Light-Modulated Exposure of the Light-Harvesting Complex II (LHCII) to Protein Kinase(s) and State Transition in *Chlamydomonas reinhardtii* Xanthophyll Mutants†

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ABSTRACT: Reversible phosphorylation of chl *a*/*b* protein complex II (LHCII), the mobile light-harvesting antenna, regulates its association and energy transfer/dissipation to photosystem (PS) II or I (state transition). Excitation of LHCII induces conformational changes affecting the exposure of the phosphorylation site at the N-terminal domain to protein kinase(s) [Zer, H., et al. (1999) *Proc. Natl. Acad. Sci. U.S.A. 96*, 8277−8282; Zer, H., et al. (2003) *Biochemistry 42*, 728−738]. Thus, it was of interest to examine whether the pigment composition of LHCII affects the light-induced modulation of LHCII phosphorylation and state transition. To this end, we have used thylakoids of wild-type *Chlamydomonas reinhardtii* and xanthophyll deficient mutants *npq1*, *lor1*, *npq2*, *npq1 lor1*, and *npq2 lor1*. Phosphorylated protein bands P11, P13, and P17 are considered components of the mobile *C. reinhardtii* LHCII complex. The protein composition of these bands has been analyzed by mass spectrometry using Qtof-2 with a nanospray attachment. P11 and P13 contain *C. reinhardtii* light-harvesting chlorophyll *a*/*b* binding protein LhcII type I. P17 contains *C. reinhardtii* LhcII types III and IV. Illumination of isolated thylakoids inhibits the redox-controlled phosphorylation of polypeptide bands P13 and P17 and to a lower extent that of P11. The light-induced inhibition of LHCII phosphorylation and the state transition process are not influenced by extensive differences in the xanthophyll composition of the mutants. Thus, LHCII can be visualized as possessing two functionally distinct, independent domains: (i) the pigment binding transmembrane domain regulating the extent of energy transfer/dissipation and (ii) the surface-exposed phosphorylation site regulating the association of LHCII with PSII or PSI.

Light absorption driving the photosynthetic electron transfer and related energy storage may be harmful to the photosynthetic apparatus when the excitation rate exceeds the rate of its utilization for photochemistry. Photosystem II (PSII)[1] is particularly prone to light-dependent oxidative damage (*1*). Light absorption can be regulated on a long-term range (days) through controlled expression of the *lhcb1* and *lhcb2* genes encoding light-harvesting complex II (LHCII) (*2*) and activation of proteolysis of light-harvesting antenna proteins (*3*−*5*). Dissipation of excess absorbed light via non-photochemical quenching (NPQ) of chlorophyll fluorescence (*6*, *7*) lowers the light stress on a short time scale. The major and fast energy-dependent NPQ (<2 min) component, qE, is related to generation of ΔpH across the thylakoid membrane via electron transfer (*8*−*10*). A second component, qT (state transition, relaxation time in the range of >10 min), is achieved by uncoupling of LHCII from PSII through the activation of redox-regulated reversible phosphorylation of the outer, mobile PSII−LHCII antennae (*1*, *11*−*13*). Phosphorylated LHCII is considered to diffuse in the thylakoid membrane plane from the appressed membranes (grana) to the nonappressed (stroma) domains where it interacts with the PsaH subunit of PSI (*14*) and increases the light absorption cross section of this complex. Thus, state transition balances the excitation rates of the two photosystems. In *Chlamydomonas* cells, the state transition process is accompanied by migration of a substantial amount of the cytochrome b_6f complex population from the appressed membrane regions toward the membrane domains exposed

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[1] Abbreviations: chl, chlorophyll; CP43, chlorophyll *a* binding proteins forming the PSII core internal antennae; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; LHCII, light-harvesting complex II; NPQ, non-photochemical quenching; PMSF, phenazine methosulfate; PSI and PSII, photosystems I and II, respectively; PVDF, polyvinylidene difluoride; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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to the stroma, thus enhancing the rate of cyclic electron flow and ATP synthesis (*15*).

A decrease in the lumenal pH induces qE through protonation of PSII proteins and activation of reversible interconversion of violaxanthin to form antheraxanthin and zeaxanthin (the xanthophyll cycle) **(***9*, *10***)**. These pigments promote thermal energy dissipation, thus reducing the rate of generation of triplet chlorophyll and related formation of harmful $^1O_2$. Together, changes in the relative amount of various xanthophylls and the protonation of specific sites in the PSII antennae (*6*, *10*, *16*) cause conformational changes of PSII characterized by a short chl lifetime and a low fluorescence yield (*17*). The majority of the NPQ processes are thought to occur in the PSII antennae (*6*−*8*). The extent of qE is highly correlated with the levels of zeaxanthin and antheraxanthin. Excessive excitation of PSII relative to PSI activates violaxanthin de-epoxidase, thus modulating the thylakoid zeaxanthin content. In addition to antheraxanthin and zeaxanthin derived from β-carotene, xanthophylls derived from α-carotene, lutein (the most abundant carotene in the thylakoid membrane) and loroxanthin, have been also implicated in qE (*18*).

Photosystem II phosphoproteins include members of the Lhcb family (*19*, *20*), the PSII core antenna CP43, the D1/D2 proteins forming the PSII reaction center, and PsbH. Recently, a 9 kDa thylakoid membrane extrinsic protein, TSP9, was reported to harbor three phosphorylation sites and is released into the stroma upon their phosphorylation (*21*). The phosphorylation of LHCII is controlled by reversible activation of thylakoid-bound protein kinase(s) involving interaction of plastoquinol with the cytochrome b_6f complex (*1*, *12*, *22*−*26*). The redox state of vicinal dithiols of thioredoxin-like proteins may affect the oxidation and/or reduction of dithiol sites of the protein kinase(s) and prevent the activation of LHCII phosphorylation (*27*).

Several protein kinases may be involved in the phosphorylation of PSII proteins (*12*). A protein kinase encoded by the *Arabidopsis TAK1* gene was demonstrated to phosphorylate LHCII. Mutants impaired in TAK1 activity exhibit a significant loss of state transition (*28*). Attempts to isolate the LHCII protein kinase have been reported (*29*). Preparations highly enriched in solubilized protein kinase(s) have been obtained from spinach thylakoids and phosphorylated specifically the threonine in the N-terminal domain of isolated LHCII (*30*). The protein kinases present in such preparation exhibit activity in the absence of reducing agents. Possibly, the redox control resulting from the interaction of the kinase(s) with the cytochrome b_6f complex is lost in the isolation process (*30*).

Recently, remarkable progress in the identification of the LHCII protein kinase(s) involved in *Chlamydomonas* LHCII phosphorylation was achieved. Two allelic independent mutants, *stt7-1* and *stt7-2*, have been isolated using screening of *Chlamydomonas* colonies by fluorescence video imaging for mutants with impaired state transition (*31*). Using a genetic approach, the stt7 gene encoding a protein kinase that could complement the *Chlamydomonas* mutants and restore state transition was identified and characterized (*32*).

We have demonstrated that illumination of isolated pea LHCII modulates the exposure of the N-terminal domain containing the phosphothreonine site to protein kinase(s) in an *in vitro* reconstituted system (*30*) as well as in intact thylakoids and detached pea leaves (*33*). These results suggest an additional mode of light regulation of LHCII phosphorylation. The questions of whether alteration of the xanthophyll composition that may affect the properties of the transmembrane domain of the LHCII complex (*34*, *35*) may influence the light-induced modulation of the LHCII exposure to protein kinase(s) and the state transition arise.

In this work, we have addressed this question using the wild-type (wt) unicellular green alga *Chlamydomonas reinhardtii* and mutants impaired in the pathways of xanthophyll synthesis and interconversion. The results show that illumination intensity above that required for saturation of electron flow induces inaccessibility of LHCII to protein kinase(s) in *Chlamydomonas* wt thylakoids as reported for higher-plant thylakoids (*33*). Alteration of the xanthophyll composition of the thylakoids does not affect significantly the phosphorylation of LHCII and state transition or the light-induced modulation of redox-controlled LHCII phosphorylation.

MATERIALS AND METHODS

Cell Culture and Preparation of Thylakoids. The wild type, Ac208, and the xanthophyll cycle mutants of *C. reinhardtii* derived from strain Cc125 were grown at 25 °C in liquid medium containing acetate (*36*) illuminated by white fluorescent light (20 μmol of photons m^{-2} s^{-1}). The following mutants were used: *npq1* (mt−) which is unable to convert violaxanthin to antheraxanthin and zeaxanthin; *npq2* (mt+) which is defective in zeaxanthin epoxidase activity, thus lacking antheraxanthin, violaxanthin, and neoxanthin (*37*) (however, zeaxanthin can be synthesized from β-carotene in this mutant); *lor1* (mt−) which is defective in lycopene ϵ-cyclase activity, thus lacking α-carotene, lutein, and loroxanthin; and double mutants *npq1 lor1* (*18*, *38*) and *npq2 lor1*, strains constructed by crossing the two single mutants. All xanthophyll mutants were backcrossed at least four times to the wt Cc-125. The list of xanthophylls that can be synthesized by the wt and mutants used in this work is given in Table 1. The plastocyanin-less mutant Ac208 (mt+) used in this work was kindly supplied by L. Mets (University of Chicago, Chicago, IL). Cells were harvested during the exponential phase of growth, and thylakoids were prepared according to the method in ref *39* and resuspended in TMN buffer [50 mM Na-Tricine (pH 7.5), 10 mM NaCl, 5 mM $MgCl_2$, 200 μM PMSF, and 1 mM benzamidine]. The thylakoid suspension was kept on ice in darkness until use for up to 1 h.

Preillumination of Thylakoid Membranes under Conditions Preventing Kinase Activity. Thylakoids (200 μg of chlorophyll/mL) were suspended in buffer containing 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 10 μM DCMU, and 10 mM NaF to inhibit phosphatase activity. The suspension was illuminated in glass tubes 8 mm in diameter and exposed to 150 μmol of photons m^{-2} s^{-1} at 25 °C using a projector equipped with a tungsten−halogen lamp, and heat filter. The effect of this treatment on the phosphorylation of the thylakoid proteins was subsequently assayed in darkness following activation of the protein kinase(s) by addition of duroquinol (*40*).

Phosphorylation Assay. Thylakoid proteins (20 μg of chlorophyll) were phosphorylated using a membrane suspen-

Table 1: Xanthophyll Synthesis Potential and Levels in wt *C. reinhardtii* and Mutants Exposed to High Light Intensities[a]

	lutein	loroxanthin	β-carotene	zeaxanthin	antheraxanthin	violaxanthin	neoxanthin
wt	24.9	13.8	28.5	14.1	1.1	4.51	13.1
npq1	26.5	16.0	24.1	–	–	19.3	14.1
npq2	20.4	12.9	21.6	45.1	–	–	–
lor1	–	–	32.9	32.1	5.5	19.2	10.3
npq1 lor1	–	–	31.4	9.5	6.1	45.8	8.3
npq2 lor1	–	–	28.3	71.7	–	–	–

[a] Carotenoid levels (% of total) were measured in acetone extracts of cells exposed for 3 h to 750 μmol of photons m^{-2} s^{-1}. Similar levels were measured in cells grown in low light; however, the zeaxanthin level of the wild-type cells was significantly lower. The carotenoid levels did not change in thylakoids extracted from cells grown in low light (Figure 4) or high light and further incubated in the light for up to 45 min (not shown).

sion in 1.5 mL Eppendorf tubes containing 100 μL of 50 mM Tris-HCl buffer with addition of 10 mM $MgCl_2$, 10 mM NaF, 0.2 mM ATP, and, when indicated, 5 μCi of [γ-^{32}P]-ATP. Following addition of 1 mM duroquinol to activate the protein kinase, the tubes were incubated at 25 °C in darkness for 20 min. Maximal LHCII phosphorylation was achieved after incubation for 10−15 min under the conditions described above. Phosphorylation was terminated by addition of SDS−PAGE sample buffer. The thylakoid proteins were resolved by denaturing electrophoresis (*41*), and their phosphorylation was detected by autoradiography or by immunodecoration of nonradioactive phosphoproteins resolved by SDS−PAGE after transfer to PVDF membranes and using anti-phosphothreonine antibodies (*42*).

LHCII Polypeptide Identification. The gel region containing the LHCII proteins was cut out, and the individual resolved stained bands were digested in gel using trypsin (*43*). Identification of the different LHCII polypeptides was carried out by mass spectrometry (Qtof2, Micromass), using the nanospray attachment (*44*). Data analysis was performed using Biolynx (Micromass), and database searches were preformed with Mascot (Matrix Science).

State transition was assessed according to the method of Fleischman et al. (*31*), with modifications as described below. Cells grown in acetate-containing medium until the end of the log phase were sedimented by centrifugation and resuspended in acetate-free growth medium. The cell suspension was incubated on a rotatory shaker (250 rpm) at 25 °C in darkness for 6 h. This procedure was carried out to allow depletion of the carbon reserves by respiration and oxidation of the plastoquinol pool, thus allowing dephosphorylation of LHCII (state I). At the end of this incubation, samples were taken and fluorescence induction was assessed after addition of 20 μM DCMU. The remaining cells were then further incubated for 1 h in darkness with addition of NaN_3 (250 μM) to prevent mitochondrial respiration and 5 mM sodium acetate to allow reduction of the plastoquinone pool and thus activation of the LHCII protein kinase(s) (state II). At the end of this incubation, samples were taken and fluorescence induction was assessed again in the presence of DCMU. The procedure described above allowed the process of state transition to occur in darkness and thus prevented the contribution of NPQ to the fluorescence measurements. Fluorescence kinetic measurements were carried out using the double modulation fluorimeter (Fluorwin, Photon Systems Instruments, Brno, Czech Republic). This instrument allows fluorescence increases to be recorded with a resolution time in the millisecond range (*45*).

Degradation of the D1 Protein. For determination of D1 protein degradation during preillumination of the thylakoid membrane suspension, samples were taken at different times of light exposure as described above, and the proteins were resolved by SDS−PAGE. Following transfer of the proteins to PVDF paper, the amount of D1 protein was detected by immunodecoration using anti-D1 protein antibodies obtained from Agrisera.

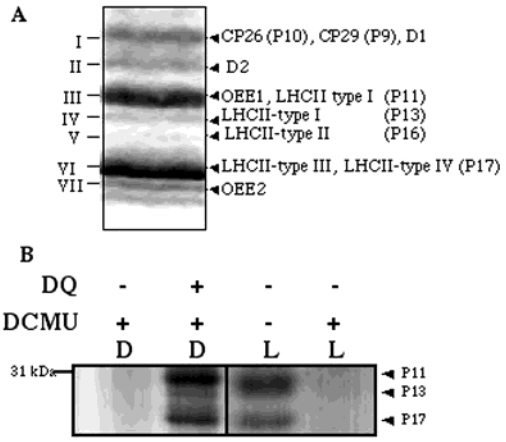

FIGURE 1: Redox-controlled phosphorylation of LHCII in isolated thylakoids of *C. reinhardtii*. (A) LHCII polypeptide pattern of *C. reinhardtii* thylakoids resolved by SDS−PAGE and stained with Coomassie brilliant blue. The separation of the stained bands in the region of 20−30 kDa is not sufficient to distinguish between polypeptides migrating close to each other. The composition of each stained band was analyzed by mass spectrometry as described in Materials and Methods. The nomenclature of the various bands, according to the system established by Bassi and Wollman (*46*), is in parentheses and will be used throughout the paper since this is the nomenclature generally used for *Chlamydomonas* thylakoid polypeptides. (B) Thylakoids from cells in state I were phosphorylated in the absence or presence of 10 μM DCMU using [γ-^{32}P]-ATP in darkness or during exposure to 150 μmol of photons m^{-2} s^{-1} for 20 min. Phosphorylation in darkness was activated by 1 mM duroquinol. Note that the phosphorylation pattern is the same in the two cases. Only the LHCII region containing polypeptides P11, P13, and P17 denoted with arrowheads is shown.

Other Procedures. The chlorophyll concentration was determined in 80% acetone extracts. The light intensity was measured with a radiometer (Li-Cor, Inc.). Carotenoid determinations were carried out in acetone extracts using the Waters-6000 HPLC apparatus.

RESULTS AND DISCUSSION

Chlamydomonas LHCII has a complex phosphoprotein composition as compared to that of the higher plants. It includes the polypeptide bands denoted P9, P10, P11, P13, and P17 (Figure 1A) (*46*) with apparent molecular masses, when resolved by Tris-glycine SDS−PAGE as in this work, of 34, 33, 30.5, 26.5, and 25 kDa, respectively (*46*). Poly-

peptides P9 and P10 are not well resolved from the D1 protein in the above system, and are considered to be the equivalents of the inner LHCII antennae proteins, corresponding to CP29 and CP26 of higher plants, respectively. Polypeptides P11, P13, and P17 (which occasionally migrate as a doublet) together with P16 are considered to be components of the oligomeric, outer mobile LHCII complex of PSII that is responsible for the state transition process (*46*).

To identify the polypeptide composition of thylakoid protein bands resolved by SDS−PAGE as performed in this work, we have used mass spectrometry analysis and the database of the *Chlamydomonas* light-harvesting antenna proteins as indicated in Materials and Methods. Figure 1A shows the polypeptide bands according to the nomenclature as used by Bassi and Wollman (P9, P10, P11, P13, P16, and P17) (*46*) which is commonly used in many publications (*31*, *32*) and the protein content of these bands obtained in this work by the mass spectrometry method (Figure 1A, bands I−VII). According to Teramoto et al. (*19*), *Chlamydomonas* LHCII consists of four different proteins that were characterized as LHCII types I−IV. LHCII type I can be resolved into three proteins that are very similar, between 92 and 97% identical. We identify band I in Figure 1A as containing polypeptides P9 and P10. These polypeptides correspond to LHCII proteins CP29 and CP26, respectively, and represent the light-harvesting chlorophyll *a*/*b* binding proteins Lhcb4 and Lhcb5, respectively (*19*). Band I was found to contain also the D1 protein which in *C. reinhardtii* is not phosphorylated (*12*). Band II contains only the D2 protein, and band III corresponding to P11 contains *C. reinhardtii* light-harvesting chlorophyll *a*/*b* binding protein LhcII type I and OEE1 (PsbO), the 33 kDa oxygen evolution enhancing protein. Band IV corresponds to P13 and was identified by mass spectrometry as LHCII type I. Band V, corresponding to P16, contains light-harvesting chlorophyll *a*/*b* binding protein LhcII type II. Band VI contains LHCII types III and IV corresponding to P17. Band VII contains OEE2 (PsbP), the 22 kDa oxygen evolution enhancing protein. Among all these bands, proteins D1 and PsbO are not phosphoproteins while D2 is phosphorylated in *Chlamydomonas*.

As in higher plants (*11*, *12*), the phosphorylation of LHCII in *C. reinhardtii* is redox-controlled (Figure 1B and ref *15*), implying that the protein kinase activity is related to the state of the plastoquinone pool reduction and cytochrome b_6f activity (*15*, *20*). In heterotrophic grown cells, due to respiratory activity, the relative ratio of thylakoid plastoquinol to plastoquinone is sufficiently high to partially activate LHCII phosphorylation even in darkness or in the absence of PSII-mediated electron flow, i.e., in cells illuminated in the presence of DCMU (*47*−*49*). In isolated thylakoids, plastoquinol is oxidized during the preparation procedure. Light-dependent reduction of plastoquinone by PSII or by addition of reducing agents in darkness is required for the activation of LHCII phosphorylation (*50*). This is demonstrated in Figure 1B. Thylakoids obtained from cells grown in the dark were phosphorylated in darkness using [γ-^{32}P]-ATP with addition of NaF to prevent LHCII dephosphorylation by thylakoid-bound phosphatase activity (*12*). The protein kinase was activated by addition of duroquinol. Addition of DCMU in this case does not inhibit the kinase activation since the PQ pool is reduced by duroquinol.

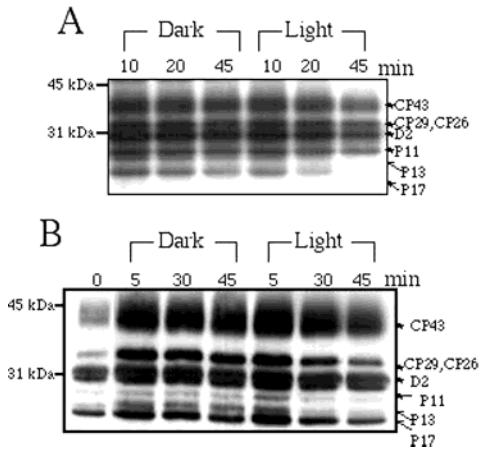

FIGURE 2: Preillumination of thylakoids modulates the subsequent level of LHCII polypeptide phosphorylation in darkness. (A) Thylakoids of cells in state I were preincubated in darkness (Dark) or exposed to 150 μmol of photons m^{-2} s^{-1} (Light) for the indicated times in the presence of 10 μM DCMU to inhibit LHCII phosphorylation. The thylakoids were then transferred to darkness; duroquinol and [γ-^{32}P]ATP were added, and phosphorylation was carried out for 20 min. (B) Thylakoids were incubated in darkness or in light as in panel A, and phosphorylation was carried out in darkness for 20 min after addition of duroquinol and nonradioactive ATP. The polypeptide proteins were resolved by SDS−PAGE, and phosphorylation was detected by autoradiography (A) or phosphothreonine antibodies (B).

DCMU inhibits the LHCII phosphorylation in the isolated thylakoids when the kinase(s) activation is achieved by light-driven PSII electron flow (Figure 1B).

We have reported recently that preillumination of thylakoids induces inaccessibility of LHCII to the protein kinase(s) assayed in darkness in pea thylakoids (*33*). To test whether preillumination has a similar effect on *Chlamydomonas* thylakoids, membranes isolated from cells grown in low light and aerated in darkness so that they would be in state I (*51*) were preilluminated in the absence of ATP and in the presence of DCMU to prevent protein kinase activation. The preilluminated thylakoids were then phosphorylated in darkness by addition of duroquinol and [γ-^{32}P]ATP or nonradioactive ATP. The phosphorylation level was assayed by autoradiography or immunodetection of phosphoproteins by anti-phosphothreonine antibodies (Figure 2). The combined use of these methods allows the estimation of both the initial level of LHCII phosphorylation prior to the isolation of the thylakoids and the maximal increase in the extent of LHCII phosphorylation using the subsequent assay of kinase activity by radioactive labeling. Preillumination of the thylakoids lowered partially the subsequent rate of phosphorylation in darkness of the CP43 and P11 polypeptide and to a larger extent that of the D2 protein as compared to the dark preincubated thylakoids. However, a drastic inhibition of the phosphorylation of LHCII P13 and P17 polypeptides was induced by the preillumination procedure (Figure 2A). A similar experiment was performed using nonradioactive ATP and detecting the final level of phosphorylation with phosphothreonine antibodies. The results of this experiment (Figure 2B) show that an initial low level of phosphorylation is present prior to the preillumination (To). The phosphorylation level of the thylakoids preincubated in darkness for up to 45 min in the absence of ATP and duroquinol increased during subsequent phosphorylation in

darkness in the presence of duroquinol and ATP (Figure 2B, panels marked dark). A slightly higher phosphorylation level was obtained in the preilluminated thylakoids phosphorylated in darkness after preillumination for only 5 min, particularly for the LHCII P11, P13, and P17 polypeptides. This increase is in agreement with previous data demonstrating that short preillumination of pea LHCII exposes the N-terminal domain containing the phosphothreonine site to the protein kinase(s) (*30*, *33*). Following preillumination for 45 min, a decrease in the level of CP43 phosphorylation could be detected. However, the phosphorylation of LHCII P11 and especially P13 and P17 was significantly inhibited compared to that of the other phosphoproteins (Figure 2B). These results match those obtained when using radioactive ATP (Figure 2A).

In evaluating the phosphorylation levels of the various phosphoproteins, one should consider that the affinity of the phosphothreonine antibodies differs for various phosphoproteins, and thus, one cannot compare in a quantitative way the phosphorylation extent among the different polypeptides. This is clearly evident when comparing the phosphorylation of CP43 as detected by radiography (Figure 2A) with that of the immunodetection system (Figure 2B). However, the changes in the signal intensity of the immunodecoration for a particular polypeptide are quantitatively related to its phosphothreonine content (*33*, *52*).

The results presented above indicate that preillumination lowers the subsequent level of phosphorylation of LHCII components to a considerably higher level for LHCII P13 and P17 than for P11 polypeptides considered to be part of the mobile LHCII complex, as compared to that of the D2 protein and CP43 PSII core polypeptide.

Persistence of Protein Kinase Activity in the Preilluminated Thylakoids. One can consider that preillumination inactivates the thylakoid protein kinase phosphorylation possibly due to downregulation of the enzyme activity by the light-modulated redox state of the thylakoid and interaction with soluble thioredoxin-like components (*52*). This possibility is compatible with the recently reported identification of the *C. reinhardtii* thylakoid protein kinase Stt7 (*32*) harboring seven cysteine residues. Two of these cysteines are four amino acid residues apart and could serve as the target for interaction with thioredoxin. Stt7 phosphorylates the P11 polypeptide of the *Chlamydomonas* LHCII complex (*32*). The phosphorylation level of P13 and P17 is also impaired in the *stt7* mutant (*31*). The polypeptides affected mostly by preillumination in our work are P13 and P17. Thus, the question of whether the phenomena reported here are due to the putative redox inactivation of Stt7 arises. One should consider that the specificity of the protein kinases involved in the phosphorylation of PSII proteins could differ for the different subunits of the complex. Light-harvesting complex II is a heteromer formed of trimers of different polypeptides (P11, P13, and P17) together with P16 (*46*).

The degree of phosphorylation of one subunit of the complex may affect the exposure of the phosphorylation sites of other subunits. Thus, the degree of phosphorylation of a particular PSII light-harvesting antenna component may be affected to various degrees due to the level of activation of the different protein kinases operating in the chloroplasts. Therefore, the light-induced inhibition of the LHCII phosphorylation observed in this work may be due to a combination of both, downregulation of Stt7 via the redox state of thioredoxins as well as LHCII/PSII conformational changes induced by illumination.

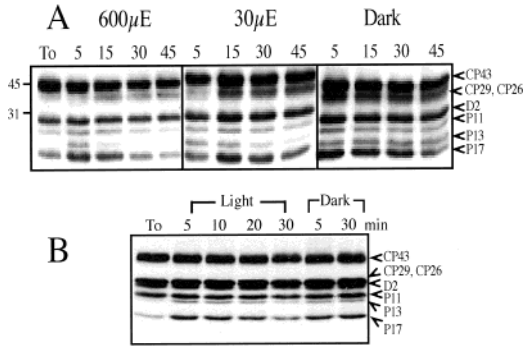

FIGURE 3: Reduction of electron carriers past cytochrome b_6f does not affect the light-induced decrease in the level of LHCII polypeptide phosphorylation. (A) Thylakoids of the *C. reinhardtii* Ac208 mutant lacking plastocyanin have been preincubated in darkness or exposed to light (30 or 600 μmol m^{-2} s^{-1}) for the indicated times and then phosphorylated in darkness for 20 min following addition of nonradioactive ATP and duroquinol. (B) wt, control thylakoids were preilluminated with addition of 1 mM methyl viologen and 30 μg/mL catalase in the absence of DCMU. The preilluminated thylakoids were then phosphorylated in darkness using duroquinol to activate the enzymes and nonradioactive ATP. The membrane polypeptides have been resolved by SDS−PAGE, and the phosphorylation level was detected by immunoblotting using phosphothreonine antibodies. Only the polypeptide region between 20 and 50 kDa is shown. The lanes labeled "To" indicate the level of polypeptide phosphorylation prior to the beginning of the preillumination.

Downregulation of the protein kinase activity by reduced thioredoxins may be hindered in thylakoids isolated from plastocyanin-less mutants since in such thylakoids thioredoxins cannot be reduced by light-driven PSI electron flow. Thus, we have used thylakoids obtained from the Ac208 mutant of *C. reinhardtii* cells lacking plastocyanin when grown in copper-containing media (*53*) as is the case for the growth medium we use for the *Chlamydomonas* cells (*36*). Thylakoids obtained from the mutant cells have been incubated in darkness or exposed to a light intensity of 30 or 600 μmol m^{-2} s^{-1} for up to 45 min in the absence of ATP followed by phosphorylation in darkness for 20 min after addition of ATP and duroquinol. The results of such an experiment (Figure 3) show that preillumination of the thylakoids with only 30 μmol m^{-2} s^{-1} reduces significantly the subsequent level of phosphorylation of polypeptides P13 and P17. This effect is greater following preillumination with a 20-fold higher light intensity. To further test the possibility that the illumination-induced changes in LHCII are responsible for the loss of LHCII phosphorylation, thylakoids isolated from wt cells were preilluminated in the presence of added methyl viologen, which is readily reduced by photosystem I. Thus, reduction of thylakoid membrane electron carriers by PSI, including bound thioredoxin, should be prevented under these experimental conditions. Results of such an experiment (Figure 3B) were similar to those obtained when using the Ac208 mutant. Notably, the same pattern of the preillumination-induced transient increase primarily in the phosphorylation of the LHCII P13 and P17 polypeptides, followed by the gradual loss of their phosphorylation potential, is obtained in the mutant thylakoids as in the wt membranes preilluminated in the presence of methyl viologen. The transient increase in the level of

phosphorylation of polypeptides P13 and P17 following a short preillumination as compared to that of the other phosphoproteins is interpreted as evidence of the light-induced modulation of the exposure of the N-terminal domains of these polypeptides as reported to occur also in pea thylakoids. In the absence of LHCII phosphorylation during illumination as is the case in the experiments described above, an initial increase in the exposure of the N-terminal domain may lead to further changes in the organization of LHCII/PSII in the thylakoid membrane plane, preventing the accessibility of the phosphorylation site to the protein kinase(s) (*30*, *33*). The results presented in Figure 3 support the conclusion that the preillumination effect on the subsequent phosphorylation of LHCII in darkness as tested in this work is due to changes induced in the organization of the LHC PSII substrates.

Changes in the superorganization of the thylakoid membrane induced by exposure of *C. reinhardtii* cells to high light intensities so as to induce photoinactivation of PSII have been reported (*54*). The results presented here indicate that such effects may occur even at relatively low light intensities if phosphorylation of LHCII is prevented during the illumination. Light-induced changes in the organization of isolated LHCII as well as the complex bound to the thylakoids or incorporated in lipid membranes as detected by CD spectroscopy (*55*, *56*) lend support to the above interpretation of the experimental data.

Effect of Preillumination on the LHCII Phosphorylation of Mutants Impaired in Xanthophyll Synthesis. We have previously reported that light absorbed by both chlorophyll *a* and *b* is involved in the modulation of the accessibility of the N-terminal domain of pea LHCII to protein kinase (*30*). Since we ascribe the effect of thylakoid preillumination mostly to light-induced changes in the LHCII conformation or aggregation state, it was of interest to consider the possibility that besides chlorophyll, carotenoids may play a role in the phenomena described above. Apart from being involved in energy transfer and/or dissipation, the carotenoids are important for the proper assembly, folding, and stability of LHC apoproteins of the complex (*9*, *57*). The prevailing LHCII carotenoids in wt *Chlamydomonas* cells exposed to relative low light intensities are lutein (up to 50% of the total), loroxanthin, β-carotene, neoxanthin, and violaxanthin (*58*). The carotenoids synthesized by wt *Chlamydomonas* and mutants *lor1*, *npq1*, *npq2*, *npq lor1*, and *npq2 lor1* (*18*, *37*, *38*) are shown in Table 1.

Thylakoids isolated from the *lor1*, *npq1*, *npq2*, and double mutants, all grown in low light, were preincubated in darkness or pre-exposed to 150 μmol of photons m^{-2} s^{-1} for up to 30 min in the presence of 10 μM DCMU to prevent the activation of the thylakoid protein kinase(s). After addition of duroquinol and ATP, the thylakoids were phosphorylated in darkness for 20 min. The phosphorylation level of the various phosphoproteins was detected by immunodecoration with phosphothreonine antibodies. Results of such an experiment are given in Figure 4, in which panel A shows a stained gel. The different LHCII polypeptides are marked according to mass spectrometry identification as in Figure 1. Panels B and C show the extent of wt and mutant thylakoid protein phosphorylation. In thylakoids of wt and single mutants, in contrast to polypeptides P13 and P17, the CP43, D2, and P11 polypeptides were more heavily phosphorylated *in vivo* prior to the isolation of the thylakoid membranes. This phosphorylation level corresponds to that marked as To in panels B and C of Figure 4. Preillumination of the *lor1* and *npq1* mutant thylakoids induces a slight loss of phosphorylation ability of CP43 (Figure 4B). However, a pronounced inhibition of phosphorylation was induced by preillumination of these thylakoids, for the P13 and P17 bands, comparable to that of the wild-type thylakoids (Figure 4B).

The *Chlamydomonas lor1* mutant is deficient in ϵ-cyclase activity, leading to a lack of xanthophylls derived from α-carotene, lutein, and loroxanthin (*18*, *38*). This drastic change in the carotenoid composition of the *lor1* thylakoids (Table 1) has apparently no effect on the light-induced inaccessibility of LHCII to the protein kinase. The *npq1* mutant cannot convert violaxanthin to antheraxanthin and zeaxanthin in high light and contains near-wt levels of lutein and loroxanthin. Thus, under our experimental conditions irrespective of whether grown in high or low light, the *npq1* mutant is similar to the wt grown in low light (*18*) (Table 1).

The *npq2* mutant synthesizes the α-carotene-derived xanthophylls and has a level of lutein close to that of wild-type cells. However, *npq2* is defective in zeaxanthin epoxidase activity (*37*), thus lacking antheraxanthin, violaxanthin, and neoxanthin, and contains constitutively high levels of zeaxanthin (Table 1).

The effect of preillumination on *npq1 lor1* and *npq2 lor1* mutants upon the subsequent phosphorylation of the LHCII polypeptides in darkness is shown in Figure 4C. Preillumination of the *npq2* thylakoids resulted in a reduction in the phosphorylation level of LHCII polypeptides P13 and P17 after preillumination for 15 min. The phosphorylation pattern of the *npq1 lor1* double mutant expressing only violaxanthin (Table 1; *18*) was also basically similar to that of the wild type and the *npq1* and *lor1* mutants. The phosphorylation level of P13 and P17 proteins of this mutant was most affected by preillumination (Figure 4C**)**. It is noteworthy that the p11 polypeptide that was reported to be essential for the state transition process in *C. reinhardtii* (*32*) is phosphorylated to a relatively high level prior to the preillumination and is less affected by the preillumination treatment (Figure 4B,C, To). Preillumination of the *npq2 lor1* double mutant induced the loss of phosphorylation potential of all phosphoproteins, and a similar phenomenon also partially occurred in the thylakoids preincubated in darkness. This effect is specific to this double mutant and may be ascribed at least partially to light-induced aggregation of the polypeptides upon illumination of the isolated thylakoids that prevented complete solubilization of the complexes during the SDS−PAGE procedure. Apparently, in this mutant, the neoxanthin binding site of LHCII is empty (*59*). Whether the unoccupancy of the neoxanthin binding site of LHCII is related to the tendency of this mutant LHCII to aggregate remains to be further investigated. Minor differences between the extent of initial phosphorylation and illumination-induced loss of phosphorylation potential of the LHCII polypeptides of the various mutants are noticeable. However, in general lines, despite the large difference in the carotenoid content of the various mutant thylakoids, the light-induced effects on the availability of the phosphorylation

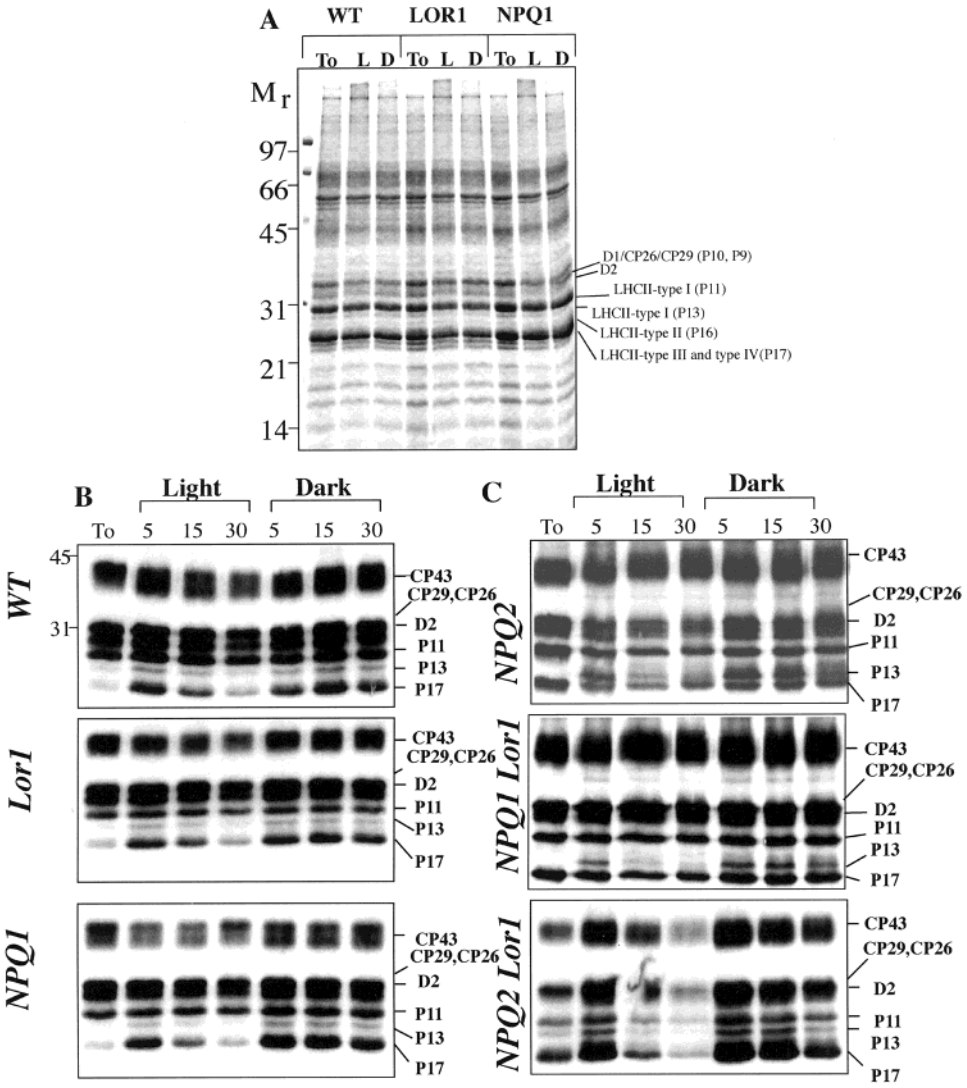

FIGURE 4: Effect of preillumination on the loss of LHCII phosphorylation in thylakoids of mutants impaired in carotenoid synthesis. Thylakoids of wt *C. reinhardtii* and mutants deficient in synthesis of different xanthophylls (shown in Table 1) were incubated in darkness or exposed to 150 μmol of photons m^{-2} s^{-1} in the presence of 10 μM DCMU for 30 min. The thylakoid polypeptides were then resolved by SDS−PAGE using 15% polyacrylamide gels and stained with Coomassie brilliant blue (A). (B and C) Immunoblots of thylakoids incubated in darkness (Dark) or preilluminated (Light) for the indicated times followed by phosphorylation for 20 min in darkness with nonradioactive ATP in the presence of duroquinol. Only the region of the polypeptides from 20 to 50 kDa is shown in panels B and C.

sites of polypeptides P13 and P17 to the protein kinase are similar.

It has been reported that extensive photoinactivation of PSII and the ensuing degradation of its core protein D1 cause dissociation of LHCII from PSII and disassembly of PSII (*60*, *61*). Such conditions occur particularly when using isolated thylakoids in which the repair of PSII is inhibited in absence of *de novo* D1 protein synthesis (*1*). The relatively low light intensities and the high chlorophyll concentration used during the thylakoid preillumination induced only a limited photoinactivation of PSII (*62*). The degradation of the D1 protein as estimated from the loss of the stained D1 band following preillumination of thylakoids of wt and the *lor1* and *npq1* mutants was weaker than the detection sensitivity by immunodecoration with anti-D1 protein antibodies. However, partial loss of the D1 protein was detected in the *npq2* thylakoids (Figure 5), and a marginal loss of the protein also occurred during the illumination of the thylakoids of the *npq2 lor1* double mutant (not shown). At present, we

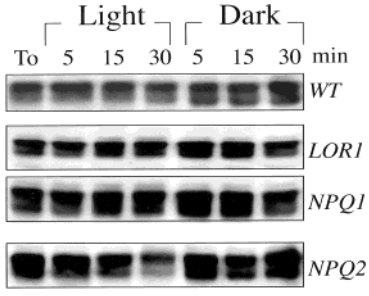

FIGURE 5: Stability of the D1 protein during preillumination of thylakoid membranes. Thylakoid suspensions were preilluminated as described in Materials and Methods, and samples were withdrawn and reactions stopped by addition of the sample buffer followed by SDS−PAGE. The gels were blotted onto nitrocellulose paper, and the D1 protein was detected by immunodecoration with anti-D1 protein antibodies.

cannot explain the loss of the D1 protein band of the *npq2* and *npq2 lor1* mutants as compared to the stability of these proteins in thylakoids of the wt and *lor1* and *npq1* mutants.

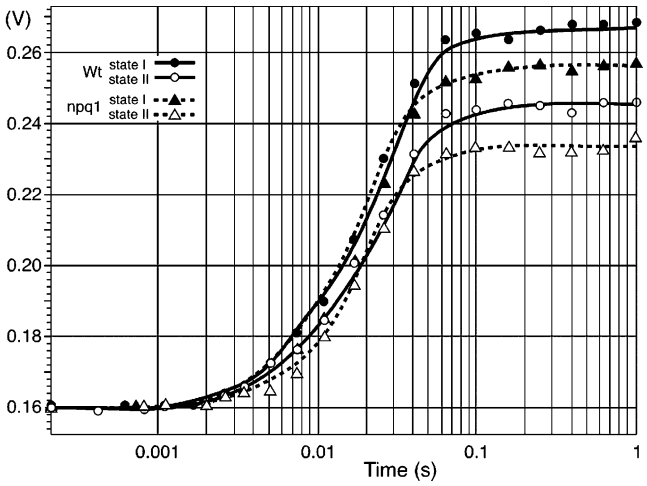

FIGURE 6: State transition in wt and mutant cells. Fluorescence induction of wt and *npq1* cells in state I and state II was recorded as described in Materials and Methods. The calculated extents of fluorescence loss following the transition from state I to state II were 24 and 22% for the wt and *npq1* cells, respectively.

Part of the D1 protein loss could be only apparent and due to aggregation of the SDS-solubilized protein. The nature of this property of the *npq2* and *npq2 lor1* mutant thylakoids requires further investigation.

Since LHCII polypeptides are phosphorylated in the xanthophyll mutants, the question of whether this process is accompanied by state transition and thus lowering of the fluorescence of cells following dissociation of LHCII from PSII arises (*31*). Transition from state I to state II resulted in reduction of the maximal fluorescence intensity of the wt and the *npq1* mutant as shown in Figure 6. Similar results were obtained for the other xanthophyll mutants used in this work. The loss of fluorescence intensity in state II relative to state I varied between 22 and 27% (data not shown). One should note that while the apparent loss of the D1 protein band may be ascribed to the protein degradation during the preillumination of the *npq2* thylakoids, the D1 protein level is maintained in the intact cells in which state transition is achieved by changes in the plastoquinone to plastoquinol ratio in darkness, and thus, the level of the D1 protein and assembly of PSII are not affected.

In the experiments described above, we have used wild-type and mutant cultures grown at relatively low light intensities (20 μmol of photons m^{-2} s^{-1}). To detect whether illumination may affect the xanthophyll composition of the thylakoids, cells grown in low light were harvested, washed free of acetate, and resuspended in acetate-free medium at a chlorophyll concentration of 30 μg/mL followed by incubation with 750 μmol of photons m^{-2} s^{-1} at 25 °C on a rotary shaker (150 rpm) for 3 h. The cells were then rapidly cooled, and thylakoids were prepared and exposed to the preillumination treatment followed by an assay of the residual phosphorylation activity. The carotenoid contents of the cells grown in low light as well as of cells at the end of the high light exposure and of the thylakoids at the end of the preillumination treatment were determined by HPLC analysis. The carotenoid content of the thylakoids following the preillumination treatment was similar to that of the whole cells at the end of the high light exposure (not shown). The zeaxanthin content of the wild-type cells exposed to high light levels increased (Table 1) as expected when compared to that of the cells grown in low light (*7*). Preliminary data indicate that the effect of preillumination of the thylakoids on the subsequent phosphorylation of the LHCII in darkness was similar to that of the thylakoids obtained from the cells grown in low light (not shown).

The light-induced effects are not altered in mutants lacking zeaxanthin and antheraxanthin (*npq1*), carotenoids that are present in limited amounts also in the wild type. Deficiency in lutein, the major xanthophyll of LHCII and loroxanthin, is balanced by the presence of the β-carotene-derived xanthophylls, as is the case in the *lor1* mutant. The most striking result is the fact that the pattern of LHCII polypeptide phosphorylation is not significantly altered in thylakoids containing only zeaxanthin, as is the case in the *npq2 lor1* mutant. These results suggest that the structural properties imposed on the LHCII transmembrane domain by the alteration of its carotenoid composition involved in energy transfer and dissipation do not influence significantly the N-terminal domain involved in LHCII phosphorylation and state transition. This is in agreement with the hypothesis that the light-induced effects on the exposure of the LHCII phosphorylation site are of a general nature and occur in the thylakoids of unicellular green alga *C. reinhardtii* as in higher-plant LHCII (*1*, *33*). The results obtained in this work indicate that exposure of *C. reinhardtii* LHCII *in situ* to the light may induce changes in the complex resulting in the inaccessibility of the phosphothreonine phosphorylation sites at the N-terminal domain of P13 and P17 to the protein kinase(s). This effect is not influenced by the xanthophyll composition and content of LHCII and may play a role in the reported reduction of the level of LHCII phosphorylation seen in pea thylakoids *in situ* or *in vivo* when exposed to saturating light intensities (*33*). It is interesting to note that the structure of spinach LHCII resolved at 2.72 Å shows only the α-carbon trace of the N-terminal domain from Ser14 to Asp54 (*63*). Thus, even in the crystal state, this domain maintains a certain degree of disorder while the freedom of orientation of the remaining N-terminal region containing the phosphorylation site completely prevented its resolution. These results support the concept that the orientation or interaction of this domain with the N-termini of adjacent monomers within the LHCII trimer and/or intertrimers may be modulated in response to variable external factors. The light-induced changes in the LHCII terminal domain preventing its phosphorylation are highly temperature dependent (*33*), suggesting the possibility that local temperature changes due to the thermal energy dissipation by the transmembrane domain may be involved in the process (*56*).

LHCII can thus be visualized as possessing two functionally distinct domains, each responsible for a specific role. The hydrophobic transmembrane domain is involved in light absorption, energy transfer, and dissipation, while the exposed stromal hydrophilic domain governs its lateral movements and interaction with the other transmembrane complexes. This function is achieved via modulation of its conformation by reversible phosphorylation and light-induced conformational changes. Since light absorption is in the realm of the transmembrane domain, handling of the absorbed energy is related to the pigment composition and structural organization of the transmembrane domain. However, the light-induced changes in the N-terminal domain are not significantly affected by the changes in these parameters.

Thus, the "autonomy" of the phosphorylation site and the processes it governs prevent the interference of the changes induced by the xanthophyll cycle in the LHCII pigment composition with the state transition process.

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