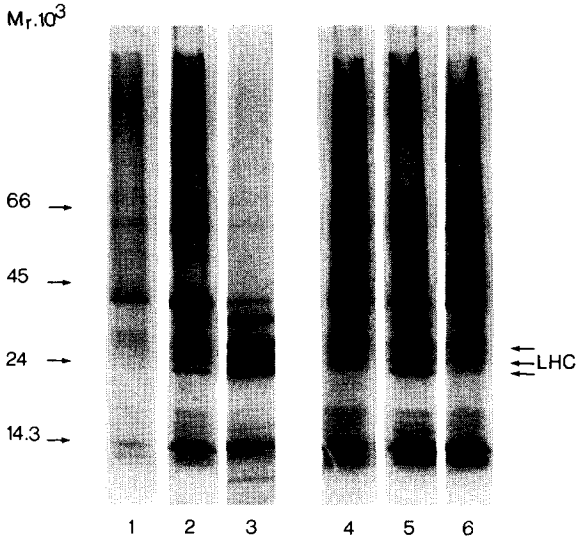

Fig. 4. Phosphorylation in vitro of thylakoids from y-1 cells during normal and perturbed chloroplast development. (1) 0 h, (2) 2 h, (3) 8 h, (4) 6 h plus chloramphenicol, (5) 8 h light repair, (6) 8 h dark repair.

Thylakoid polypeptide phosphorylation during perturbed development

Pulse labeling of cells greened in the presence of chloramphenicol revealed that the 22–24 kDa polypeptides of the LHC, which were synthesized in the presence of the drug, were not phosphorylated. Conversely, the residual amount of 44–47, 32–34 and 26 kDa polypeptides, whose synthesis

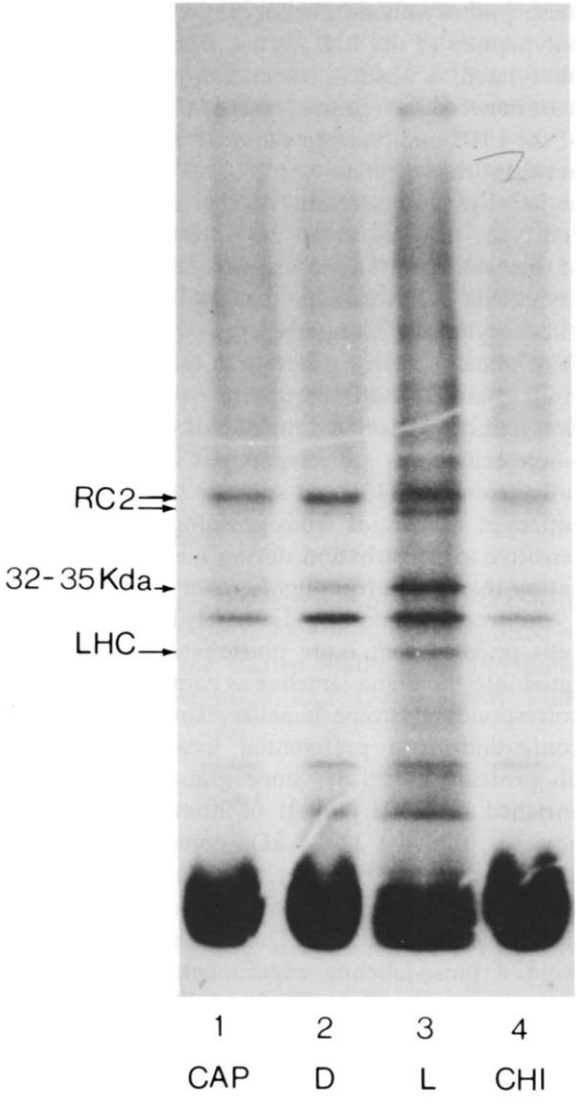

Fig. 5. Autoradiograph showing the pattern of phosphorylation in cells greened in chloramphenicol for 6 h (1), and subsequently repaired for 8 h in the dark (2), light (3), or light plus cycloheximide (4). The pulse labeling was performed in the absence of any translation inhibitors. The double arrow indicates reaction center II (RC2) polypeptides of 44–47 kDa, and the single arrow denotes the LHC polypeptides 22–24 kDa.

is sensitive to chloramphenicol, were labeled in these cells (Fig. 5). The inhibitor was washed out of a population of unlabeled cells greened in the presence of chloramphenicol and the cells were divided into three aliquots. The samples were exposed to light, dark, or light plus cycloheximide for 8 h, then pulse labeled with [^{32}P]orthophosphate. As shown in Fig. 3, significant labeling of the 22–24 kDa component of the LHC only occurred in the cells repaired in the light. However, the 44–47 kDa polypeptides were also phosphorylated in these cells.

It has been demonstrated that protein phosphorylation is stimulated by light in vitro via PS II. Consequently, as expected, thylakoids prepared from cells greened in chloramphenicol gave results analogous to the photochemically inactive membranes from dark-grown cells (Fig. 4). Again, however, a discrepancy was evident between the results obtained with intact cells and with isolated thylakoids.

Discussion

It has been previously shown that in fully developed thylakoids of *C. reinhardii* there are a number of phosphoproteins which appear to be exclusively associated with PS II [10]. In pulse-labeling experiments of light-grown y-1 cells, the 44–47 kDa reaction center II polypeptides are not phosphorylated. However, the results presented here demonstrate that in the early phase of thylakoid development, these polypeptides are labeled. Various studies have shown that PS II units are functionally separated in the early phase of greening [26–29], and it is possible that phosphorylation of these reaction center II components at this stage of development might provide a structural basis for this separation.

Dark-grown *Chlamydomonas* y-1 cells did not incorporate phosphate in vivo or in vitro into the residual thylakoid polypeptides present in these cells. It is possible that the protein kinase is not synthesized in dark-grown cells, thus accounting for this observation.

In isolated thylakoids from light-grown cells, the protein kinase is obligatorily dependent upon the reduction of plastoquinone by PS II [30,31]. This might explain why the protein kinase, if present, is inactive in vitro in residual thylakoids from dark-grown cells. The same reasoning applies to chloroplast membranes from cells greened in chloramphenicol. In this case, the finding of protein phosphorylation in vivo in these cells indicates that synthesis of the protein kinase is not sensitive

to chloramphenicol, and supports the contention that linear electron flow is not a necessary condition for protein kinase activity in vivo [10]. Alternatively, the protein kinase could have been present in the residual thylakoid of the dark-grown cells but in an inactive state. In vivo, the protein kinase is active in dark-adapted, light-grown cells, probably due to an alternative pathway of plastoquinone reduction in the dark [10,32]. It has been shown that there are significant amounts of all components of the electron-transfer chain in dark-grown cells [13,33]. In the case of cytochrome *f*, this amounts to 40% of the total quantity found in green cells. Nevertheless, all of this cytochrome was found in a reduced state and could not be photooxidized until the cells were illuminated [33]. It is thus possible that some rearrangement of the electron-transfer chain takes place, which requires a small amount of chlorophyll synthesis before any redox reactions can occur, leading to activation of the protein kinase in vivo. Such a situation would argue in favor of a unique membrane-bound protein kinase activity in vivo, coupled in some way to the electron-transfer chain, as opposed to additional soluble protein kinases which might be expected to be active in dark-grown cells.

Another interesting feature of the pattern of phosphorylation during plastid differentiation concerns the LHC. In a previous study it was found that the LHC was not phosphorylated when the 44–47 kDa polypeptides were missing due to mutation in a temperature-sensitive T_{44} mutant [10]. The small amount of the 22–24 kDa polypeptides of the LHC present early in the greening process was not phosphorylated in vivo. It might be speculated that, as in the case of the T_{44} mutant, the LHC is spatially disconnected from PS II and is not available to the protein kinase [34–36]. Phosphorylation of the 22–24 kDa polypeptides, together with the almost complete loss of phosphorylation of the 44–47 kDa polypeptides, occurred late in the greening, and coincided with the formation of the antenna of PS I [22,23].

Light-saturation studies of PS II photochemistry and PS II-dependent photophosphorylation have shown that the antenna in cells from the early phase of greening [18,37] or following repair in the dark, or light plus cycloheximide [14], is poorly connected to the reaction center. In all these photosynthetic phenotypes, the 22–24 kDa polypeptide of the LHC is not significantly phosphorylated. A positive correlation might therefore exist between the relative levels of phosphorylation of the LHC and reaction center II, and interaction between the reaction center and antenna. The higher light requirement of the aforementioned cell types to bring about an equivalent reduction of the electron-transfer chain to that of normal thylakoids might explain the low-light-stimulated phoshorylation manifested in vitro in such thylakoids. A puzzling feature of the in vitro assay is the completely different pattern of phosphorylation found with isolated thylakoids compared with whole cells. Such a discrepancy is not found with fully green cells [10], and suggests that the organization of thylakoids from greening cells is more sensitive to perturbation during membrane purification than those from light-grown cells.

Subfractionation of pulse-labeled, fully greened cells revealed that more phosphate was incorporated into the grana lamellae as compared with the corresponding stroma lamellae. This supports the contention of a preferential location of phosphoproteins in PS II, since grana lamellae are enriched in PS II [20]. It is interesting to note, however, that the 22–24 kDa components of the LHC were less labeled in the grana than in the stroma, despite the predominance of the LHC in the former fraction [38]. This result was obtained from a pulse-labeling experiment, and the low phosphorylation of the 22–24 kDa LHC polypeptides in the grana might be explained in two ways: either a part of these polypeptides is not available to the protein kinase or there is a lower turnover rate of the esterified phosphate on these polypeptides in the grana. If the former supposition is correct, then some involvement in membrane stacking might be envisaged. The requirement of the LHC for membrane stacking in vitro has been shown [39,40], and models for grana formation involving the interaction of cations with fixed negative changes have been proposed [41,42]. Furthermore, the negative surface charge density of grana lamellae is lower than that of stroma lamellae [43]. The lower phosphorylation of these components of the LHC in grana may contribute to this lateral charge heterogeneity.

In summary, differences in the pattern of

thylakoid polypeptide phosphorylation have been observed in vivo during plastid differentiation. The changes can be considered to reflect alterations in membrane topography such that different sites are exposed to the protein kinase and/or phosphatase, depending upon the organization of the membrane. It would appear that the antenna of PS I is a major factor in bringing about these topographical changes. It may be tentatively concluded, therefore, that altered charge distribution on membrane polypeptides, resulting from the absence of specific nonphosphorylated components, might play an active role in determining the structural association between antennae and reaction centers in the PS II unit.

Acknowledgments

G.C. Owens is the recipient of a NATO/SERC Overseas Studentship. This work was supported by a grant from the United States-Israel Binational Agricultural Research and Development Fund (BARD).

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