Phosphorylation−Dephosphorylation of Light-Harvesting Complex II as a Response to Variation in Irradiance Is Thiol Sensitive and Thylakoid Sufficient: Modulation of the Sensitivity of the Phenomenon by a Peripheral Component†

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ABSTRACT: Downregulation of phosphorylation of chlorophyll *a*/*b*-binding proteins (LHCII) of the photosystem II at high irradiance could only be demonstrated with leaf discs but not in isolated thylakoids. The present view suggests this phenomenon to be regulated by stromal thioredoxin. Here, we show that high-light inactivation of LHCII phosphorylation can be reproduced in isolated thylakoids and have explained the apparent absence of inactivation in vitro to be due to the derepressed activity of a peripheral kinase. We investigated this phenomenon with *Arachis hypogea* thylakoids prepared with (*Th:A*) or without (*Th:B*) tricine, where tricine is known for removing peripheral proteins from thylakoids. While LHCII remained phosphorylated at high irradiance in *Th:B*, the response of *Th:A* mimicked *Arachis* leaflets where LHCII was transiently phosphorylated with irradiance. LHCII phosphorylation in *Th:A* was sensitive to thiol reducing conditions, but in *Th:B*, the phenomenon became insensitive to thiol reduction following illumination. Washing *Th:B* with tricine made them resemble *Th:A*, and conversely, *Th:A* reconstituted with the Tricine extract resembled *Th:B* with respect to both irradiance response and thiol sensitivity. In vitro phosphorylation reactions indicated a thiol insensitive kinase activity to be present in the Tricine extract that was capable of phosphorylating histone H1 as well as purified LHCII. This peripherally associated kinase activity explained the sustenance of LHCII phosphorylation as well as its thiol insensitivity at high irradiance in *Th:B* thylakoids. Contrary to the current view, our results clearly show that irradiance dependent phosphorylation and dephosphorylation of LHCII is a thylakoid sufficient phenomenon, although it remained open to regulation by thiol redox state modulation.

The chlorophyll *a*/*b*-binding proteins Lhcb1 (27 kD) and Lhcb2 (25 kD), designated together as the light-harvesting complex II (LHCII),[1] are the most abundant chlorophyll binding proteins in thylakoid membranes (*1*). A neat balance between the amount of light harvested with the amount utilized and dissipated is thought to be brought about by reversible phosphorylation of these proteins by thylakoid kinases mediating various environmental cues. Primarily, the LHCII proteins are implicated in State I−State II transitions at low light intensities where the consequence of phosphorylation appears to be the redirection of excitation to photosystem I (PSI) at the expense of photosystem II (PSII), thereby balancing the excitation energy distribution between these photosystems (*2*−*5*). In the presence of excess energy, state transitions appear to have less significance, and LHCII dephosphorylation is speculated to favor its aggregation to promote energy dissipation through nonphotochemical quenching as a protection against photodamage (*6*). LHCII phosphorylation also helps in long-term acclimation of plant cells to different levels of light (*7*) and in optimizing the production of ATP and NADPH in relation to metabolic requirements of the cell (*2*).

The literature on LHCII phosphorylation at present suggests three different branches in the signaling system that determines the net level of phosphorylated LHCII in the thylakoids. The first branch constitutes the LHCII kinase(s). The primary signal transduction loop, which connects the light driven electron flow with the activation of LHCII kinase(s), involves the interaction of reduced plastoquinone with the quinol oxidation site of the cytochrome *bf* complex, where the electron carriers of the high potential path, the rieske Fe−S center, and cytochrome *f* are reduced (*2*, *8*, *9*). The action of this kinase(s) initiates the chain of events that determines the level of phosphorylated LHCII in response to changing environmental cues. Multiple LHCII kinases (e.g., TAKs in *Arabidopsis* (*10*, *11*) and Stt7 in *Chlamydomonas* (*12*)) are now identified. All these kinases have been demonstrated to have a role in light harvesting through the photosystems, but molecular dissection of their activities has not been possible, and which of them is the direct target of the redox system is yet to be found. Light-induced inaccessibility of the phosphorylation site of LHCII to the LHCII kinase(s) appears to be the second branch of the

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[1] Abbreviations: LHCII, chlorophyll *a*/*b*-binding proteins of photosystem II antenna; DTTred, reduced dithiothreitol; DTTox, oxidized dithiothreitol; NEM, *N*-ethylmaleimide; p-, phosphorylated form of proteins; μE m^{-2} s^{-1}, μmol of photons m^{-2} s^{-1}; *Th:A*, thylakoid A; *Th:B*, thylakoid B; PSI, photosystem I; PSII, photosystem II; NPQ, nonphotochemical quenching.

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signaling system that determines the level of phosphorylated LHCII (*13*, *14*). This phenomenon has recently been claimed to substantially contribute to the transient loss of LHCII phosphorylation observed under high light conditions in vivo. The substrate mediated regulation is presumed to be further complicated considering differential phosphorylation of various other LHCII polypeptides in response to irradiance or environmental cues (*2*). The third branch of the signaling system is the thiol redox state of the chloroplasts where the mediators are chloroplast thioredoxins (*15*, *16*). Thioredoxins, when reduced in the presence of light by ferredoxin−thioredoxin reductase, induce reduction of dithiol groups of LHCII kinase(s), thereby inactivating them. This mechanism of LHCII kinase inactivation has also been claimed to be the molecular principle behind the downregulation of LHCII phosphorylation under high light conditions in vivo (*15*−*18*). How the different branches of the signaling network interact in response to environmental cues, to modulate the net level of phospho-LHCII for determining the light harvest by the individual photosystems, is far from being understood.

We have investigated the irradiance response of LHCII phosphorylation in *Arachis hypogea*, both in intact leaflets and in isolated thylakoids. It was found that the phenomena significantly differed depending on peripherally associated proteins in the isolated thylakoids. While the present view suggests a downregulation of LHCII phosphorylation at higher irradiance to be regulated by the ferredoxin−thioredoxin system, we have shown that LHCII phosphorylation−dephosphorylation as a function of irradiance is a thylakoid sufficient phenomenon. Stromal assistance was not required for its downregulation at higher irradiance, although it remained open to regulation by thiol redox state modulation. The apparent absence of the downregulation of LHCII phosphorylation at higher irradiance and its insensitivity to thiol reduction in illuminated thylakoids were found to be coupled properties of isolated thylakoids. These properties of isolated thylakoids appeared to be due to another peripherally associated LHCII kinase activity that was thiol insensitive in nature. Our investigation has thus been able to dissect two distinct activities that lead to LHCII phosphorylation in thylakoid membranes in response to irradiance.

MATERIALS AND METHODS

Plant Material. A. hypogea plants were grown in a growth chamber at 25 °C with a 16 h photoperiod under 40 μmol of photons m^{-2} s^{-1}. Leaflets of an average diameter of 1 cm were collected from 3−4 week old plants.

Light Treatment of Arachis Leaflets. Leaflets were collected from *A. hypogea* plants dark-adapted for 16 h. They were floated on distilled water in a Petri dish with the adaxial surface up and were illuminated in a light chamber at 25 °C under various irradiances for different time periods as indicated. The leaves were then rapidly frozen in liquid nitrogen until the isolation of the thylakoid membranes.

Isolation of Thylakoid Membranes. Thylakoids were isolated from dark-adapted leaflets by two methods: (i) thylakoid A (*Th:A*)—via intact chloroplast according to Bennett et al. (*19*) and (ii) thylakoid B (*Th:B*)—direct isolation according to Rintamaki et al. (*20*). The final pellets of *Th:A* and *Th:B* were washed and suspended in storage buffer-A (0.1 M sorbitol, 10 mM Tricine-NaOH, pH 7.8, 10 mM $MgCl_2$, 1 mM KCl) and storage buffer-B (10 mM Hepes-NaOH, pH 7.5, 100 mM sucrose, 5 mM NaCl, 10 mM $MgCl_2$). They were snap cooled in liquid N_2 and stored at −80 °C. Both the preparations were protected from light and kept ice-cold during the isolation procedure.

Detection of LHCII Phosphorylation in Intact Arachis Leaflets (in Vivo) and in Isolated Thylakoids (in Vitro). (i) In Vivo. Thylakoids isolated according to Rintamaki et al. (*20*) from differentially light-treated leaflets were solubilized in SDS−PAGE sample buffer, and the polypeptides were analyzed in 12% SDS−PAGE (*21*) containing 6 M urea. Routinely, 10 μg of chlorophyll was loaded into each well. LHCII phosphorylation was immunodetected using polyclonal phosphothreonine specific antibody (New England Biolabs) (*17*). All blots were developed using a BM Chromogenic Western blotting kit (Roche Biochemicals).

(ii) In Vitro. Thylakoids isolated from dark-adapted leaves (*19*, *20*) were subjected to in vitro phosphorylation reactions (*22*, *23*) by incubating them in respective storage buffers supplemented with 0.2 mM ATP and 200 μCi of [γ-^{32}P]-ATP mL^{-1} at a chlorophyll concentration of 0.4 mg mL^{-1} at 25 °C under various intensities of light and for various periods as indicated in the figures and analyzed in 12% SDS−PAGE (*21*) containing 6 M urea followed by autoradiography.

To chase the LHCII kinase activity, thylakoids were subjected to illumination at 200 μmol of photons m^{-2} s^{-1} for various periods, [γ-^{32}P]ATP was added at indicated periods, and incubation continued for two more minutes in the presence of light. To prevent the phosphatase activity during these experiments, thylakoids were preincubated with 10 mM NaF for 20 min in the dark prior to light treatment. Lhcb2 was identified through Western blot using polyclonal anti-Lhcb2 antibody (Agrisera).

Washing of Th:B with 10 mM Tricine and Reconstitution of Th:A and Tricine Washed Th:B (Th:B^{TW}) with the Tricine Extract. Th:B was gently suspended in 10 mM Tricine-NaOH, pH 7.8 (1:2.5, v/v). The suspension was centrifuged at 3000*g* for 3 min. The washing was repeated once again, and the supernatants were saved. The washed thylakoids were suspended in storage buffer-B and subjected to in vitro phosphorylation reactions under indicated conditions.

The supernatants from the above washings were pooled, and the extract was concentrated by dialysing against storage buffer-B containing 50% glycerol (Pharmacia). The concentrated extract was dialyzed against 0.1X storage buffer-B and then further concentrated using a speed vacuum to reduce its volume by 20 times its initial volume. For reconstitution experiments, *Th:A* and tricine washed *Th:B* (*Th:B*^{TW}) were incubated with this Tricine extract (1:1 v/v) by keeping in ice at 4 °C for overnight. The reconstituted thylakoids were then subjected to in vitro phosphorylation reactions under the indicated conditions.

In Vitro Phosphorylation Reactions by the Tricine Extract. For histone phosphorylation, reactions were performed according to DasGupta (*22*) in 50 mM Tris, pH 8.0, 10 mM $MgCl_2$, 10 mM NaCl, 10 mM NaF supplemented with 0.2 mM [γ-^{32}P]ATP (4000 cpm/pmol) using histone H1 (0.4 μg/μL) (Life Technologies) as substrate. Tricine extract was added at a volume of 1/3 of the final reaction mixture. The reactions were carried out for 20 min and stopped by SDS−PAGE sample buffer.

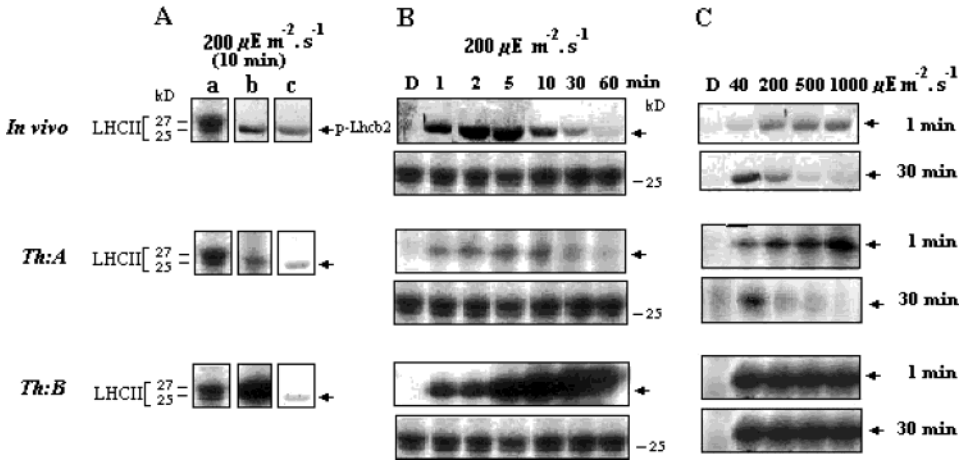

FIGURE 1: Irradiance dependent phosphorylation and dephosphorylation of LHCII. *A. hypogea* leaflets (in vivo) or thylakoids (*Th:A* and *Th:B*) were illuminated at different irradiances for indicated periods. In vivo p-LHCII was detected by immunoblotting with phosphothreonine specific antibody. In vitro p-LHCII was detected by [γ-^{32}P]ATP. (A) Among the LHCII polypeptides (Lhcb1 and Lhcb2), Lhcb2 is phosphorylated. Coomassie blue stained gel pattern of isolated thylakoids (lanes a); detection of p-Lhcb2 (lanes b); and immunoblot using Lhcb2 specific antibody (lanes c). LHCII phosphorylation as a function of (B) duration of incident irradiance—lower panels indicate Coomassie blue stained gel patterns, and upper panels indicate detection of p-LHCII, (C) intensity of incident irradiance—upper and lower panels indicate detection of p-LHCII at 1 and 30 min, respectively. All results are representative of duplicate sets of experiments with triplicate isolations. ← indicates p-Lhcb2.

For LHCII phosphorylation, native LHCII was purified from 3−4 week old *A. hypogea* leaflets according to Burke et al. (*24*) and Mullet (*25*) and stored at −80 °C in 0.5% octyl-β-D-glucoside. The phosphorylation reaction mixture contained 50 mM Tris, pH 8.0, 10 mM $MgCl_2$, 10 mM NaCl, 10 mM NaF, 0.5 mM 3-[(chloroamido propyl)dimethylammonio]-1-propansulfonate, 0.01% Triton X-100 supplemented with 0.2 mM [γ-^{32}P]ATP (4000 cpm/pmol), and chl-protein substrate (LHCII) equivalent to 5 μg of chlorophyll (*13*). Tricine extract was added at a volume of 1/3 of the final reaction mixture. The reactions were carried out for 20 min and stopped by SDS−PAGE sample buffer.

Chlorophyll Determinations. Chlorophyll was extracted in 80% buffered acetone and determined according to Hipkins and Baker (*26*).

RESULTS

Irradiance Dependent Phosphorylation and Dephosphorylation of LHCII: in Vivo and in Vitro. LHCII phosphorylation and dephosphorylation were monitored as a function of irradiance both in vivo and in vitro by subjecting intact leaflets or two different thylakoid preparations (*Th:A* and *Th:B*) to variable light treatments (Figure 1). While Lhcb1 (27 kD) and Lhcb2 (25 kD) (collectively referred to as LHCII) together were the most abundant proteins in the Coomassie stained gels (Figure 1A, lanes a), only Lhcb2 (25 kD) was phosphorylated in response to 200 μmol of photons m^{-2} s^{-1} for 10 min both in vivo and in vitro (Figure 1A, lanes b). The identity of Lhcb2 was confirmed through its cross-reaction with Lhcb2 specific antibody (Figure 1A, lanes c). When monitored as a function of duration of 200 μmol of photons m^{-2} s^{-1} irradiance, the maximal phosphorylation of LHCII was observed in intact leaflets after 2−5 min of light treatment. By 10 min, it started dephosphorylating and was completely dephosphorylated by 60 min (Figure 1B, in vivo). Under similar light treatments, LHCII phosphorylation and dephosphorylation in *Th:A* were identical to its in vivo response (Figure 1B, *Th:A*), but with *Th:B* no downregulation of LHCII phosphorylation was observed even after 60 min (Figure 1B, *Th:B*). As a function of intensity of irradiance, LHCII phosphorylation was monitored for two different time periods: 1 min, at the onset of the phosphorylation, and 30 min, with the dephosphorylation in progress (Figure 1C). The results were again same for intact leaflets and *Th:A*. In both these cases, at the early time point the level of LHCII phosphorylation was found to increase with the increase in intensity of light. The observation was reverse in the later time point, where with the increase of intensity of light, the level of phosphorylated LHCII (p-LHCII) decreased (Figure 1C, in vivo and *Th:A*). The total time required for LHCII phosphorylation and subsequent dephosphorylation thus appeared to be inversely proportional to the intensity of incident irradiance. However, results obtained with *Th:B* were distinctly different. There was no change in the level of LHCII phosphorylation with the change of intensity of light both in the early and in the later time period (Figure 1C, *Th:B*). Interchanging the storage buffers during in vitro phosphorylation reactions did not affect the nature of irradiance response in *Th:A* and in *Th:B*, indicating the thylakoid components per se to be responsible for differential LHCII phosphorylation in the preparations (data not shown). As per the current view, which suggests stromal factors to have a role in the downregulation of LHCII phosphorylation at higher irradiance, it is possible that *Th:A* is copurified with stromal factors that positively influenced the phenomenon. The other possibility of *Th:B* being copurified with factors that negatively influenced the phenomenon remained open at this point.

LHCII Kinase versus Phosphatase Activity in Th:A and Th:B. The inability of *Th:B* to show the downregulation of LHCII phosphorylation under higher or prolonged irradiance could be due to (i) failure in inactivating the LHCII kinase, or (ii) failure in activating the LHCII phosphatase, or (iii) both. To clarify these possibilities, the phosphorylation of LHCII was monitored for periods of 2 min at various intervals with both *Th:A* and *Th:B* in the presence and absence of phosphatase inhibitor NaF under 200 μmol of photons m^{-2} s^{-1} (Figure 2). In *Th:A*, p-LHCII was detectable

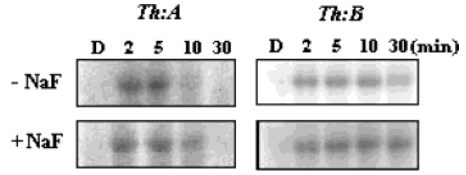

FIGURE 2: LHCII kinase vs phosphatase activity in *Th:A* and *Th:B*. To chase the LHCII kinase activity, LHCII phosphorylation was monitored with [γ-^{32}P]ATP in 200 μmol of photons m^{-2} s^{-1} or in dark (D) for 2 min at the indicated periods in the absence and presence of NaF as described in the Materials and Methods. Results are representative of duplicate sets of experiments with triplicate isolations.

up to the 5−7th min interval, which extended to 10−12th min in the presence of NaF indicating that the LHCII kinase remains active for 10−12 min after being exposed to light (Figure 2, *Th:A*). It also suggested the activation of the cognate phosphatase to precede inactivation of the kinase. In similar chasing experiments with *Th:B*, p-LHCII was detectable over the entire span of 30 min, indicating the kinase to be active throughout the period of investigation in these thylakoids (Figure 2, *Th:B*). The relative increase in p-LHCII in the presence of NaF in *Th:B* indicated the phosphatase to be active in these thylakoids along with the kinase. The sustenance of LHCII phosphorylation in *Th:B* under prolonged light exposure thus appears to be due to persistence of the LHCII kinase activity in these thylakoids, which become inactivated in *Th:A* (Figure 2, *Th:A*) and in intact leaves as well (Figure 1B).

Effect of Thiol Redox State on LHCII Phosphorylation in Th:A and Th:B. Recently, it has been reported that LHCII phosphorylation is regulated by the thiol redox state of the thylakoids and is suggested to occur in vivo through the stromal ferredoxin−thioredoxin system (*15*−*17*). We found that *Th:A* and *Th:B* significantly differed in their sensitivity to the thiol redox state (Figure 3). DTTred added in the dark-adapted state inhibited LHCII phosphorylation in both the thylakoids (Figure 3A), but it could inhibit the phenomenon only in *Th:A*, when added in illuminated thylakoids (Figure 3B). Under similar conditions, DTTox did not have any effect on LHCII phosphorylation, indicating the inhibition in the presence of DTTred to be due to its thiol reducing property (Figure 3A,B). Also, the addition of *N*-ethylmaleimide (NEM) in the dark-adapted thylakoids did not have any inhibitory effect on LHCII phosphorylation in any of the two thylakoid preparations, indicating that the regulatory thiols involved in the inhibition are oxidized in dark-adapted thylakoids and thus are not accessible for reaction with NEM (Figure 3A). With the preilluminated thylakoids, substituting DTTred for NEM did not affect LHCII phosphorylation in either of the thylakoid preparations (Figure 3B). In *Th:A*, this indicated that the target regulatory thiols maintain their oxidized state even after the thylakoids were illuminated maintaining their inaccessibility to NEM. The insensitivity of preilluminated *Th:B* to DTTred as well as NEM indicated thiol groups to have no positive role in determining LHCII phosphorylation in the illuminated form of these thylakoids. To understand if thiol reduction was associated with the downregulation of LHCII phosphorylation at prolonged irradiance in *Th:A*, we next investigated whether DTTox had any inhibitory effect on this phenomenon. As shown in Figure 3C, DTTox added in preilluminated thylakoids could

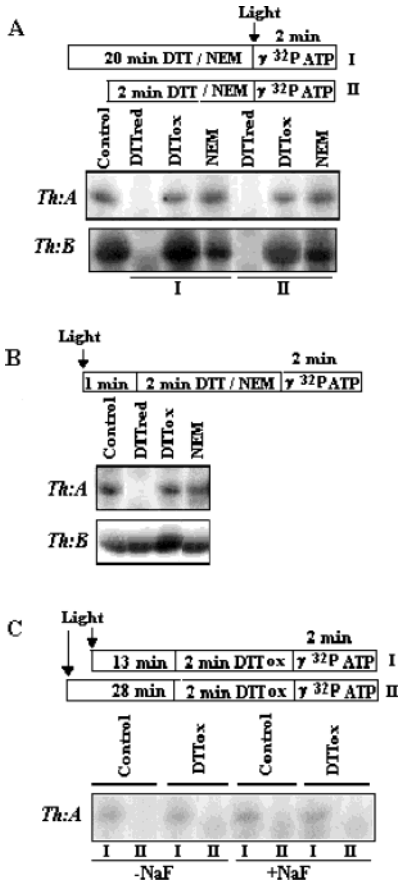

FIGURE 3: Effect of thiol reagents on LHCII phosphorylation in *Th:A* and *Th:B*. (A) In dark-adapted thylakoids. Thylakoids were preincubated without (control) or with 10 mM DTTred or 10 mM DTTox or 0.1 mM NEM in the dark for 20 min (I) or 2 min (II) prior to the phosphorylation assay in the presence of 200 μmol of photons m^{-2} s^{-1} and [γ-^{32}P]ATP for 2 min. (B) In illuminated thylakoids. Thylakoids were illuminated at 200 μmol of photons m^{-2} s^{-1} for 1 min. Thiol reagents were subsequently added, and incubation continued for two more minutes before initiation of the phosphorylation assay. (C) DTTox fails to prevent the dephosphorylation of LHCII of *Th:A* in prolonged irradiance. *Th:A* preincubated with or without NaF in the dark was illuminated in 200 μmol of photons m^{-2} s^{-1} for 13 min (I) or 28 min (II). DTTox was subsequently added (lanes marked DTTox), and incubation continued for two more minutes before initiation of the phosphorylation assay. Control lanes indicate incubation under identical conditions without DTTox. The results are representative of duplicate sets of experiments with triplicate isolations.

neither prevent nor delay the downregulation of LHCII phosphorylation both in the presence and in the absence of NaF under prolonged irradiance. This entire experiment was also performed using thylakoids that were preincubated with DTTox for 20 min in dark keeping DTTox present throughout the period of illumination. The results were same (data not shown), indicating that even prolonged DTTox incubation failed to prevent downregulation of LHCII phosphorylation at prolonged irradiance. Our results thus suggest that the kinase in isolated thylakoids (*Th:A*) is irreversibly inactivated by a mechanism that is distinct from a simple thiol reduction.

Restoration of Irradiance Dependent LHCII Phosphorylation After Inactivation of LHCII Kinase upon Prolonged Irradiation: in Vivo and in Vitro. Results in the preceding section have shown that the irradiation response of LHCII phosphorylation in *Th:A* thylakoids in vitro mimicked the response observed in vivo in *Arachis* leaflets (Figure 1). In

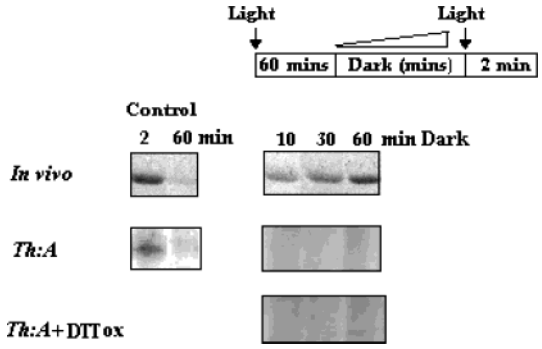

FIGURE 4: Restoration of the phosphorylation capacity of LHCII polypeptides in the dark after inactivation of the LHCII kinase on prolonged irradiance. *A. hypogea* leaflets (in vivo) or thylakoids (*Th:A*) were illuminated in 200 μmol of photons m^{-2} s^{-1} for 60 min followed by dark incubation for indicated time periods. Restoration of LHCII phosphorylation was then followed for 2 min in 200 μmol of photons m^{-2} s^{-1}. In vivo p-LHCII was detected by immunoblotting with phosphothreonine specific antibody. In vitro p-LHCII was detected by adding [γ-^{32}P]ATP during rephosphorylation where dark incubation was done in the absence and presence of 10 mM DTTox. Control lanes indicate LHCII phosphorylation in the presence of 200 μmol of photons m^{-2} s^{-1} for indicated periods. The results are representative of duplicate sets of experiments with triplicate isolations.

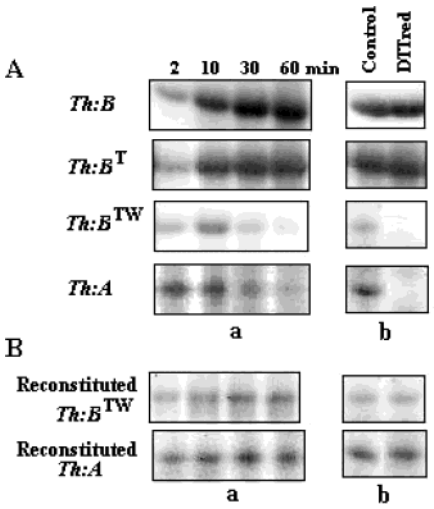

FIGURE 5: Peripheral factor(s) copurified with *Th:B* determine the irradiance and thiol sensitivity of the isolated thylakoids. (A) Irradiance and thiol sensitivity of tricine washed *Th:B* (*Th:B*^{TW}) as compared to *Th:A* and *Th:B*. *Th:B* washed with 10 mM tricine suspended in storage buffer-B was subjected to in vitro phosphorylation reactions with [γ-^{32}P]ATP under 200 μmol of photons m^{-2} s^{-1} for indicated time periods (panels a) and in the presence of DTTred added in preilluminated thylakoids (panels b) as described in Figure 3B. *Th:B*^{T} represents irradiance and thiol sensitivity of *Th:B* in the presence of 10 mM Tricine. (B) Irradiance and thiol sensitivity of *Th:A* and *Th:B*^{TW} reconstituted with the Tricine extract. Preparation of the Tricine extract from *Th:B* and reconstitution with this extract was described in Materials and Methods. The reconstituted thylakoids were subjected to in vitro phosphorylation reactions with [γ-^{32}P]ATP under 200 μmol of photons m^{-2} s^{-1} for indicated periods (panels a) and in the presence of DTTred added in preilluminated thylakoids (panels b). The results are representative of two different attempts of washing and reconstitution with two different isolates.

both cases, LHCII phosphorylation was downregulated in the presence of prolonged/high irradiance. These isolated thylakoids, however, were distinctly different from the leaflets in their ability to restore LHCII phosphorylation after being inactivated in the presence of prolonged irradiance (Figure 4). While incubation in darkness for 60 min could completely revive the irradiation dependent LHCII phosphorylation in vivo in *Arachis* leaflets (Figure 4, in vivo), such treatments failed to show any restoration of the phenomenon in vitro in *Th:A* thylakoids even after prolonged incubation in darkness (Figure 4, *Th:A*). The presence of DTTox during dark incubation did not have any effect in restoring LHCII phosphorylation in isolated *Th:A* thylakoids, suggesting again that the mode of inactivation of the LHCII kinase in *Th:A* under prolonged irradiance was different from simple thiol reduction (Figure 4, *Th:A* + DTTox). These observations clearly demonstrate that the complete regulation of LHCII phosphorylation in response to irradiance is regulated by a complex network involving both thylakoid bound and stromal factors. While *Th:A* thylakoids appear to be complete with respect to sensing the intensity and/or duration of irradiance (Figure 1B, *Th:A*), without stromal association, they fail to recover their potential to rerespond to irradiance after being inactivated (Figure 4). On the other hand, experiments on the rephosphorylation of LHCII could not be undertaken with *Th:B* as it failed to show any appreciable dephosphorylation even after prolonged incubation in darkness (data not shown). Such a variation of irradiance response in isolated thylakoids attests to the view that the protein composition of the isolated thylakoids has important roles in the irradiance response of the photosynthetic machinery.

Peripheral Factor(s) Copurified with Th:B Determined the Irradiance and Thiol Sensitivity of the Isolated Thylakoids. The difference in irradiance response and thiol sensitivity of LHCII phosphorylation in illuminated thylakoids was felt to be due to the nature of the peripheral proteins that were copurified with them. Following the present view, we expected stromal factors associated with *Th:A* to enable it to mimic the in vivo irradiance response. We therefore subjected *Th:A* to treatments such as 10 mM sodium-pyrophosphate, pH 7.8 (*27*), 2 M NaCl (*28*), 2 M KSCN (*28*) that are known to remove peripheral proteins from thylakoid membranes and then investigated the irradiance response and thiol sensitivity of the washed thylakoids. Contrary to such expectations, these treatments did not allow *Th:A* to behave like *Th:B* (data not shown). Instead, a simple wash with 10 mM tricine, which is also known for removing thylakoid peripheral proteins (*27*, *28*), enabled the washed *Th:B* (*Th:B*^{TW}) to mimic *Th:A* both with respect to its irradiance (Figure 5A, panels a) and with respect to its thiol sensitivity (Figure 5A, panels b). The irradiation response of *Th:B*^{TW} was analyzed in storage buffer-B in the absence of tricine, eliminating the possibility of the presence of tricine being inhibitory toward LHCII phosphorylation at prolonged irradiance. Attesting to this fact, the irradiation response of LHCII phosphorylation in *Th:B* did not change in the presence of 10 mM tricine (Figure 5A, *Th:B*^{T}). At this point, our data indicated negatively acting factors to be associated with *Th:B*, whose removal ensured the downregulation of LHCII phosphorylation at prolonged irradiance as well as its sensitivity to thiol reducing conditions in preilluminated thylakoids. We next investigated whether reconstituting the *Th:A* or *Th:B*^{TW} with the tricine extracted peripheral proteins could impose the properties of *Th:B* in them with respect to these properties. As shown in Figure 5B, reconstituted *Th:A* and *Th:B*^{TW} allowed LHCII phosphorylation to persist in

these thylakoids under prolonged irradiance (Figure 5B, panels a) and turn insensitive to thiol reducing conditions in the illuminated state (Figure 5B, panels b). Both these reconstituted thylakoids, however, remained sensitive to DTTred in their dark-adapted forms (data not shown). Apart from proving LHCII phosphorylation−dephosphorylation as a function of irradiance to be a thylakoid sufficient phenomenon as demonstrated in *Th:A*, these results clearly indicate a parallel between the thiol sensitivity and the irradiation response of LHCII phosphorylation in isolated thylakoids.

It should be noted that during preparation, *Th:A* was washed and subsequently stored in 10 mM tricine buffer (*19*). It is possible that tricine selectively eliminated negatively acting factor(s) associated with *Arachis* thylakoids allowing *Th:A* to mimic the in vivo results and demonstrate the downregulation of LHCII phosphorylation at higher irradiance. Our results suggest that the same factor(s) are washed out from *Th:B* in the presence of tricine enabling it to behave like *Th:A* with respect to irradiance and thiol sensitivity. The fact that reconstituted *Th:B*^{TW} and *Th:A* were analyzed for irradiation response in storage buffer-B (Hepes) and storage buffer-A (tricine), respectively (Figure 5B, panels a), indicated that the mere presence of tricine is unable to dictate the abatement of LHCII phosphorylation in response to irradiance in isolated thylakoids.

Protein Kinase Activity in the Tricine Extract. In the preceding section, we have demonstrated that a simple wash with 10 mM tricine enabled *Th:B* to show the downregulation of LHCII phosphorylation at prolonged irradiance, thus mimicking the irradiation response of *Th:A* (Figure 5A). Alternatively, reconstitution of the washed *Th:B* (*Th:B*^{TW}) or *Th:A* with the Tricine extract prevented the downregulation of LHCII phosphorylation, or in other words, the Tricine extract restored the phosphorylation of LHCII at high irradiance in these thylakoid preparations (Figure 5B). These observations raised the possibility of the Tricine extract containing another LHCII kinase whose irradiance insensitive activity led to the persistence of LHCII phosphorylation in reconstituted *Th:A* or in *Th:B*^{TW} at prolonged irradiance. To check this possibility, we investigated for kinase activity in the Tricine extract. As shown in Figure 6A, lane 2, the extract showed a strong kinase activity toward the exogenous substrate histone H1 under standard assay conditions. The activity was found to be insensitive to thiol reagents, as it was not affected in the presence of DTTred or DTTox (Figure 6A, lane 3 and 4). The presence of NEM also had no effect on histone phosphorylation, indicating that reduced thiol groups have no influence on the catalytic activity of the kinase (Figure 6A, lane 5). The kinase reactions appeared to be light independent as these in vitro phosphorylation reactions showed similar results in the absence and presence of light (data not shown). The Tricine extract could also phosphorylate the Lhcb2 polypeptide of the purified native LHCII from *Arachis* leaflets indicating the kinase to be a LHCII kinase (Figure 6B). These results indicate the sustenance of LHCII phosphorylation under prolonged irradiance in vitro in *Th:B* to be due to this kinase activity in the extract. Its thiol insensitive activity explains the thiol insensitivity of LHCII phosphorylation in illuminated *Th:B* or reconstituted *Th:A*. These findings substantiate our results described in Figure 2, where we have shown that the absence

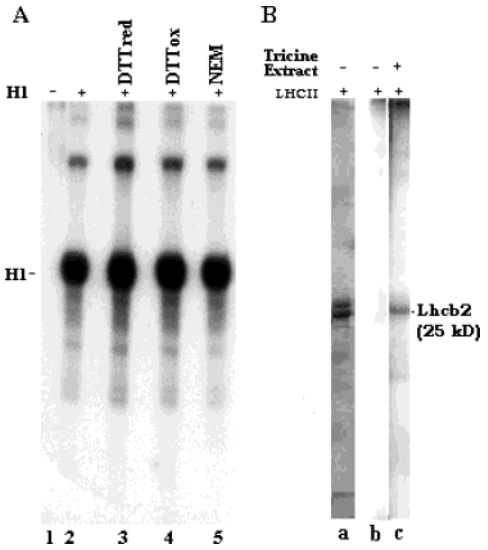

FIGURE 6: Kinase activity of the Tricine extract. (A) In vitro phosphorylation of histone H1 by the Tricine extract. Phosphorylation of histone H1 by the Tricine extract in the absence (lane 2) and presence of 10 mM DTTred (lane 3), 10 mM DTTox (lane 4), and 0.1 mM NEM (lane 5). (B) Phosphorylation of purified LHCII by the Tricine extract. Lane a: Coomassie blue stained gel patterns of isolated LHCII. Lanes b and c: phosphorylation of purified LHCII in the presence of [γ-^{32}P]ATP without or with the Tricine extract, respectively. Results are representative of duplicate sets of experiments with two different preparations of Tricine extract.

of the downregulation of LHCII in *Th:B* was due to the persistence of LHCII kinase activity.

DISCUSSION

In this paper, we investigated LHCII phosphorylation as a function of irradiances in intact *Arachis* leaflets and in isolated thylakoids. On the basis of our observations, we have delineated the properties of two distinct kinase activities that phosphorylate LHCII in the isolated thylakoids and have proposed a possible relation in their action with physiological relevance (Figure 7). The kinase activity associated with the irradiation response of *Th:A* is referred to as **K1,** and the peripheral kinase activity that is washed out in the presence of tricine from *Th:B* is referred to as **K2**. **K1** appeared to be an integral kinase of both *Th:A* and *Th:B* as the removal of **K2** from *Th:B* allowed it to mimic the irradiation response of *Th:A* (Figures 5A and 7A). Again, *Th:A* mimicked the irradiation response of intact *Arachis* leaflets, indicating that **K1** mediates the transient phosphorylation of LHCII in response to irradiance in vivo (Figure 1). Both in *Arachis* leaflets and in isolated thylakoids (*Th:A* and *Th:B*), LHCII phosphorylation occurred only in the presence of irradiance, indicating that **K1**is a strictly light-activated LHCII kinase (Figure 7B: **K1**, Figure 1). Thiol reducing conditions prevented the light-dependent activation of **K1**, indicating that oxidized thiol groups are essential in determining the associated conformational change (Figure 7B: **K1**, Figure 3A). The kinase remained sensitive to thiol reducing conditions even after being subjected to illumination, indicating that oxidized thiol groups are essential for also maintaining the active conformation of **K1** (Figure 7B: **K1**, Figure 3B). The downregulation of LHCII phosphorylation under prolonged irradiance was due to the inactivation of **K1** (Figure 7B: **K1**, Figure 2, *Th:A*). As the thiol oxidizing conditions

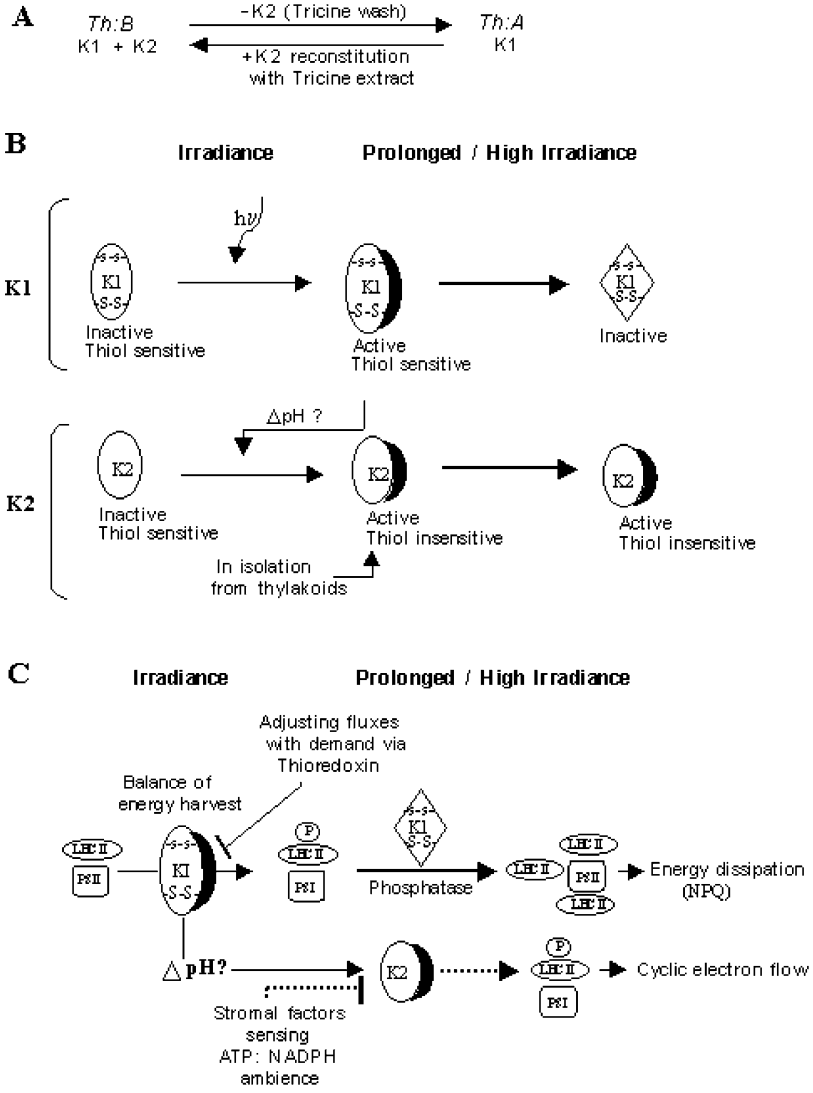

FIGURE 7: Schematic representation of the regulation of LHCII phosphorylation in response to irradiance in vitro and in vivo. (A) **K1** and **K2** are two different kinases regulating LHCII phosphorylation in response to irradiance. The irradiance sensing kinase firmly associated with isolated thylakoids (*Th:A* and *Th:B*) is termed as **K1**, and the peripheral kinase that is washed out in the presence of tricine from *Th:B* is termed as **K2**. (B) Regulation of **K1** and **K2** in response to irradiance in vitro. **K1** is an irradiance sensitive kinase with oxidizing thiol groups being important determinants of its activity both in dark-adapted and illuminated thylakoids. It is inactivated under prolonged/high irradiance by a mechanism distinct from simple thiol reduction. **K2** is irradiance and thiol insensitive in isolation from thylakoids. The irradiance and thiol sensitivity of **K2** in association with the thylakoids is explained by suggesting **K1** activity to be a prerequisite for **K2** activation. (C) Regulation of LHCII phosphorylation by **K1** and **K2** in vivo. In the presence of normal irradiance, **K1** phosphorylates LHCII to balance the net light harvest by the two photosystems (state transition). Although **K1** is inactivated in the presence of prolonged/high irradiance by a mechanism distinct from thiol reduction, it may be negatively regulated by a ferredoxin−thioredoxin system coordinating the illumination response with the metabolic signals. At high irradiance, dephosphorylation favors aggregation of LHCII to promote energy dissipation through NPQ from PSII. A cellular demand of ATP under high irradiance activates the irradiance and thiol insensitive kinase **K2** to maintain the phosphorylated state of LHCII to favor cyclic electron transport through PSI, which otherwise remains inactivated by stromal factors in ambient conditions. **K2** may be activated directly by **K1** dependent phosphorylation or indirectly through the ΔpH generated across the thylakoids under illuminated conditions. In panels B and C, thin and bold lines indicate reactions that occur in normal and prolonged irradiance, respectively. Dotted lines indicate irradiance independent reactions.

could not reverse this downregulation in *Th:A*, the mode of inactivation of **K1** does not appear to be due to simple thiol reduction (Figure 3C). Another independent mode of inactivation of **K1** is suggested to work in conjunction with thiol reduction, for downregulating **K1** in response to prolonged/high irradiance. A recent paper has demonstrated that the downregulation of LHCII phosphorylation at higher irradiance is substantially contributed by the occlusion of the phosphorylation site of the LHCII polypeptides under illumination (*14*). This does not appear to be a possibility in our results as *Th:B* thylakoids continued to show LHCII kinase activity under prolonged irradiance using the endogenous LHCII polypeptides as substrates (Figure 2, *Th:B*).

The properties of the peripherally associated kinase **K2** has been deciphered from the irradiation response of *Th:B* containing both **K1** and **K2** as compared to *Th:A*, which contains only **K1**. Stripping *Th:B* of **K2** allowed it to show the downregulation of LHCII phosphorylation in response to irradiance like *Th:A* (Figures 5A and 7A). In consonance, the reconstitution of *Th:A* with **K2** allowed LHCII to be phosphorylated under prolonged irradiance in these thylakoids where otherwise it was downregulated (Figures 5B and 7A). These observations indicated that **K2** maintains its active conformation at high irradiance and is responsible for

LHCII phosphorylation under such conditions (Figure 7B: **K2**). **K2** activity in isolation from thylakoids was insensitive to light as well as thiol reducing conditions. This is consistent with its maintenance of activity in the presence of high irradiance and also explains the thiol insensitivity of LHCII phosphorylation in *Th:B*, after being subjected to illumination (Figure 7B: **K2**, Figure 3B). The question, however, remained as to how LHCII phosphorylation in *Th:B* was strictly dependent on light and was sensitive to thiol reduction in dark conditions. We explained this conflict by suggesting that **K1** and **K2** act in series where the active state of irradiance dependent, thiol sensitive, and thylakoid bound kinase **K1** is a prerequisite for the thiol and irradiance insensitive peripherally associated kinase **K2** to be activated (Figure 7B). How **K1** activation could lead to **K2** activation is not clear, but such mediation is possible to be undertaken by ΔpH as it has been demonstrated to induce LHCII phosphorylation in isolated thylakoids (*29*).

Taken together, we demonstrated that LHCII phosphorylation as a function of irradiance in isolated thylakoids significantly varied with the nature of the peripheral proteins associated with the membranes that differs depending on the method adapted for their preparation (Figure 1). Irradiance response of *Th:B* containing both **K1** and **K2** is consistent with earlier observations of Rintamaki et al. (*17*), where the downregulation of LHCII phosphorylation at higher irradiance could not be demonstrated with isolated thylakoids leading to the general belief that the phenomenon is regulated by stromal factors (*17*, *18*). On the other hand, the irradiance response of *Th:A* containing **K1** only has clearly indicated LHCII phosphorylation−dephosphorylation as a function of irradiance to be regulated in a thylakoid sufficient manner without stromal assistance (Figures 1 and 5). We suggest that the phosphorylation of LHCII by **K2** in *Th:B* masked the normal rhythm of LHCII phosphorylation−dephosphorylation by **K1**, giving the apparent impression of the absence of downregulation of LHCII phosphorylation at high/prolonged irradiance in vitro in isolated thylakoids. Results with *Th:B* are also consistent with another observation of Rintamaki et al. (*16*), where LHCII phosphorylation in illuminated thylakoids turned insensitive to DTTred (Figure 3B). As discussed earlier, the absence of downregulation at higher irradiance and thiol insensitivity in illuminated thylakoids were coupled properties of LHCII phosphorylation (Figure 5). On the basis of our data, these properties can clearly be attributed to the presence of thiol insensitive **K2** activity in *Th:B*. The removal of **K2** from *Th:B* not only brought back the normal response of LHCII phosphorylation in response to irradiance, but it also restored thiol sensitivity to the phenomenon (Figure 5A). Thus, we have been successful in dissecting a thiol sensitive **K1** (*15*) and a thiol insensitive **K2** (*30*) component of LHCII phosphorylation. **K1** is firmly bound to thylakoid membranes and mediates the irradiation intensity to the photosynthetic machinery by transient phosphorylation of LHCII. Earlier reports have suggested LHCII kinase activities to be associated with PSII complexes (*30*) and in all probability, **K1** appears to be associated with the PSII complex of the thylakoids guarding the entry of the incident irradiation. The thiol insensitive component of LHCII phosphorylation appears to be due to the peripheral kinase **K2**. Our continued investigation on the Tricine extract harboring the **K2** activity revealed it to contain a single multiprotein complex. This was evidenced by the presence of a single band in nondenaturing condition, which yielded several bands in a denaturing gel. Attempts to dissociate this complex even under mild conditions led to a drastic loss of kinase activity. LC-MS/MS analysis of a major band present in the Tricine extract indicated it to represent the α and β subunits of the CF_1 component of the ATPase complex. These preliminary observations suggest that the peripheral kinase **K2** is associated with the unappressed region of the thylakoids through the multiprotein assembly of the ATPase complex.[2]

Finally, we propose a working hypothesis to explain the possible significance of the action of **K1** and **K2** in vivo (Figure 7C). **K1** is suggested to undertake the transient phosphorylation of LHCII for balancing the net light-energy harvest by the two photosystems. As the span of transience was inversely proportional to the intensity and/or duration of the incident irradiance (Figure 1B,C), this kinase appeared to have a perfect sensor for the number of incoming photons possibly via the reduction of plastoquinone and the cytochrome *bf* complex. Apart from having a distinct mode of inactivation in the presence of high or prolonged irradiance, **K1** is suggested to be negatively regulated in vivo by the ferredoxin−thioredoxin system too, as indicated by its persistent sensitivity to thiol reducing conditions in both dark-adapted and illuminated thylakoids. Thioredoxin is one of the competing electron acceptors at the terminal end of PSI (*31*). The others are plastoquinone and NADP, where the former favors ATP formation through PSI mediated cyclic electron transport and the latter drives the system toward the Calvin cycle (*32*−*34*). Thiol sensitivity of **K1** thus enables the system to coordinate the illumination response with the metabolic signals for determination of the net light harvest at any time point throughout its reign of active state for adjusting fluxes to the actual demand. The question arises as to what may be the relevance of the action of **K2** in vivo, where **K1** activity in the isolated thylakoids (*Th:A*) could mimic the irradiation response of LHCII phosphorylation in vivo. The significance of **K2** activity is believed to be associated with the properties that it does not share with **K1**, for example, its insensitivity to high irradiance and thiol reducing conditions. **K2** dependent LHCII phosphorylation is only detected in isolated thylakoids (*Th:B*), suggesting that the kinase is under negative regulation in vivo in the presence of stromal factors under ambient conditions. It is possible that such stromal factors release the negative regulation upon sensing a decrease in the ATP:NADPH ratio because LHCII phosphorylation is known to be favored when PSI mediated cyclic electron flow is required by a photosynthetic organism for the generation of ATP (*32*). Thus, phosphorylated LHCII helps the system to adapt to changes to demands of ATP even under adverse illumination conditions. Normally, under high irradiance LHCII associated with PSI in the unappressed thylakoids tends to be rapidly dephosphorylated, and the dephosphorylated LHCII population is believed to bind to PSII and undertake the task of excess energy dissipation (*6*). A cellular demand of ATP under high irradiance would require a **K2** type kinase to take over and maintain the phosphorylated state of LHCII to favor the cyclic electron transport through PSI. The thiol insensitivity of **K2** also

[2] A. Hazra, K. Parsawar, and M. DasGupta, unpublished results.

makes sense because under high light conditions, ferredoxin is expected to keep all its acceptors including the thioredoxin system in reduced form. Our propositions are consistent with the long-held views on physiological roles of LHCII phosphorylation where phospho-LHCII is suggested to have a role in metabolic adaptation of a photosynthetic cell (*2*, *32*). A role of LHCII phosphorylation is also described in relation to long-term acclimation to high irradiance or photoinhibitory mechanisms, and **K2** having a role in these processes is also a strong possibility (*6*, *7*).

In summary, we bring into light through this paper how misleading it might become while working on the regulation of a phenomenon like LHCII phosphorylation with in vitro preparations of thylakoids (*19*, *20*) that can completely change its behavior depending on its peripheral protein family. Several LHCII kinases are already in the literature (*10*−*12*, *30*, *35*, *36*), and they certainly are members of intricate regulatory circuits that connect light harvest with changing metabolic demands of a photosynthetic cell. Which of these kinases are turned on or off under in vitro conditions in isolated thylakoids is a difficult judgment. Against the current view, we have been able to demonstrate that irradiance dependent phosphorylation and dephosphorylation of LHCII is regulated in a thylakoid sufficient manner without stromal assistance. We have also shown that LHCII phosphorylation is subjected to both thiol sensitive as well as thiol insensitive regulations through distinct kinases.

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