Light Affects the Accessibility of the Thylakoid Light Harvesting Complex II (LHCII) Phosphorylation Site to the Membrane Protein Kinase(s)†

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ABSTRACT: Redox-controlled, reversible phosphorylation of the thylakoid light harvesting complex II (LHCII) regulates its association with photosystems (PS) I or II and thus, energy distribution between the two photosystems (state transition). Illumination of solubilized LHCII enhances exposure of the phosphorylation site at its N-terminal domain to protein kinase(s) and tryptic cleavage in vitro [Zer et al. (1999) *Proc. Natl. Acad. Sci. U.S.A. 96*, 8277−8282]. Here we report that short illumination (5−10 min, 15−30 μmol m^{-2} s^{-1}) enhances the accessibility of LHCII phosphorylation site to kinase(s) activity also in isolated thylakoids. However, prolonged illumination or higher light intensities (30 min, 80−800 μmol m^{-2} s^{-1}) prevent phosphorylation of LHCII in the isolated membranes as well as in vivo, although redox-dependent protein kinase activity persists in the illuminated thylakoids toward exogenous solubilized LHCII. This phenomenon, ascribed to light-induced inaccessibility of the phosphorylation site to the protein kinase-(s), affects in a similar way the accessibility of thylakoid LHCII N-terminal domain to tryptic cleavage. The illumination effect is not redox related, decreases linearly with temperature from 25 to 5 °C and may be ascribed to light-induced conformational changes in the complex causing lateral aggregation of dephosphorylated LHCII bound to and/or dissociated from PSII. The later state occurs under conditions allowing turnover of the phospho-LHCII phosphate. The light-induced inaccessibility of LHCII to the membrane-bound protein kinase reverses readily in darkness only if induced under LHCII-phosphate turnover conditions. Thus, phosphorylation prevents irreversible light-induced conformational changes in LHCII allowing lateral migration of the complex and the related state transition process.

Photosystem II (PSII)[1] is subject to photoinactivation due to light-induced oxidative damage (*1*−*3*). Reversible phosphorylation of the PSII light-harvesting complex II (LHCII) (*4*−*8*) by thylakoid bound protein kinase(s) (*9*, *10*) regulates the association of LHCII with PSI or PSII allowing energy transfer from the antenna to the photochemical reaction centers I or II. This process (state transition) balances the rate of the two photosystems excitation. Phosphorylation of LHCII is considered to induce its dissociation from PSII. Recent results indicate that the association of phospho-LHCII with PSI involves docking of the complex via the PsaH-subunit of PSI (*11*). At this stage, it is not known which among the reported thylakoid-associated protein kinases (*8*) are redox controlled and involved in the phosphorylation of LHCII. Reduced plastoquinone and the cytochrome *bf* complex play a major role in the signal transduction path involved in the activation of the thylakoid protein kinase(s) (*12*−*18*). Activation of the protein kinase(s) and LHCII phosphorylation occur already at low light intensities when only a fraction of the cytochrome *bf* population is reduced. Phosphorylation of LHCII reaches a plateau with increasing light intensity and reduction of the plastoquinone pool in isolated thylakoids. However, LHCII phosphorylation is drastically lowered in leaves exposed to light intensities close to or above those that saturate electron flow (*19*). This phenomenon is not yet well understood at the mechanistic or physiological levels.

So far, the redox regulation of the thylakoid protein kinase-(s) is considered as the major mechanism controlling the phosphorylation of the PSII chl-proteins (*6*, *8*, *20*, *21*). We have recently demonstrated that illumination of isolated LHCII induces reversible exposure of its phosphorylation site at the N-terminal domain to protein kinase(s) in an in vitro reconstituted system (*22*). A similar situation occurs

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[1] Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propansulfonate; chl, chlorophyll; CP43, CP47, chlorophyll *a* binding proteins forming the PSII-core internal antennae; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethyl-urea; LHCII, light harvesting complex II; NPQ, nonphotochemical quenching; PMSF, phenazine methosulfate; P-LHCII, phosphorylated LHCII; PSI, PSII, photosystem I and II, respectively; PVDF, polvinylidene difluoride; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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in isolated PSII-core complexes exposing the phosphorylation site(s) of CP43 to protein kinase(s) activity associated with the complex (*23*). The discovery of the light-induced activation of chlorophyll-proteins phosphorylation at the substrate level in vitro raises the question whether this phenomenon occurs in situ and if so, what is its physiological significance.

In this work, we have explored the light-dependent activation of LHCII phosphorylation at the substrate level in isolated thylakoids as well as in detached pea leaves. The results corroborate that short illumination of thylakoids induces a transient increase in the exposure of the N-terminal domain of LHCII, thus enhancing its phosphorylation. However, surprisingly, prolonged illumination induces further changes in the thylakoid membrane preventing the accessibility of the LHCII phosphorylation site to the protein kinase. This phenomenon may contribute to the observed lowering of LHCII phosphorylation by high light intensities seen under physiological conditions (*21*, *24*).

MATERIALS AND METHODS

Plants (*Pisum sativum*) were grown for 10−12 days on vermiculite at 22 °C with a 12/12 h light/dark period. For experiments in vivo, leaves were detached and placed in Petri dishes over three layers of Whatman 3 paper soaked in water. The leaves were covered with a single layer of thin lens-cleaning paper (Kodak) to maintain humidity and punctured in a few places with a 16-gauge needle. This procedure allowed fast penetration of DCMU (5−10 min) when added to inhibit the light-dependent reduction of the plastoquinone pool in the leaves and thus prevent the activation of the protein kinase(s). The activity of PSII in the DCMU treated leaves and whole chain electron flow as well as nonphotochemical quenching (NPQ) and LHCII phosphorylation activity in absence of DCMU were maintained in the punctured leaves for at least 10 h as assayed by fluorescence kinetics measurements. Similar results were obtained if the punctured pea leaves were floated on water. However, the handling of the leaves set on the filter paper was more convenient when exposing the leaves to different light treatments. Pea leaves were harvested at the end of the dark period before the onset of illumination of the plants to ensure a low basal phosphorylation level of PSII proteins. Thylakoids were prepared according to ref *22* and LHCII was isolated as previously described (*25*) and stored at −70 °C at a concentration of 4 mg of chl mL^{-1} in 0.5% nonyl-β-D-glucoside (Sigma).

In some experiments, isolated LHCII solubilized in 0.5% nonyl-β-D-glucoside was added as a phosphorylation substrate to the thylakoid membrane suspension. In this case, the final concentration of the detergent was 0.025% w/v. Attempts to rapidly separate the mixture into isolated LHCII and thylakoid membranes by sucrose density centrifugation so as to estimate accurately their phosphorylation level were not successful. Thus, the contribution of the exogenous added LHCII to the total LHCII phosphorylation in the mixture was calculated by subtraction of the phosphorylation level of a sample containing only thylakoid membranes from that of a sample containing both, membranes and added, isolated LHCII.

Phosphorylation Assay. To determine the maximal potential level of thylakoid LHCII phosphorylation the assay (100 μL) contained 50 mM Tris-HCl pH 8.0, 10 mM NaCl, 10 mM $MgCl_2$, 10 mM NaF, 0.2 mM ATP without or with addition of 5 μCi ^{32}P-γ-ATP and thylakoid membranes (25 μg of chlorophyll). When indicated, isolated exogenous LHCII (25 μg of chl) was added to the thylakoid membranes. In this case, the phosphorylation of both the thylakoid as well as the exogenous added LHCII in the mixture was carried out by the thylakoid bound protein kinase(s) that required redox-activation, achieved by illumination or addition of 1 mM duroquinol in darkness (*26*). Phosphorylation of isolated LHCII (total 25 μg of chlorophyll) in 100 μL of the phosphorylation mixture as above was carried out by addition of a solubilized membrane extract enriched in protein kinase(s) activity obtained from spinach thylakoids (5 μg of protein) (*22*). The solubilized protein kinase(s) was constitutively active, and thus there was no need to add duroquinol to the assay (*22*, *23*). All incubations were carried out in Eppendorf tubes at 25 °C. The phosphorylation of thylakoid bound or isolated LHCII reached a plateau in about 10−15 min irrespective of whether the membrane-bound kinase(s) activation was induced by illumination or in darkness by addition of duroquinol (not shown) as well as when using the solubilized protein kinase(s) preparation (*22*). In all phosphorylation assays, the incubation time was extended to 20 min to ensure that all the LHCII available sites were phosphorylated. Phosphorylation was terminated by addition of denaturing sample buffer and the sample proteins were resolved by SDS−PAGE (*27*). The extent of phosphorylation when using ^{32}P-γ-ATP was assayed by autoradiography and quantified by scanning of autoradiograms exposed for times compatible with a linear response of the film. The LHCII phosphorylation level was demonstrated to be detectable by phosphothreonine antibodies (*19*). Thus, LHCII phosphorylation using nonradioactive ATP was measured by immunodecoration with phosphothreonine specific antiserum (Cell Signaling Technology Inc.) (*19*) of SDS−PAGE resolved polypeptides transferred to PVDF membranes.

Light treatment. Thylakoid membranes or isolated LHCII (250 μg of chl mL^{-1}) suspended in 50 mM Tris-HCl pH 8.0, 10 mM NaCl, and 10 mM $MgCl_2$ with or without addition of ATP and NaF, as indicated in the figures legends, were illuminated with stirring by magnetic stirrers in glass tubes (8-mm diameter) at 25 °C. Illumination was provided by a projector equipped with a tungsten−halogen lamp. When indicated, the light was filtered through interference filters (644 nm, 42% transmission, and 430 nm, 55% transmission, 20 nm half bandwidth). The above filters were used despite their relative broad transmission bands to obtain sufficient light to elicit the changes in the accessibility of LHCII to the protein kinase. Light intensity measured with a Radiometer (Li-Cor, Inc., USA) was adjusted to equal incident photon fluxes at the sample level by changing the projector distance. In some experiments, the protein kinase(s) activation was inhibited by addition of 10 μM DCMU to prevent the reduction of the membrane electron carriers by PSII activity. The effect of thylakoid preillumination on the subsequent phosphorylation in darkness was similar in experiments in which DCMU was present or absent if LHCII phosphorylation was prevented during the light exposure by omitting addition of ATP.

Detached leaves set on wet filter paper in Petri dishes as described above were incubated in darkness or illuminated

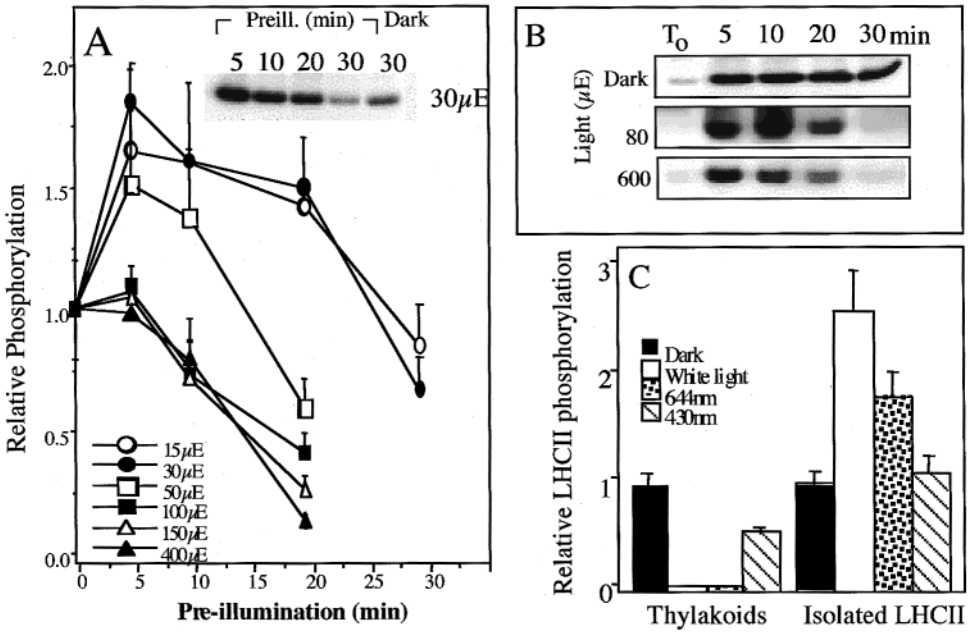

FIGURE 1: Effect of preillumination on thylakoid and isolated LHCII phosphorylation. Thylakoids (panels A and B) were incubated in darkness or preilluminated in the presence of DCMU and NaF for times and light intensities as indicated and then phosphorylated in darkness for 20 min following addition of duroquinol and ^{32}P-γ-ATP (panel A) or nonradioactive ATP (panel B); panel C, isolated LHCII or thylakoids (250 μg of chl mL^{-1}) were preilluminated for 20 min with white light or light filtered through interference filters (430 or 644 nm) as indicated in the figure insert (the light intensity at the sample level was in all cases 80 μmol of photons m^{-2} s^{-1}) and then phosphorylated in darkness for 20 min following addition of ^{32}P-γ-ATP and NaF; duroquinol was added to the thylakoids sample to activate the membrane protein kinase(s); phosphorylation of the isolated LHCII was carried out using a solubilized protein kinase(s) as described in Methods; the extent of LHCII phosphorylation detected in SDS−PAGE resolved polypeptides was measured by scanning of autoradiograms (panels A and C) or by immunodecoration with phosphothreonine antibodies (panel B); insert in panel A, radiogram; the level of phosphorylated LHCII of thylakoids prior to all incubations is indicated as T_o (panel B).

by a tungsten−halogen lamp providing 800 μmol of photons m^{-2} s^{-1} at the leaf surface and were protected from heating by a double-walled glass filter maintained at 10 °C by cool water circulation. The dishes were placed in a water bath maintained at 25 °C. The temperature of the light-exposed leaves did not exceed 30 °C under the above conditions (*28*).

Tryptic Digestion. Thylakoids equivalent to 25 μg of chl were suspended in a final volume of 100 μL of 50 mM Tris-HCl pH 8.0, containing 5 mM $MgCl_2$ and 10 mM NaCl. The suspension was incubated for 5 min in the light (30 μmol of photon m^{-2} s^{-1}) with addition of 10 μM DCMU or in darkness to prevent the redox mediated protein kinase(s) activation. Trypsin (0.1 μg mL^{-1}) (Sigma, Type XIII, bovine pancreas) was added and incubation was continued in darkness for 10 min at 25 °C. Proteolysis was terminated by addition of 0.4 μg mL^{-1} soybean trypsin inhibitor (Sigma).

RESULTS

Effect of Preillumination on the Accessibility of LHCII to Protein Kinase(s). Since illumination increases the exposure of the N-terminal domain of isolated LHCII to the protein kinases in vitro (*22*), the question arises whether illumination of thylakoids will have the same effect on the phosphorylation of LHCII in situ. To distinguish between the effect of illumination of the LHCII complex from that of the redox-dependent activation of the protein kinase, the following rather complex experimental protocol had to be used. Thylakoids were first exposed to light of various intensities (preillumination) for different times in the presence of DCMU, NaF, and without addition of ATP, to prevent LHCII phosphorylation. The membranes were then phosphorylated in darkness for 20 min using ^{32}P-γ-ATP and duroquinol. The latter was added to activate the thylakoid protein kinase(s). The phosphorylation level of a thylakoid sample that was not preilluminated served as a control. The results of such an experiment are shown in Figure 1 panel A. The extent of LHCII phosphorylation in the thylakoids illuminated prior to the phosphorylation assay increased as compared to the dark control in accordance with the results obtained using isolated LHCII in vitro (*22*). Maximal LHCII substrate activation occurred during short preillumination and could be detected only in thylakoids preilluminated at light intensities not exceeding 100 μmol of photons m^{-2} s^{-1}. Following exposure to higher light intensities and/or for longer times, the substrate activation relative to the nonilluminated control thylakoids decreased and finally LHCII phosphorylation became strongly inhibited. The inhibitory effect was practically saturated at light intensities of 100−150 μmol of photons m^{-2} s^{-1} (Figure 1A). To avoid the possible effect of light-induced increase in the activity of thylakoid LHCII phosphatase, both preillumination and the subsequent phosphorylation in darkness were carried out in the presence of NaF. Thus, the final phosphorylation level depended only on the extent of accessible LHCII phosphorylation sites and kinase(s) activity.

We have previously reported that PSII proteins may be partially phosphorylated in leaves taken from dark-adapted plants prior to the isolation of thylakoids and phosphorylation assay (*23*). To ascertain that the above results are not due to changes in the phosphorylation of a minor nonphosphorylated fraction of LHCII that does not represent the entire LHCII population, a similar experiment was performed using

nonlabeledATP and detecting the phosphorylation level by immunodecoration with phosphothreonine antibodies. Thylakoids were preilluminated in the presence of DCMU and NaF with light intensities and for times as indicated (Figure 1B). The availability of phosphorylation sites was subsequently assayed in darkness following addition of ATP, and duroquinol. The results obtained using the phosphothreonine immunodecoration method show that the initial level of LHCII phosphorylation in the thylakoids used in this experiment was almost not detectable (Figure 1B, lane T_o). Phosphorylation of these thylakoids (carried out in darkness for 20 min) after preincubation of the membranes in darkness for up to 30 min resulted in an equal degree of LHCII phosphorylation in all samples (Figure 1B, lanes marked "dark"). Preillumination with 80 μm photons m^{-2} s^{-1} for up to 10 min increased considerably the phosphorylation level as compared to the dark control, followed by complete loss of LHCII phosphorylation after 30 min of illumination. Preillumination of the thylakoids with 600 μmol of photons m^{-2} s^{-1} lowered the LHCII phosphorylation level already after 10 min. These results show that the preillumination affects the entire thylakoid LHCII population.

As opposed to the above results, illumination of isolated LHCII in vitro prior to phosphorylation in darkness induces only an increase in the exposure of the complex N-terminal domain to phosphorylation. This substrate activation is saturated at about 200 μmol of photons m^{-2} s^{-1} (*22*). LHCII encoded by the *lhcb1,2* genes and forming the mobile chl *a*/*b* complex binds seven molecules of chl *a* and five molecules of chl *b* (*29*). Since thylakoids contain besides LHCII the core complexes of PSII and PSI which bind only chl *a*, it was of interest to compare the effect of preillumination by light absorbed by chl *a* and *b*, on the subsequent phosphorylation of isolated and membrane-bound LHCII. Thylakoids and isolated LHCII were exposed to 80 μmol of photons m^{-2} s^{-1} incident white light or light of similar intensity transmitted by interference filters of 430 and 644 nm absorbed by chl *a* and *b*, respectively. Illumination was continued for 20 min, an exposure time that is in the linear range of the illumination effect as shown in Figure 1A. The samples were then phosphorylated in darkness for 20 min. Results of such experiments (Figure 1C) are expressed as increase or loss of LHCII phosphorylation relative to the dark control and normalized to the light absorbed by the samples at the wavelengths transmitted by the above filters. The results indicate that the light-induced changes affecting the increase in the exposure of the isolated LHCII N-terminal domain to protein kinase(s) or preventing phosphorylation of membrane-bound LHCII can be ascribed to absorption by both chl *a* and *b*. However, light absorbed by chl *b* seems to be more effective than that absorbed by chl *a* (Figure 1C).

The LHCII Phosphorylation Activity Persists in the Preilluminated Thylakoids. The transition from light activation to the unexpected light-induced inactivation of LHCII phosphorylation in situ presented above could be due to (a) light-induced down-regulation of the thylakoid protein kinase(s); (b) changes in the LHCII organization in the membrane after the light-induced exposure of the N-terminal domain of the complex. To distinguish between these possibilities, thylakoids were preilluminated with addition of DCMU in absence of ATP for 10 and 20 min, then transferred to darkness, and phosphorylated for 20 min following addition of ^{32}P-γ-ATP, NaF, and duroquinol. As a control, thylakoids were also preincubated in darkness prior to the phosphorylation assay. Exogenous, isolated LHCII was added to part of the thylakoids only during the phosphorylation assay in darkness (Figure 2, +/−exo.LHCII, lanes 1, 2 and 5, 6). To a second part of samples, no exogenous LHCII was added (Figure 2, lanes 3, 4 and 7, 8). Preillumination for 20 min induced complete loss of LHCII phosphorylation in the sample containing only thylakoid membranes (Figure 2, lane 4). As opposed to that, LHCII phosphorylation occurred in the preilluminated thylakoid sample to which exogenous isolated LHCII was added during the phosphorylation assay in darkness (Figure 2, lane 2). These results demonstrate that the membrane-bound protein kinase(s) retained at least part of its activity during the preillumination of the thylakoid membranes. Thus, the complete loss of LHCII phosphorylation following preillumination of thylakoids under conditions preventing the phosphorylation of LHCII may be ascribed to a large extent to the light-induced inaccessibility of membrane LHCII phosphorylation site to the protein kinase. The final phosphorylation level in the sample containing thylakoids preincubated in darkness did not increase when exogenous isolated LHCII was added during the phosphorylation assay (Figure 2, lanes 5, 6 compare with lanes 7, 8). This can be due to competition by the endogenous membrane-bound LHCII that has not been affected by illumination and is a more accessible substrate to the membrane-bound protein kinase(s).

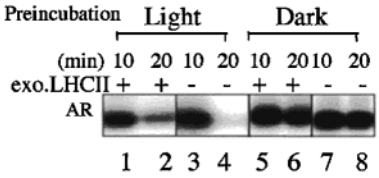

FIGURE 2: Effect of preillumination on the protein kinase(s) activity. Thylakoids were preincubated in the light (80 μmol of photons m^{-2} s^{-1}, lanes 1−4) or darkness (lanes 5−8) for times as indicated followed by phosphorylation with ^{32}P-γ-ATP in darkness for 20 min in the presence of duroquinol and NaF. The phosphorylation assay in darkness was carried out with addition of isolated exogenous LHCII (exo.LHCII, 250 μg mL^{-1}) (lanes 1, 2 and 5, 6) or without (lanes 3, 4 and 7, 8); the radiogram (AR) shows the LHCII region of SDS−PAGE resolved thylakoid proteins; note that LHCII is still phosphorylated in the sample containing exo.LHCII and illuminated for 20 min (lane 2) but not in the sample containing only thylakoid membranes (lane 4).

Effect of Preillumination on the Accessibility of the Thylakoid-Bound LHCII. N-Terminal Domain to Trypsin. The results presented above indicate that illumination of LHCII in situ, under conditions that prevent its phosphorylation induces changes in the organization of the membrane complexes that prevent subsequent access of the protein kinase(s) to the LHCII phosphorylation site.

It has been reported that tryptic digestion of isolated or membrane-bound LHCII results in the cleavage of a fragment of about 1.5 kDa at the N-terminal domain containing also the phosphothreonine site (*30*). We have shown previously that illumination of isolated LHCII exposes the N-terminal domain not only to protein kinase(s) but also to cleavage by trypsin causing complete loss of the LHCII phosphorylated domain (*22*). Since illumination affects the thylakoid LHCII exposure to the protein kinase(s), similar effects may be

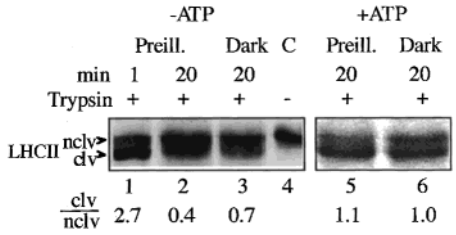

FIGURE 3: Effect of thylakoids preillumination in the presence or absence of ATP on the exposure of LHCII N-terminal domain to tryptic cleavage. Thylakoids were preilluminated (30 μmol of photons m^{-2} s^{-1}) for 1 min (lane 1), or 20 min (lanes 2 and 5) in the presence of DCMU or incubated in darkness for 20 min (lanes 3, 4, and 6). ATP and NaF were added to samples 5 and 6. Duroquinol was added to sample 6 to allow activation of the protein kinase(s) and LHCII phosphorylation in darkness; the membranes were then incubated for 15 min in the presence (+) or absence (−) of trypsin in darkness as well; proteolysis was terminated by addition of trypsin inhibitor followed by SDS−PAGE and staining; C, control, (lane 4), trypsin nontreated thylakoids. Only the LHCII region is shown. Note the appearance of a lower stained band derived from the noncleaved complex (LHCII nclv.) by proteolysis of LHCII N-terminal domain (LHCII clv.). The ratio of the relative densities of the cleaved/noncleaved LHCII (clv./nclv.) following the various treatments was calculated by scanning the stained gel.

expected to occur also at the level of its accessibility to trypsin in situ.

In interpreting the results of the experiments presented below, one should take into consideration that trypsin diffusion to the intermembrane space within the grana domains may be hindered and thus the LHCII complexes close to the grana margins may be more readily accessible to proteolysis as compared to LHCII located inside the grana stacks. Furthermore, proteolysis may induce unstacking and thus a progressive increase in the accessibility of LHCII located inside the grana region to the enzyme. In the experiments described below, proteolysis was carried out in darkness after preillumination of the membranes and we have used low trypsin concentrations to slow the changes induced by cleavage of the LHCII N-terminal domain in the grana stacking and minimize changes induced in the organization of LHCII in the membrane plane that are nonrelated to the illumination. Despite these precautions, we consider the results as semiquantitative and only indicative of the illumination effect.

To test the effect of illumination on the cleavage of LHCII by trypsin, thylakoids were incubated in the light for 1 or 20 min (Figure 3 lanes 1 and 2) or in darkness for 20 min (Figure 3, lane 3) in the presence of NaF without addition of ATP. The thylakoids were then exposed to mild tryptic proteolysis for 15 min and the polypeptide pattern was resolved by SDS−PAGE. As a control, a sample of thylakoids that was not exposed to trypsin was included in the electrophoretic run (Figure 3, lane 4, C). The degree of light-induced changes in the exposure of the LHCII N-terminal domain to trypsin could be estimated by measuring the ratio of the cleaved (clv) LHCII to the noncleaved complex (nclv) as shown in Figure 3, left panel. The ratio of cleaved LHCII to the noncleaved complex was 2.7 after 1 min of preillumination but only 0.4 to 0.7 after 20 min preillumination or dark incubation (Figure 3, left panel, lane 1, compare to lanes 2, 3). These results indicate that short preillumination increases the exposure of the N-terminal domain of membrane-bound LHCII to trypsin. As opposed to that, prolonged illumination in absence of ATP, that prevents the subsequent LHCII N-terminal domain exposure to the protein kinase-(s), reduces the amount of the trypsin cleaved LHCII. The fact that short illumination increases the proteolysis while prolonged illumination has an opposite effect indicates that the increase in the LHCII exposure to trypsin following short illumination is not due to light-induced unstacking.

The question arises whether phosphorylation of LHCII may affect the light-induced accessibility of the LHCII N-terminal domain to trypsin. To test this possibility, thylakoids were preilluminated or incubated in darkness with addition of duroquinol to activate the protein kinase. ATP was added to both samples and NaF was included to inhibit phosphatase activity. Following the incubation, tryptic proteolysis was performed in darkness for 15 min as above, followed by SDS−PAGE resolution of the thylakoid polypeptides. The results of this experiment show that proteolysis of LHCII was enhanced in the samples that were phosphorylated in the light or in darkness as compared to similar sample incubated in absence of ATP (Figure 3, lanes 5 and 6, ratio LHCII cleaved/noncleaved, 1.1 and 1.0, respectively, as compared to lanes 2 and 3, ratio LHCII cleaved/noncleaved, 0.4 and 0.7, respectively). These results indicate that, like short illumination, phosphorylation that induces dissociation of LHCII from PSII (*6*), may also unfold the N-terminal domain of LHCII. However, unlike the nonphosphorylated complex, the phosphorylated LHCII is not subject to light-induced inaccessibility of the site to the soluble trypsin. Phosphorylation of LHCII induces state transition, i.e., dissociation from PSII, thus allowing its interaction with PSI (state 2, 11). This is due to migration of P-LHCII to the stroma membrane domains (6). The possibility that upon LHCII phosphorylation partial grana unstacking may occur is still a controversial issue (*31*). The fact that under these conditions the N-terminal domain of LHCII is accessible to trypsin cleavage indicates however that phosphorylation prevents the light-induced changes in the folding of the LHCII N-terminal that protects it from the enzyme access. Taken together, the results presented in this section support the conclusion that preillumination of the thylakoids in absence of phosphorylation activity induces a transient increase followed by inaccessibility of the complex to the membrane-bound protein kinase(s).

Effect of Temperature on the Light-Induced Exposure of LHCII to the Protein Kinase(s). Light-induced conformational changes exposing the phosphorylation site of isolated LHCII to the protein kinase(s) in vitro are not affected by temperatures in the range of 4 to 25 °C (*22*). However, a different situation may occur for the membrane-bound LHCII that interacts with the lipid layer and other proteins or protein−chl complexes. To test this possibility, thylakoids were preilluminated with 600 μmol of photons m^{-2} s^{-1} in the presence of DCMU or incubated in darkness, for times and temperatures as indicated. NaF was added to all samples (Figure 4). The high light intensity was used to achieve maximal effect on the loss of LHCII phosphorylation (see Figure 1). The thylakoids were then phosphorylated in darkness following addition of ATP and duroquinol. The extent of LHCII phosphorylation was detected following resolution of the proteins by SDS−PAGE and immunodecoration with phosphothreonine antibodies. A low level of LHCII phosphorylation was detected in the thylakoids prior

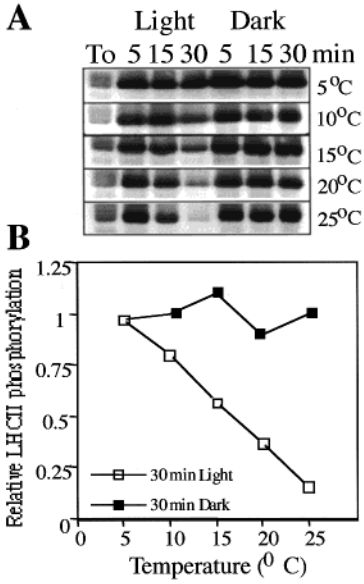

FIGURE 4: Temperature dependence of the light-induced inaccessibility of LHCII to the protein kinase(s). Thylakoids were preilluminated (600 μmol of photons m^{-2} s^{-1}) in the presence of DCMU or incubated in darkness (panel A, light or dark, respectively) in the presence of NaF at temperatures and for times as indicated. The membranes were then phosphorylated in darkness for 20 min at 25 °C with addition of ATP and activating the kinase(s) by addition of duroquinol; LHCII phosphorylation was detected by immunodecoration with phosphothreonine antibodies; T_o, indicates the LHCII phosphorylation level prior to the preincubation; (A) immunoblot; (B) loss of LHCII phosphorylation as a function of temperature during 30 min of preincubation in the light or darkness calculated from densitometry measurements of the immunoblots.

to all treatments (Figure 4A, T_o). Preillumination at increasing temperatures from 5 to 25 °C and for increasing times induced significant loss of the subsequent LHCII phosphorylation in darkness at 25 °C. However, the temperature of membrane preincubation in darkness had practically no effect on the subsequent LHCII phosphorylation level. The level of LHCII phosphorylation potential as a function of the temperature during the 30 min of preillumination (Figure 4B) shows a linear decrease with the increase of temperature from 5 to 25 °C (Figure 4B). These results indicate that in situ, light may affect not only the LHCII complex but possibly its dynamic interaction with other chlorophyll proteins. This in turn may affect the complex organization within the membrane, resulting in the inaccessibility of the phosphorylation site to the protein kinase(s).

Light-Induced Inhibition of LHCII Phosphorylation under Conditions Allowing Turnover of the Phosphothreonine Phosphate. The results presented so far indicate that illumination of thylakoids in absence of ATP and in the presence of NaF (preventing the LHCII phosphothreonine-phosphate turnover) induces inaccessibility of the LHCII phosphorylation site to the redox controlled protein kinase(s). The target of this process could be the population of nonphosphorylated LHCII bound to the PSII complex or LHCII dissociated from PSI following dephosphorylation of P-LHCII by the thylakoid protein phosphatase (*5*, *7*). The steady-state level of the later LHCII population in light exposed thylakoids depends on the relative rates of the LHCII phosphorylation/dephosphorylation and thus it is expected to reach a steady-state level under conditions allowing the phosphothreonine-phosphate turnover. This situation occurs under physiological conditions in vivo. The question thus arises whether illumination may induce inaccessibility of LHCII to the protein kinase(s) under P-LHCII phosphate turnover conditions and, if so, whether this phenomenon may reverse in darkness. To answer this question thylakoids (250 μg of chl mL^{-1}) were incubated in the presence of ATP in the light (350 μmol of photons m^{-2} s^{-1}) or darkness with addition of duroquinol to allow equal activation of the protein kinase(s) in both conditions but without addition of NaF and thus allowing phosphatase activity. The results (Figure 5 A) show a significant decrease in the level of LHCII phosphorylation after 15 min of illumination as compared to the sample incubated in darkness. This could be due to light-induced inaccessibility of LHCII to the kinase(s) and/or to light-induced increase in the activity of thylakoid protein phosphatase and thus to increased LHCII dephosphorylation rate in the light incubated sample. To distinguish between these two possibilities, thylakoids were prephosphorylated in darkness in the presence of ATP and duroquinol for 20 min. Then hexokinase(s) and glucose were added and incubation was continued for 10 min in darkness to exhaust all residual ATP and thus prevent further phosphorylation. The level of LHCII phosphorylation was measured at the end of this incubation (Figure 5B, T_o). The membranes were then further incubated in the light or darkness and the dephosphorylation of LHCII was measured as a function of time for up to 30 min. The results of such an experiment show that the rate of LHCII dephosphorylation was equal in both the illuminated and the dark-incubated thylakoids (Figure 5B). Therefore, the decrease in the phosphorylation level observed in the light-exposed thylakoids for 15 min in Figure 5A cannot be ascribed to an increase in the phosphatase activity.

To ascertain that the decrease in the LHCII phosphorylation under conditions allowing phospho-LHCII phosphate turnover is not due to light-induced inactivation of the kinase(s) (*20*, *21*), the following experiment was carried out. Thylakoids were preilluminated in the presence of ATP for 15 min at the same light intensity as in Figure 5A or incubated in darkness with addition of duroquinol to allow the LHCII phosphothreonine phosphate turnover in both conditions. Then ^{32}P-γ-ATP, duroquinol, and NaF were added and the thylakoids were further phosphorylated for 20 min in the light (Figure 5C, panel a, (L > L), lanes 1 and 4) or darkness (Figure 5C, panel a, (L > D) lanes 2 and 5). The control thylakoids in which turnover of LHCII phosphate occurred in darkness were further incubated in darkness (Figure 5C, panel a, (D > D), lanes 3 and 6, respectively). Exogenous, isolated LHCII was added (250 μg mL^{-1}) to half of the samples during this second phosphorylation period to serve as a potential, alternative substrate for the thylakoid bound kinase (Figure 5C, panel a, +exo.LHCII, lanes 1−3). The level of phosphorylated LHCII was detected after this incubation by autoradiography of thylakoid polypeptides resolved by SDS−PAGE. The results show that the final phosphorylation level of LHCII was lower in the light-incubated samples containing only thylakoid membranes as compared to that of the sample containing both thylakoids and exogenous LHCII (Figure 5C, panel a, compare lanes 1 and 4). This result indicates that the phosphorylation of the thylakoid LHCII was inhibited

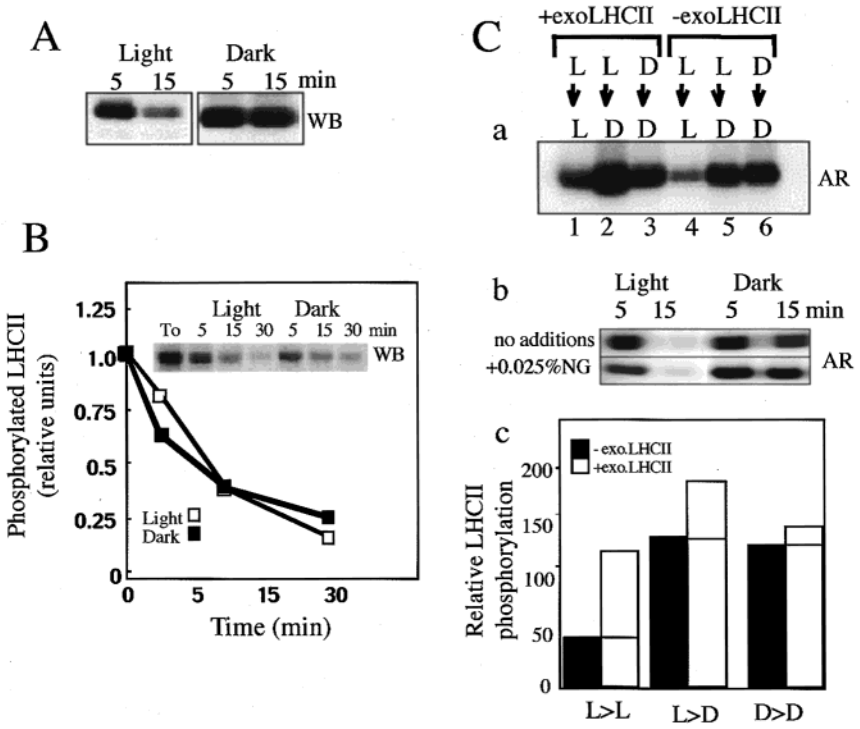

FIGURE 5: Effect of LHCII phosphothreonine turnover on the light-induced inaccessibility of LHCII to the protein kinase(s). (A) Loss of LHCII phosphorylation during light exposure in the presence of ATP. Thylakoids (250 μg mL^{-1}) were incubated in the light (350 μmol of photons m^{-2} s^{-1}) or in darkness for 5 or 15 min with addition of ATP. Duroquinol was added to the dark incubated samples to allow the kinase(s) activation and the phosphorylation level was detected by immunodecoration with phosphothreonine antibodies. Note the decrease of the LHCII phosphorylation after 15 min of illumination as compared to the dark control; (B) Kinetics of LHCII dephosphorylation in the light or darkness. Thylakoids were pre-phosphorylated in darkness for 20 min in the presence of nonradioactive ATP and duroquinol. The kinase(s) was activated by addition of duroquinol. Glucose (2 mM) and heoxinase (5 units/mL, Sigma,) were then added and incubation continued in darkness for 10 min to hydrolyze the residual ATP. The LHCII phosphorylation level was then determined (T_o). The thylakoid suspension was then further incubated in the light or darkness for times as indicated, thus allowing continuation of LHCII dephosphorylation by the thylakoid bound phosphatases; P-LHCII level was detected by immunodecoration with phosphothreonine antibodies (insert) and measured by scanning the blot; (C) Effect of thylakoid illumination in the presence of ATP on kinase(s) activity. Panel a: Thylakoids were preincubated as in panel A for 15 min in the light or darkness in the presence of ATP and in absence of NaF to allow turnover of the phosphothreonine phosphate of LHCII; duroquinol was added to all the samples incubated in darkness to allow the activation of the kinase; the light preincubated samples continued phosphorylation either in the light (L > L) or darkness (L > D). The dark preincubated samples were further phosphorylated in darkness (D > D); phosphorylation of all the above samples was carried out for 20 min with addition of ^{32}P-γ-ATP and NaF to inhibit phosphatase activity and thus allow detection of the maximal potential of LHCII phosphorylation. The phosphorylation assay was carried out with or without (±) addition of exogenous LHCII (exo.LHCII). The phosphorylation level of LHCII was detected by radiography of SDS−PAGE resolved polypeptides; only the LHCII region of the gel is shown; panel b: thylakoids were incubated as in panel a, without addition of exogenous LHCII or with addition of β-nonyl-D-glucoside (NG) at the same final concentration as used when exogenous LHCII was added; AR, radiograms; panel c: data calculated from scanning the radiogram of panel a; the upper part of the open bar graphs is ascribed to the extent of the exo.LHCII phosphorylation.

but not that of the added exogenous LHCII. The phosphorylation level increased in both samples that were transferred from light to darkness indicating recovery of the membrane-bound LHCII accessibility to the protein kinase(s) (Figure 5C, panel a, lanes 2 and 5, compare to lanes 1 and 4, respectively). The phosphorylation level of all samples that have been incubated only in darkness was similar (Figure 5C, panel a, lanes 3, 5, and 6).

To rule out the possibility that the presence of the detergent added together with the solubilized exogenous LHCII may modify the interaction of the kinase with the endogenous LHCII resulting in recovery of its phosphorylation, the following experiment was carried out. Thylakoids were incubated as in Figure 5C, panel a, without or with addition of β-D-nonylgluocoside alone at the same final concentration as in panel a. The results of this control experiment (Figure 5C, panel b) show that presence of the detergent does not prevent the light induced loss of the endogenous LHCII phosphorylation. Thus, we conclude that illumination under conditions allowing the turnover of the LHCII phosphoryl group induces inaccessibility of the endogenous LHCII to the protein kinase but does not prevent the phosphorylation of exogenous LHCII.

To estimate the contribution of exo.LHCII to the phosphorylation level of the mixture containing both, thylakoids and exo.LHCII, the radiogram of Figure 5C, panel a was scanned and the values of the samples without added exogenous LHCII were subtracted from those of the samples to which exogenous LHCII was added. The results of this calculation (Figure 5C, panel c) show that illumination did not inactivate the phosphorylation of the exogenous added LHCII by the thylakoid protein kinase(s). The chl concentration in samples containing exogenous LHCII was twice that of the samples containing only thylakoids. To ascertain that the results obtained are not affected by the higher chl concentration and thus relatively lower light excitation, the inactivation of thylakoid phosphorylation was measured under similar light intensity in samples containing thylakoid

membranes equivalent to 250 or 500 μg of chl mL^{-1}. The extent of light-induced loss of LHCII phosphorylation was similar in both samples (not shown). Furthermore, the light effect is saturated already in the range of 100 μmol of photons m^{-2} s^{-1} in samples containing 250 μg of chl mL^{-1} (Figure 1A) while in this experiment we have used 350 μmol of photons m^{-2} s^{-1}. Thus, the results obtained in Figure 5C are not due to the higher chl concentration in the samples containing added exogenous LHCII as compared to those containing only thylakoid membranes. The extent of the exogenous LHCII phosphorylation was lower in the samples incubated in darkness (Figure 5C, panel c, open bars, compare L > L with L > D). This can be due to an increase in the exposure of the phosphorylation site of the isolated LHCII to illumination (Figure 1C and ref *22*) as well as to competition with the thylakoid endogenous LHCII that may occur when the membrane-bound LHCII became inaccessible while the exogenous LHCII may diffuse more easily in the intergrana space. This is indicated by the marginal phosphorylation of the exogenous LHCII in the control sample that was not exposed to the light at all (Figure 5C, panel c, compare open bars L > D to D > D).

On the basis of the results presented in Figure 5, we propose that the level of thylakoid bound LHCII accessible to the protein kinase(s) decreases upon illumination and is reversed in darkness under conditions allowing the light-induced phosphothreonine-phosphate turnover.

Effect of the Phosphothreonine Phosphate Turnover on the Light-Induced Loss and Recovery of LHCII Accessibility to Protein Kinase(s) Activity in vivo. So far, we have shown that illumination may induce reversible inaccessibility of LHCII to the protein kinase(s) under phosphothreonine phosphate turnover conditions in situ. The question arises whether this phenomenon may also occur in vivo. To answer this question, we have exposed detached pea leaves in the absence or presence of DCMU to 800 μmol of photons m^{-2} s^{-1} at 25 °C for 30 or 60 min. We have used this light intensity since it is in the same range as that reported to induce inhibition of LHCII phosphorylation in vivo (*19*). To estimate the extent of recovery of LHCII exposure to the kinase, leaves were illuminated for 60 min, then further incubated for 30 min in darkness. As a control, leaves were also incubated only in darkness for 60 min. To quantify the extent of LHCII phosphorylation in vivo at the end of the leaves incubation, thylakoids were rapidly prepared in the cold in dim green light and the phospho-LHCII level was determined by immunodecoration with phosphothreonine antibodies in part of the membranes (Figure 6 A). The second part of the membranes was further phosphorylated in vitro in darkness with addition of ATP, duroquinol, and NaF to inhibit phosphatase activity and thus measure the maximal potential of LHCII phosphorylation in all samples (Figure 6B). A relatively high initial level of LHCII phosphorylation was detected in the thylakoids of leaves prior to all incubation (Figure 6A, T_o). This could be due to the fact that the selection and detachment of leaves of similar size from the dark adapted plants and setting them correctly on the Petri dishes upper side up (eight leaves/dish, total 14 dishes) could not be carried out in complete darkness. The green light (500 nm, half bandwidth, 45 nm) used during the preparation time (about one 1.5 h) was about 2 μmol of photons m^{-2} s^{-1}. The phosphorylation level decreased in all illuminated leaves

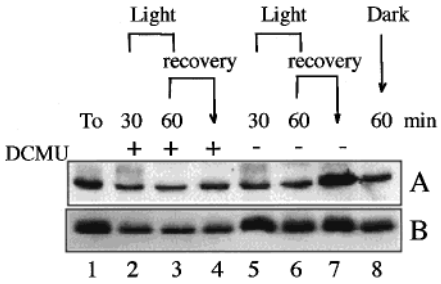

FIGURE 6: Reversibility of the light-induced inaccessibility of LHCII to the protein kinase(s) in vivo. Detached pea leaves, maintained in constant humidity in absence or presence of DCMU as described in Methods, were exposed to 800 μmol of photons m^{-2} s^{-1} or incubated in darkness for 30 or 60 min. Part of the leaves exposed to the light in the presence or absence of DCMU for 60 min were transferred to darkness (recovery) and further incubated for 30 min. At the end of the incubations, thylakoids were rapidly prepared from all samples in dim green light in the cold. In part of the membranes the level of LHCII phosphorylation following the in vivo incubation was determined (panel A), while a second part was further phosphorylated for 20 min in darkness following addition of ATP, NaF and duroquinol (panel B). The phosphorylation level of all samples was measured by immunodecoration with phosphothreonine antibodies. Only the LHCII region of the immunoblots is shown.

(Figure 6A, lanes 2−6) and, as expected, did not recover in darkness in the DCMU treated leaves in absence of kinase activation (Figure 6A, compare lanes 3 and 4). However, recovery of LHCII phosphorylation occurred in the leaves illuminated in absence of DCMU after transfer to darkness in which the LHCII kinase(s) was activated (Figure 6A, recovery, compare lanes 6 and 7). The LHCII phosphorylation in this case could be ascribed to the slow kinase deactivation in darkness following illumination ($t_{1/2}$ 4 min (*15*)). Following incubation of the thylakoids obtained from the above leaves in the presence of ATP, duroquinol, and NaF in darkness (Figure 6B), a significant increase in the level of LHCII phosphorylation of the control thylakoids (T_o) was observed and to some extent also in that from leaves that have been maintained in darkness for 60 min (Figure 6B, lane 8). Only a relatively small increase occurred in the level of P-LHCII, following the phosphorylation of the thylakoids isolated from leaves illuminated in the presence of DCMU before or after incubation in darkness (recovery) for 30 min (Figure 6B, lanes 2 and 3, compare to lane 4). As opposed to that, a significant increase of LHCII phosphorylation occurred in the thylakoids obtained from the leaves illuminated in absence of DCMU in which the phosphothreonine phosphate turned over during the illumination (Figure 6B, lanes 5 and 6). No further increase in the LHCII phosphorylation level was observed in thylakoids obtained from the above leaves after incubation in darkness (recovery) (Figure 6B, compare lanes 5 and 6, with lane 7, respectively) since in these thylakoids, LHCII was already fully phosphorylated during the recovery period in the leaf (Figure 6A, lane 7 compare with panel B, same lane). These results indicate that illumination may induce inaccessibility of LHCII to the protein kinase(s) in vivo irrespective of whether the phosphothreonine phosphate of LHCII turns over during the illumination or if LHCII is dephosphorylated. However, the accessibility of LHCII to the protein kinase(s) is rapidly recovered in darkness already during the time needed for the thylakoid preparation (<20 min) from leaves

illuminated under conditions allowing LHCII phosphorylation/dephosphorylation. As opposed to that, LHCII accessibility to the protein kinase(s) was not recovered in the thylakoids of leaves exposed to illumination while phosphorylation of LHCII was prevented, even if the leaves were further incubated in darkness for 30 min prior to the isolation of the thylakoids membranes.

DISCUSSION

Light-Induced Changes in the LHCII Accessibility to the Protein Kinase(s). The signal transduction system connecting the light-dependent redox state of the thylakoid membrane with the protein kinase(s) activation is generally considered as the major regulatory mechanism responsible for the rate and extent of PSII and LHCII proteins phosphorylation and thus, for the related state transition process (*6*, *32*). Under physiological conditions the level of LHCII phosphorylation is the result of the balance between the rate of membrane-bound kinase(s) and phosphoprotein phosphatase activities (*4*, *7*). However, illumination of leaves that should have led to maximal redox activation of the thylakoid protein kinase-(s) actually lowers significantly the LHCII phosphorylation level in vivo (*8*, *19*, *21*).

Down-regulation of the protein kinase(s) activity following reduction of dithiol groups of the enzyme(s) by a reduced thioredoxin-like soluble chloroplast component was proposed as the mechanism responsible for this phenomenon in vivo (*20*, *21*). The light-induced effect described in this work occurs in isolated thylakoids in absence of the chloroplast matrix containing the thioredoxin-like components that may induce the kinase(s) inactivation (*21*) as well as in vivo, in the presence of DCMU which upon continuous illumination induces oxidation of the reduced electron carriers past the PSII Q_B site (*33*). The thylakoid redox-regulated protein kinase(s) activity toward exogenous LHCII persisted while the membrane-bound LHCII phosphorylation was inhibited in the illuminated thylakoids. Illumination affected the exposure/occlusion of the LHCII N-terminal domain harboring the phosphothreonine site not only toward the protein kinase(s) but also toward its proteolytic cleavage by added trypsin. Thus, the light-induced inhibition of thylakoid LHCII phosphorylation does not correlate with the redox state of the thylakoid electron carrier components and can be ascribed to inaccessibility of the phosphothreonine site of LHCII to the protein kinase(s). This phenomenon may contribute substantially to the transient loss of LHCII phosphorylation observed under high light conditions in vivo.

As mentioned in the introduction, we have reported that illumination of isolated LHCII induces exposure of the phosphorylation site at the N-terminal domain to solubilized protein kinase(s) in an in vitro reconstituted system (*22*). In this work, we could demonstrate in situ a light-induced transient increase in the exposure of LHCII to the membrane-bound kinase(s) as well as to trypsin. Unlike in the case of isolated LHCII, increasing light excitation of the thylakoids was followed by inaccessibility of the LHCII phosphothreonine site to protein kinase(s). The light-induced folding of the LHCII N-terminal domain, that causes its exposure to protein kinase(s) or trypsin activity, may further affect the organization of the complex in the light-exposed membrane causing aggregation of the modified free LHCII or PSII bound to LHCII (*34*−*36*) which may hinder accessibility to the LHCII N-terminal domain.

Exposure of the phosphorylation site of isolated LHCII in vitro is affected by chl *b* excitation but only marginally by light absorbed by chl *a*. The accessibility of the membrane-bound complex to the thylakoid protein kinase-(s) in situ is lowered following preillumination by chl *b* and to a lesser extent by chl *a* absorbed light. The lower effect of chl *a* excitation in thylakoids (Figure 1C) could be only apparent and is ascribed to the fact that only about one-third of the total chl *a* in the thylakoid membranes is bound to LHCII. The difference in the effect of light absorbed by chl *a* between isolated LHCII and the membrane-bound complex may be due to LHCII interaction with the chl *a* containing PSII in the membrane and requires further investigation.

Temperature Effect and Light-Induced Structural Change. The light-induced exposure of the N-terminal domain of isolated, solubilized LHCII to protein kinase(s) in vitro occurs at temperatures down to 5 °C (*22*). However, the light-induced inaccessibility of thylakoid-bound LHCII to the membrane-bound kinase(s) is strongly temperature dependent. Fluorescence emission by PSII is not significantly altered in thylakoids in which LHCII is rendered inaccessible to the protein kinase(s) following illumination under conditions preventing LHCII phosphorylation as used in this work (not shown). This indicates that energy transfer from LHCII to PSII is not altered and thus the light-induced inaccessibility of LHCII to phosphorylation under the above conditions may be related to aggregation of PSII-LHCII complexes. Light-induced conformational changes of LHCII integrated in lipid membranes as well as in isolated thylakoids, indicating lateral aggregation and formation of macrodomains as detected by CD spectroscopy, has been reported (*34*−*36*). Fluidity of thylakoid lipids is maintained down to 5 °C (*37*). However, phosphatidyl glycerol, one of the lipids required for the formation of LHCII trimers (*38*), the natural state of LHCII in situ (*39*), has a high phase transition temperature (*40*) and thus could be involved in the above phenomena. The mobility of phosphorylated LHCII is severely restricted below 12 °C (*37*). The linear relation between the temperature of preillumination and accessibility of the phosphorylation site to the kinase(s) indicate that the temperature effect is not simply related to a membrane lipid phase transition. This could be compatible with a light-induced thermal effect within the complex due to the light absorption by the LHCII pigments (*41*).

Physiological Significance. The data presented in this work support the conclusion that illumination of thylakoids induces structural changes that may promote or hinder the accessibility of the phosphorylation site of LHCII to the protein kinase. This phenomenon may affect LHCII association with PSII or PSI and thus could be detrimental to the state transition process. The question arises as to what may be the mechanism of this phenomenon under physiological conditions in vivo. The discrepancy between the expected increase in LHCII phosphorylation with increasing light intensity and thus, redox-activation of the protein kinase(s), and the observed lowering of LHCII phosphorylation under such conditions can be explained based on the results of this work. According to the hypothesis presented schematically in Figure 7, in light-exposed chloroplasts part of the LHCII population has not yet been phosphorylated and is still bound

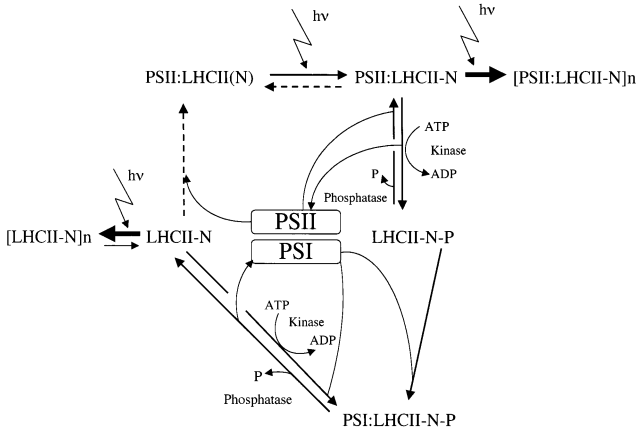

FIGURE 7: Schematic representation of the role of turnover of P-LHCII in its light-induced inaccessibility to the protein kinase(s). Illumination of LHCII bound to PSII, (PSII:LHCII(N)), increases the exposure of the LHCII N-terminal domain to the protein kinase(s) in situ (PSII:LHCII-N); further exposure to the light under conditions preventing LHCII phosphorylation may induce an aggregation state ($[PSII:LHCII-N]_n$) in which LHCII is inaccessible to the protein kinase(s) as well as to trypsin cleavage; this state reverses only slowly to its initial condition in darkness; upon illumination inducing LHCII phosphorylation, (LHCII-N-P) the phosphorylated LHCII dissociates from PSII and following lateral migration binds to PSI (PSI:LHCII-N-P); dephosphorylation of the PSI-bound LHCII generates nonphosphorylated, free LHCII (LHCII-N) which following further illumination leads to formation of the kinase-inaccessible state ($[N-LHCII]_n$); this state could be a less stable aggregate form which may reverse readily to the free, kinase-accessible LHCII(N) in darkness; heavy arrows indicates reactions that occur only in the light; plain arrows indicate reactions that may occur in the light or in darkness in the presence of duroquinol to activate the kinase; dashed arrows indicate reaction that occur only in darkness; thin arrows indicate lateral migration and association of complexes.

to PSII while part of the P-LHCII that was associated with PSI has been already dephosphorylated and is on its way to rebind to PSII. The dephosphorylated LHCII can be rephosphorylated prior to its reassociation to PSII. In this case, it may rebind to PSI. Recent results (*11*, *42*) indicate that P-LHCII interacts via the PsaH subunit with PSI, thus inferring that the dissociation of the PSI-bound P-LHCII occurs following its dephosphorylation. Under light intensities causing down-regulation or partial photoinactivation of PSII, PSI activity oxidizes the plastoquinone pool lowering the protein kinase(s) activity and resulting in LHCII dephosphorylation (*6*). Thus, the steady-state levels of free LHCII, LHCII bound to PSII, free P-LHCII, and P-LHCII bound to PSI will depend on the rate of P-LHCII phosphate turnover as well as the lateral mobility of the various LHCII states in the membrane plane. The dynamics of this process may be affected by the strength of the interaction/binding of LHCII and P-LHCII with PSII or PSI. The steady-state level of the nonphosphorylated free or PSII-bound LHCII in which the N-terminal domain has been "unfolded" by illumination and thus may be the "target population" of the light-induced effects should be related to the phosphoryl turnover rate of the P-LHCII. Slow reversibility of the above light-induced changes may acts as a "sink", accumulating LHCII forms inaccessible to the protein kinase(s), thus contributing to the observed lowering of LHCII phosphorylation in high-light exposed leaves. Therefore, the time course of the accumulation of kinase(s) inaccessible LHCII in light-exposed chloroplasts in vivo will depend on the light intensity and time of exposure. The light-induced inaccessibility of LHCII to the protein kinase(s) in vivo occurring under conditions preventing phosphorylation of the complex did not reverse in darkness for 30 min (Figure 6) and not even after 1 h (not shown). However, the inaccessibility occurring under illumination conditions allowing turnover of the LHCII phosphorylation reversed readily upon cessation of the light exposure in vitro as well as in vivo (Figures 5 and 6). These results indicate that kinase-inaccessible LHCII aggregates containing free LHCII, as is the case under conditions allowing P-LHCII phosphate turnover, reverse more readily to the phosphorylation-accessible state than those containing LHCII bound to PSII. Possibly, illumination may induce partial dissociation of LHCII trimers to monomers. However, the resulting mixture of trimers and monomers should still be phosphorylated if the phosphorylation sites remain accessible to the kinase(s) (*22*).

The results of this work do not exclude the possibility that illumination may down-regulate partially the LHCII protein kinase(s) activity in vivo (*43*). The fast reversion of the LHCII inaccessibility to the membrane protein kinase(s) under phospho-LHCII phosphate turnover, indicate that this effect may be limited since inactivation of the protein kinase(s) under strong illumination conditions would have led to formation of irreversible, kinase(s)-inaccessible LHCII forms. However, this is not the case as demonstrated in Figure 6.

Light-induced conformational changes may be a general characteristic property of chl-proteins. Illumination was demonstrated to enhance the exposure of CP43 chl-protein of the PSII-core complex to phosphorylation by a core-associated protein kinase (*23*). To reduce the complexity of the scheme presented in Figure 7, the light effect on the PSII core proteins phosphorylation was not included.

In eukaryotes, the products of the *lhcb* 3−6 genes encoding chlorophyll *a*-binding proteins are tightly associated with the PSII core complex. The ubiquitous chl protein complexes of the PSII core, CP43 and CP47 do not appear as free, stable entities in vivo. When not associated with the D1/D2 proteins forming the heterodimer of the PSII reaction center, these complexes are mostly degraded unless reassembled in a functional PSII complex (*3*, *44*). LHCII is the only chlorophyll protein component of photosystem II that can assume a stable form when dissociated from the complex. The appearance of "free" chl *a*/*b* LHCII complexes encoded by the *lhcb1*,*2* genes seems to parallel in evolution that of the redox-controlled LHCII protein kinase(s). The results presented here suggest that the phosphorylation of LHCII may have additional functions besides the dissociation of the trimeric complex from PSII and promoting its binding to PSI in the process of state transition, as understood presently (*42*, *45*). On the basis of the results of this work, we propose that LHCII phosphorylation prevents or minimizes the light-induced tendency of "free" or PSII-bound LHCII complexes to aggregate and form macrodomains that may hinder the lateral mobility required for efficient thylakoid membrane functions.

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