APPLICATION OF PHOSPHOTHREONINE ANTIBODIES TO ANALYSIS OF THYLAKOID PHOSPHOPROTEINS*

II Core Proteins Shows Different Irradiance-dependent Regulation in Vivo

APPLICATION OF PHOSPHOTHREONINE ANTIBODIES TO ANALYSIS OF THYLAKOID PHOSPHOPROTEINS*

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An immunological approach using a polyclonal phosphothreonine antibody is introduced for the analysis of thylakoid protein phosphorylation in vivo. Virtually the same photosystem II (PSII) core phosphoproteins (D1, D2, CP43, and the psbH gene product) and the light-harvesting chlorophyll a/b complex II (LHCII) phosphopolypeptides (LHCBL1 and LHCBL2), as earlier identified by radiolabeling experiments, were recognized in both pumpkin and spinach leaves. Notably, the PSII core proteins and LHCII polypeptides were found to have a different phosphorylation pattern in vivo with respect to increasing irradiance. Phosphorylation of the PSII core proteins in leaf discs attained the saturation level at the growth light intensity, and this level was also maintained at high irradiances. Maximal phosphorylation of LHCII polypeptides only occurred at low light intensities, far below the growth irradiance, and then drastically decreased at higher irradiances. These observations are at variance with traditional studies in vitro, where LHCII shows a light-dependent increase in phosphorylation, which is maintained even at high irradiances. Only a slow restoration of the phosphorylation capacity for LHCII polypeptides at the low light conditions occurred in vivo after the high light-induced inactivation. Furthermore, if thylakoid membranes were isolated from the high light-inactivated leaves, no restoration of LHCII phosphorylation took place in vitro. However, both the high light-induced inactivation and low light-induced restoration of LHCII phosphorylation seen in vivo could be mimicked in isolated thylakoid membranes by incubating with reduced and oxidized dithiothreitol, respectively. We propose that stromal components are involved in the regulation of LHCII phosphorylation in vivo, and inhibition of LHCII phosphorylation under increasing irradiance results from reduction of the thiol groups in the LHCII kinase.

Photosystem (PS)1 II is a multiprotein complex of the thyla-

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1 The abbreviations used are: PS, photosystem; DTT, dithiothreitol; LHCII, light-harvesting chlorophyll a/b complex of PSII; Thr(P), phosphothreonine; PFD, photon flux density; P-, phosphorylated form.

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thylakoid membranes, but is not so easily applicable to in vivo phosphorylation studies of leaves due to the uneven uptake of the radioactive label into higher plants. Furthermore, radioactive labeling does not give an accurate value of the total phosphorylation level due to phosphorylation pre-existing to the labeling experiment. Here, we introduce a new analytical tool to study the reversible phosphorylation of thylakoid proteins in vivo by using a phosphothreonine (Thr(P)) antibody, which is shown to give a specific immunological reaction with all the major thylakoid phosphoproteins. Taking advantage of this new analytical tool, we demonstrate that phosphorylation of PSII core proteins and LHCCI polypeptides in vivo is differentially regulated in response to increasing light intensities. The results will be discussed in terms of possible mechanisms for differential regulation of thylakoid protein phosphorylation including the involvement of the thiol-redox state.

EXPERIMENTAL PROCEDURES

Plant Material and Growth Conditions—Pumpkin (Cucurbita pepo L.) plants were grown in a greenhouse at a photon flux density (PFD) of 200 μmol of photons m⁻² s⁻¹ 16-h light/8-h dark rhythm at 25 °C. Hybrid cultures of spinach (Spinacia oleracea L.) were grown in a greenhouse at a PFD of 400 μmol of photons m⁻² s⁻¹ 10-h light/14-h dark rhythm at 25 °C. Fully expanded leaves were used in the experiments.

Light Treatments of the Leaf Discs—Leaf discs (diameter 2.7 cm), punched from dark-adapted, fully expanded leaves and floating on distilled water in a Petri dish, were illuminated in a growth chamber under various PFDs at 25 °C. A metal-halide lamp HQI-T 250 W/daylight served as a light source. For analysis of thylakoid phosphoproteins, the leaf discs were rapidly frozen in liquid nitrogen and stored at −80 °C until isolation of thylakoid membranes. Thylakoid membranes were isolated as described by Rintamäki et al. (21), resuspended in a small amount of storage buffer consisting of 10 mM Hepes-NaOH, pH 7.5, 100 mM sucrose, 5 mM NaCl, 10 mM NaF, and 10 mM MgCl₂, and frozen in liquid nitrogen.

Detection of Thylakoid Phosphoproteins by PolyonalThr(P) Antibody—Thylakoids were solubilized in the presence of 6 M urea and polyepitides separated by SDS-polyacrylamide gel electrophoresis (23) using 15% (w/v) acrylamide gels with 6% urea. Routinely, 0.5–1.0 μg of chlorophyll was loaded in each well. The polyepitides were transferred to an Immobilon-P membrane (Millipore), which was blocked with 1% bovine serum albumin (fatty acid-free, Sigma). Phosphoproteins were immunodetected using an Immuno-Lite Assay Kit (Bio-Rad). Three different commercial antibodies to phosphoproteins were tested: rabbit polyclonal antibody and monoclonal antibody (Zymed Laboratories Inc.) and mouse monoclonal anti-phosphothreonine (Sigma). Thylakoid phosphoproteins (Fig. 2 A, B, C) were identified with polyclonal D1 (21), D2 (24), CP43 (a generous gift from Dr. R. Barbato), and LHCB2 (a generous gift from Dr. S. Jansson) specific antibodies.

RESULTS

Immunological Detection of Thylakoid Phosphoproteins with Phosphothreonine Antibodies—The cross-reaction of thylakoid phosphoproteins was tested with three different commercial Thr(P) antibodies. The best cross-reaction with several thylakoid phosphoproteins, from both spinach and pumpkin leaves, was obtained with a rabbit polyclonal antibody (Zymed Laboratories Inc.) (Fig. 1). The main phosphothreonine bands were identified as phosphorylated CP43, D2 protein, D1 protein, and LHCB2 protein by using the specific antibodies (Fig. 1B). Phospho-LHCB1 was identified as the most abundant polypeptide of LHCCI in Coomassie Brilliant Blue-stained gels (Fig. 1B). Two smaller polypeptides of 20 and 10 kDa were also detected after longer exposure times of the x-ray films (Fig. 1A), the latter probably representing the phosphorylated psbH gene product. These phosphoproteins are the same thylakoid proteins, which generally become phosphorylated in ³²P-labeling experiments (see, e.g., Ref. 20).

All the main PSI core phosphoproteins and LHCCI proteins, are known to be phosphorylated at an N-terminal threonine residue (5). However, the immunological cross-reactivity with the various phosphoproteins was different, with the strongest signal obtained for the D1 protein (Fig. 1C). These differences in signal intensity can be explained by different affinity of the antibody to epitopes flanking the phosphothreonine. Nevertheless, all the phosphoproteins gave a linear response with increasing amount of samples applied on the gel (Fig. 1C), indicating that the antibody can be used for relative quantification of these phosphoproteins.

The suitability of the Thr(P) antibody for quantitative analysis of thylakoid phosphoproteins was further tested by monitoring the phosphorylation level of the D1 protein under different light intensities, both with the Thr(P) antibody and the D1-specific antibody, the latter recognizing both the phosphorylated and nonphosphorylated D1 forms (see Ref. 21). A similar phosphorylation pattern was obtained for D1 protein phosphorylation with both antibodies (Fig. 2, A and C).

Steady-state Phosphorylation of LHCCI and PSII Core Proteins at Different Irradiances—Discs punched from dark-adapted mature leaves of pumpkin and spinach plants were subjected to different irradiances for 3 h (pumpkin) or for 1 h (spinach) and the relative amounts of phosphorylated CP43, D2, D1, LHCB1, and LHCB2 in the isolated thylakoid membrane were analyzed with the Thr(P) antibody (Fig. 2). Accumulation of the D1 (Fig. 2A, B, and C) and the other PSII core phosphoproteins (Fig. 2C) occurred with increasing irradiance up to the growth light level and maximal phosphorylation level was maintained also at higher irradiances. In sharp contrast to the PSI core protein phosphorylation, a strong accumulation of P-LHCCI occurred only at the relatively low light intensity; around 100 μmol of photons m⁻² s⁻¹ (Fig. 2, A and B). At increasing light intensities, a reduction of LHCCI phosphorylation was observed, commencing well below the growth conditions. Only residual phosphorylation of LHCCI occurred at high irradiances (Fig. 2, A and B). Both main LHCCI phosphoproteins, P-LHCB1 and P-LHCB2, showed the same transient phosphorylation behavior under increasing irradiance (data not shown). In conclusion, a pronounced phosphorylation of LHCCI occurs only under low light intensities, whereas phosphorylation of the PSI core proteins prevails under moderate and high light intensities.

High Light-induced Reduction in LHCCI Phosphorylation—The steady-state level of P-LHCCI dropped drastically at increasing irradiances, especially at PFDs exceeding 500 μmol of photons m⁻² s⁻¹ (Fig. 2). Kinetic studies on the accumulation of P-LHCCI at high irradiance (Fig. 3, HL), with the first samples withdrawn already after 2.5 min of illumination, did not reveal even transient accumulation of P-LHCCI. In a comparative experiment at optimum irradiance for LHCCI phosphoryl-
The maximum accumulation of P-LHCII was achieved within 30 min with no reduction in the phosphorylation level during prolonged illumination.

In theory, the lack of P-LHCII accumulation at high light could be caused by an efficient LHCII dephosphorylation activity induced by high light. Therefore, the in vitro dephosphorylation rate of P-LHCII was determined both in darkness and at high light. Accumulation of P-LHCII was first induced by incubating the leaves at low light, and subsequently the loss of P-LHCII was recorded after shifting the leaves either to high light or to darkness. The rate constant for P-LHCII dephosphorylation was 2 times higher in high light (0.020 min$^{-1}$) than in darkness (0.011 min$^{-1}$) (Table I). Nevertheless, even in high light, the half-life of P-LHCII was as long as 34 min (Table I).

The dephosphorylation rate of P-LHCII at low light in vivo cannot be estimated with Thr(P) antibody because of strong LHCII phosphorylation activity. However, the dephosphorylation rate of P-LHCII in vitro in phosphorylated thylakoids was very similar at both low and high light, the rates being very close to that estimated in vivo at high light (Table I).

In light of the above results, it can be concluded that the low level of LHCII phosphorylation at high irradiances cannot be explained by enhanced dephosphorylation activity but is mainly due to a reduced phosphorylation activity. Kinetics for restoration of the LHCII phosphorylation activity at low light, after the high light-induced inactivation, is presented in Fig. 4. Maximal phosphorylation of LHCII was first induced by illumination of leaves at 30 μmol of photons m$^{-2}$ s$^{-1}$ and subse-
quently the leaves were transferred to 1000 μmol of photons m$^{-2}$ s$^{-1}$, which leads to dephosphorylation of P-LHCII. The leaves were then transferred to low light conditions, where restoration of LHCII phosphorylation activity was followed. The restoration of the phosphorylation activity of LHCII at low light, after the high light inactivation, took several hours (Fig. 4), a considerably longer time than that required to attain the same phosphorylation stage in dark-adapted leaves (Fig. 4).

This suggests, that LHCII phosphorylation becomes reversibly inactivated at high irradiances and that restoration occurs slowly at low light (Fig. 4) or dark conditions (data not shown).

To obtain further insight into the mechanism of high light-induced inactivation/low light-induced reactivation of the LHCII phosphorylation, we performed in vitro phosphorylation...
The experiments described in Figs. 4 and 5 indicate that the high light inactivation of LHCBII phosphorylation was reversible in vivo (Fig. 4) but not in vitro (Fig. 5B), suggesting that stromal compounds may be involved in the inactivation/reactivation of LHCBII phosphorylation. To test whether the thiol-redox state is involved in the high light-induced inactivation of LHCBII phosphorylation (see Ref. 26), we treated thylakoid membranes isolated from dark-adapted leaves (Fig. 5B, lanes Hl 30' and Dark 30' in immunoblot).

The experiments described in Figs. 4 and 5 indicate that the high light inactivation of LHCBII phosphorylation was reversible in vivo (Fig. 4) but not in vitro (Fig. 5B), suggesting that stromal compounds may be involved in the inactivation/reactivation of LHCBII phosphorylation. To test whether the thiol-redox state is involved in the high light-induced inactivation of LHCBII phosphorylation (see Ref. 26), we treated thylakoid membranes isolated from high light-illuminated leaves with reduced and oxidized DTT. The preincubation with thiol reagents was performed in darkness before phosphorylation in vitro. The preincubation with oxidized DTT readily reactivated the LHCBII phosphorylation activity of thylakoid membranes isolated from high light-illuminated leaves (Fig. 5B). Reduced DTT, on the other hand, had a completely opposite effect, since it totally prevented the phosphorylation of LHCBII in vitro (Fig. 5B). Contrary to the LHCBII polypeptides, the thiol reagents had no significant effect on the phosphorylation of the PSII core proteins in vitro (data not shown).

**DISCUSSION**

Thylakoid protein phosphorylation plays a key role in the regulation of the light-harvesting process, as well as the turnover of thylakoid proteins under varying environmental conditions. Studies of protein phosphorylation have been mainly conducted by labeling experiments using 32P, which, when applied to higher plants in vivo, on one hand meets penetration problems and on the other hand always fails to detect the
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endogenous phosphorylation level of a protein at a given condition prevailing in the beginning of the experiment. The phosphothreonine antibody, introduced in the present study, overcomes such problems. This antibody recognizes the same PSII and LHCII phosphoproteins of the thylakoid membrane, which can be labeled with radioactive phosphorus, and gives a linear response with increasing sample concentration in immunodetection (Fig. 1). Accordingly, this immunooassay also has an important advantage compared with radioactive labeling, in revealing the endogenous amounts of phosphoproteins. It is worth noting that even a prolonged incubation in darkness does not always result in complete dephosphorylation of PSII phosphoproteins, particularly in stressed leaves (data not shown).

By using the Thr(P) antibody, we could explicitly demonstrate that the PSII core proteins and LHCII polypeptides have an opposite phosphorylation behavior in response to increasing light intensities in vivo. PSII core proteins are less phosphorylated at low light where the phosphorylation of LHCII polypeptides is at its maximum. Conversely, the phosphorylation of PSII core proteins attains the maximal level at the light intensity, where phosphorylation of LHCII polypeptides already is reduced to a very low level (Fig. 2).

Phosphorylation of LHCBl and LHCB2 polypeptides induces the migration of the outer LHCII antenna from the apressed to stroma-exposed thylakoid membrane region, thereby reducing the light-harvesting capacity of PSII (1, 16, 27). According to our present studies (Fig. 2), the lateral migration should only be occurring at low light intensity, where the state transitions typically occur. Therefore, organizational rearrangements of the PSII antenna, based on LHCII phosphorylation, should not account for protection against photoinhibition at high light where dephosphorylation, rather than phosphorylation of LHCII occurs (Fig. 3, Table I).

Our results on differential light regulation of PSII core and LHCII protein phosphorylation give weight to the concept of kinase heterogeneity (1–3). Phosphorylation of both the PSII core proteins and the LHCII polypeptides is redox-mediated via the activation of the kinase(s) (3, 9, 28, 29). It has been reported, however, that the light-dependent phosphorylation of LHCII polypeptides can be prevented by inhibiting the electron transport at the site of cytochrome b/f complex, either by specific inhibitors or by using cytochrome b/f-deficient mutants (9, 10, 30), whereas the phosphorylation of PSII core proteins is not affected under such experiments (9).

The distinct loss of P-LHCII at irradiances exceeding the growth light level can mainly be attributed to the inactivation of LHCII kinase. Although light, as compared with darkness, enhanced the dephosphorylation rate of P-LHCII, the intensity of the light seemed to be of minor importance (Table I). A decrease in the phosphorylation level of LHCII polypeptides has also been observed during photoinhibitory illumination of Chlamydomonas reinhardtii in vivo (31), and in isolated chloroplasts at moderate and high irradiances (32). In both cases the plastoquinone pool remained reduced (31, 32), as also observed for pumpkin leaves under high light conditions.5 In light of these results, the redox activation of the LHCII kinase, via occupation of the Qo site of cytochrome b/f complex by plastoquinol (see Refs. 10 and 11), is likely to be functional also at high irradiances. A direct involvement of thylakoid membrane energization (high ΔpH) in the reduction of LHCII phosphorylation can also be excluded by the experiment presented in Fig. 4; the restoration of LHCII phosphorylation capacity at low light in vivo after the high light-induced inactivation was slow, whereas the relaxation of ΔpH is known to occur rapidly (33). Furthermore, the high light-induced inactivation of LHCII phosphorylation did not occur in isolated thylakoid membranes (Fig. 5A), indicating that some stromal components, rather than a membrane compound, are involved in this high light-induced inactivation process. Therefore, a superimposed regulatory mechanism, distinct from the activation process of the LHCII kinase, seems to become functional in declining the phosphorylation level of LHCII at increasing light intensities.

In the in vitro phosphorylation assays conducted in the presence of thiol reagents (Fig. 5B) suggested the involvement of thiol groups in the high light-induced inactivation of LHCII phosphorylation. We have also recently shown that LHCII phosphorylation in isolated thylakoids is very sensitive to small changes in the equilibrium ratio between oxidized and reduced thiol reductants, so that only a small increase in the reduction level causes strong inhibition of LHCII phosphorylation.3 LHCII phosphorylation has earlier been observed to become modulated by thiol-directed reagents such as N-ethylmaleimide, which could be prevented by preincubation of thylakoid membranes with ATP and ADP before the addition of thiol-directed reagents (26). Furthermore, similar modulation in the phosphorylation activity of thylakoid membranes was also observed when histones were used as a substrate (26).

Taken together, these results suggest that the LHCII kinase is the target of this modulation by thiol reagents rather than a modifications at the substrate level. Based on these observations, we propose that the LHCII kinase has thiol groups in the high light-induced inactivation of the enzyme. Light, despite controlling the redox state of thylakoid electron carriers, also exerts a strict regulation on the thiol redox state. The ferredoxin/thioredoxin system is a general mechanism of light-dependent enzyme regulation in chloroplasts (see Ref. 34). Reduction of thioredoxin is generally saturated at relatively low light intensities in vivo (35), in analogy with the inactivation of LHCII phosphorylation (Fig. 2). We therefore suggest that reduced thioredoxin may be involved in the regulation of LHCII phosphorylation.

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