Ascorbate-Mediated LHCII Protein Phosphorylation−LHCII Kinase Regulation in Light and in Darkness†

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ABSTRACT: A freeze−thaw cycle of isolated thylakoids in darkness in the presence of ascorbate was employed as a novel experimental system to activate the light-harvesting complex (LHC) II kinase. Under these conditions ascorbate reduces Q_A, the primary quinone electron acceptor of photosystem (PS) II, and the subsequent reduction of plastoquinone and the cytochrome (cyt) b_6f complex results in the activation of the LHCII kinase. Using this activation system, several facets of regulation of LHCII protein phosphorylation were unravelled. (i) Myxothiazol inhibited the activation of LHCII protein phosphorylation, thus being a potent inhibitor of electron flow not only in cyt *bc* complexes but in darkness also in cyt b_6f complexes. (ii) Oxygen, the only electron acceptor in darkness, was required for LHCII kinase activation demonstrating that after a full reduction of the cyt b_6f complex, an additional plastoquinol oxidation cycle in the quinol oxidation (Qo) site is required for LHCII kinase activation. (iii) In the absence of electron flow, when the intersystem electron carriers are reduced, the activated LHCII kinase has a half-life of 40 min, whereas the fully activated LHCII kinase becomes deactivated in a time scale of seconds upon oxidation of the cyt b_6f complex, indicating that the kinase constantly reads the redox poise of the cyt b_6f complex. (iv) The LHCII kinase is more tightly bound to the thylakoid membrane than the PS II core protein kinase(s). It is concluded that oxidation of plastoquinol at the Qo site of the reduced cyt b_6f complex is required for LHCII kinase activation, while rapid reoccupation of the Qo site with plastoquinol is crucial for sustenance of the active state of the LHCII kinase.

Reversible phosphorylation of thylakoid proteins is an intriguing phenomenon related to photosynthetic redox reactions. Following the pioneering work of Bennett (*1*, *2*), our understanding of the physiological role of thylakoid protein phosphorylation and of the biochemical mechanisms of kinase reactions has greatly advanced, besides the fact that the identity of the kinase(s) still remains elusive.

Reversible phosphorylation of the light-harvesting complex II (LHCII)[1] proteins has provided a molecular clue to the mechanism of state transitions as plant short-term chromatic adaptation (for reviews, see refs *2* and *3*). Recently, the molecular base was established (*4*) for the phosphorylated LHCII proteins to bind the photosystem (PS) I complex upon state transitions to balance the excitation energy between the two photosystems. In vivo studies with *Chlamydomonas*, on the other hand, have demonstrated that the state transitions are also regulated by the intracellular demand for ATP (*5*).

Studies using phosphothreonine antibodies (P-Thr) for monitoring the in vivo steady state of LHCII protein phosphorylation in higher plant leaves revealed that maximal LHCII protein phosphorylation occurs at low light intensities, 1/5 to 1/10 of that experienced during acclimated growth of the plant (*6*). Under acclimated growth conditions, the phosphorylation of LHCII proteins was already down-regulated, and doubling of the light intensity completely abolished LHCII protein phosphorylation. Moreover, not only the light conditions but also the metabolic state of chloroplasts, particularly the sugar metabolism, was recently shown to exert a strict control over LHCII protein phosphorylation (*7*). Thus, the LHCII protein phosphorylation in vivo in higher plants is likely to function also in the sensing and signaling of the balance between production of reducing equivalents in the light reactions of photosynthesis and the utilization of reducing equivalents for metabolic needs (*8*).

Induction of LHCII protein phosphorylation in the thylakoid membrane reflects the activation of the respective kinase. The activation mechanisms of the LHCII kinase are strictly regulated by the redox state of plastoquinone and the cytochrome (cyt) b_6f complex (*9*−*11*), whereas the inhibition of the LHCII kinase in vivo by high light and reducing metabolites is functioning on the top of this basic activation/deactivation system and is likely mediated by the thiol redox state of chloroplasts (*6*−*7*, *12*). Studies with inhibitors (*13*) of the cyt b_6f complex as well as the use of cyt b_6f complex mutants (*11*, *14*−*15*) have unambiguously demonstrated that the activation of the LHCII kinase requires

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[1] Abbreviations: PS, photosystem; LHC II, light-harvesting chlorophyll *a*/*b* complex of PSII; cyt, cytochrome; Q_o, the quinol oxidation site of the cyt b_6f and the cyt *bc* complex; Q_A, the primary quinone electron acceptor of PS II; DPI, diphenylene iodonium; NEM, *N*-ethylmaleimide; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DNP-INT, 2-iodo-6-isopropyl-3-methyl-2′,4,4′-trinitrodiphenyl ether; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; PVDF, poly(vinylidene difluoride); P-Thr, phosphothreonine.

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a functional cyt b_6f complex and involves a binding of reduced plastoquinone to the plastoquinol oxidation site (Qo) of the cyt b_6f complex (*9*−*10*). Progress based on studies with *Chlamydomonas* further suggested that the activation of the kinase involves a dynamic movement of the Rieske protein between its distal and proximal positions in the cyt b_6f complex (*16*−*17*). The movement from distal to proximal position was hypothesized to activate the kinase, whereas the movement back to the distal position would, in turn, release the activated kinase for LHCII protein phosphorylation (*16*).

LHCII protein phosphorylation in higher plants shares many similarities with *Chlamydomonas*, but also many differences have been revealed by recent studies. Among them, a complete shift to cyclic electron transfer upon LHCII protein phosphorylation (*18*) and concomitant redistribution of the cyt b_6f complex in the thylakoid network (*19*) are unique for *Chlamydomonas* and may be related to the extent of LHCII protein phosphorylation upon state transitions. In higher plants, the LHCII proteins are more modestly phosphorylated, and the dynamic regulatory processes have been far more difficult to target experimentally than in the unicellular *Chlamydomonas*.

We report here an experimental system in which the LHCII kinase activation is induced by freezing and subsequent thawing treatment of thylakoid membranes in the presence of ascorbate in darkness. In this system, ascorbate is shown to donate electrons to the plastoquinone pool via Q_A, the primary quinone electron acceptor of PSII, rather than by direct reduction of plastoquinone pool, thereby initiating the phosphorylation of thylakoid membrane bound proteins. This approach provides a simplified in vitro system for mechanistic studies of the LHCII kinase activation and deactivation in higher plant thylakoid membranes with respect to changes in the redox state and conformation of various thylakoid components.

MATERIALS AND METHODS

Plant Material. Pea (*Pisum sativum*) plants were grown for three weeks in a greenhouse at 22 °C with a 16-h photoperiod at a photon flux density of 300 μmol m^{-2} s^{-1}. Before experiments, the plants were transferred to darkness for 16 h to ensure full dark-acclimation and dephosphorylation of LHCII proteins.

Isolation of Intact Chloroplasts. Intact chloroplasts were isolated from plants acclimated for 16 h in darkness as described by Zhang et al. (*20*) excluding bovine serum albumin, DTT, and ascorbate from all isolation buffers. Chloroplasts were resuspended in an assay buffer consisting of 50 mM HEPES [4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid]-NaOH, pH 8.0, 0.33 M sorbitol, 5 mM NaCl, 10 mM $MgCl_2$, and 10 mM NaF, and all the reactions with isolated chloroplasts were carried out at a final chlorophyll concentration of 0.4 mg mL^{-1}.

Isolation of Thylakoid Membranes. Thylakoid membranes were rapidly isolated from dark-treated leaves as described earlier (*8*), including 10 mM NaF as a phosphatase inhibitor in all buffers.

Phosphorylation Assay. Before phosphorylation assays, the isolated chloroplasts or thylakoids from dark-treated plants were preincubated at 20 °C in darkness for 10 min with each of the chemicals indicated in the figure legends. For freeze−thaw treatment, samples were frozen in liquid nitrogen, and after thawing in darkness, 0.4 mM ATP, or certain concentrations of ATP as indicated in Figure 1, was added to each sample. Phosphorylation took place during further incubation for 15 min in darkness. Reaction was stopped by adding

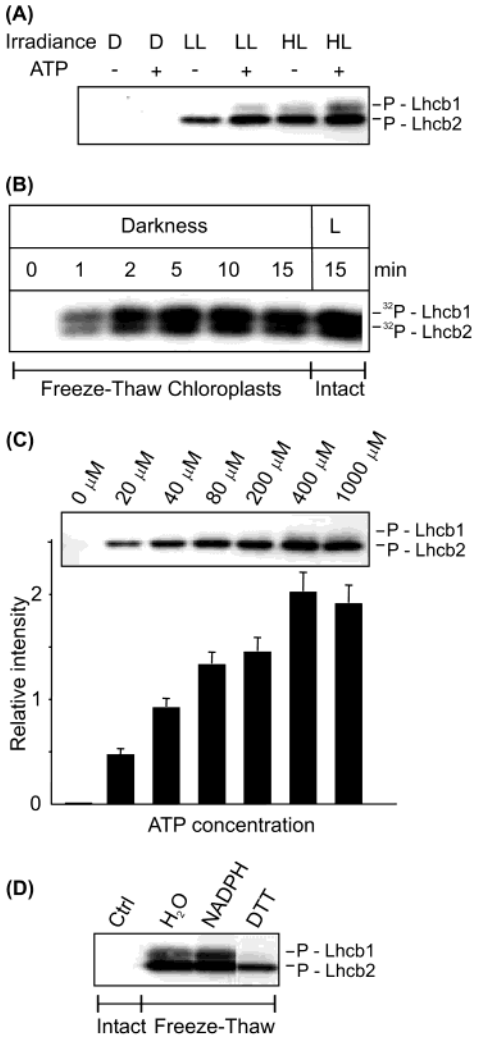

FIGURE 1: LHCII protein phosphorylation in isolated chloroplasts, induced by illumination or by a freeze−thaw treatment. (A) P-Thr immunoblots demonstrating the effect of light intensity on the phosphorylation of LHCII proteins in intact chloroplasts. Chloroplasts were isolated from dark-acclimated pea leaves and subsequently incubated in darkness (D) or illuminated at 50 (LL) or 600 (HL) μmol photons m^{-2} s^{-1} for 15 min in the presence or absence of exogenous ATP (0.4 mM). (B) Autoradiogram demonstrating the time course of LHCII protein phosphorylation in darkness in the freeze−thaw-treated chloroplasts. After freeze−thaw treatment, the chloroplasts were supplemented with 0.4 mM ATP (γ-^{32}P-ATP at 0.02 Ci/g chlorophyll) in darkness for 0−15 min. Illuminated intact chloroplasts are presented as a positive control. (C) Dependence of LHCII protein phosphorylation on ATP concentration. The freeze−thaw-treated chloroplasts were incubated with various concentration of ATP for 15 min in darkness, and the phosphorylation of LHCII proteins was analyzed by immunoblotting with P-thr antibody. (D) Effect of NADPH and DTT on freeze−thaw activated phosphorylation of LHCII proteins in chloroplasts. Isolated chloroplasts were incubated with 5 mM NADPH or 10 mM DTT in darkness for 10 min prior to freeze−thaw treatment, and LHCII protein phosphorylation was assayed in darkness as described above. Dark incubated intact chloroplasts are shown as a control (Ctrl). LHCII phosphorylation was visualized by immunoblotting (A, C, and D) or by autoradiography (B).

Laemmli solubilization buffer. The following inhibitors were used: stigmatellin from Fluka, 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB), rotenone, myxothiazol, diphenylene iodonium (DIP), *N*-ethylmaleimide (NEM) from Sigma, and 2-iodo-6-isopropyl-3-methyl-2′,4,4′-trinitrodiphenyl ether (DNP-INT) kindly provided by Prof. A. Trebst (Ruhr-University, Bochum, Germany). When indicated, thylakoid membranes were illuminated at low light (50 μmol photons m^{-2} s^{-1}) or at high light (600 μmol photons m^{-2} s^{-1}). For γ-^{32}P-labeled phosphorylation, the radioactivity supplied to the reaction system was 0.02 Ci/g chlorophyll.

Oxygen was removed from the samples using either a glucose/glucose oxidase trap added to dark-treated thylakoids before any further treatments or by flushing dark-treated thylakoids with argon first for 15 min and continuing flushing during the entire reaction process. All anaerobic treatments were repeated with these two alternative oxygen depletion methods to ensure the reliability of obtained results.

Detection of LHCII Protein Phosphorylation. Samples were solubilized in the presence of 6 M urea, and polypeptides were fractionated by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE, ref *21*) in 15% (w/v) acrylamide gels containing 6 M urea and electroblotted to a poly(vinylidene difluoride) (PVDF) membrane (Immobilon P, Millipore, Bedford, MA). For identification of Lhcb1 and Lhcb2 proteins, the membrane was probed with protein specific antibodies. The detection of respective phosphoproteins by polyclonal P-Thr antibody (New England BioLabs) was performed as described previously (*6*). To analyze the ^{32}P-labeled phosphoproteins, the gels were dried, exposed to X-ray films, and visualized by autoradiography. An IMAGE program (Imaging Research Inc.) was used for quantification of LHCII protein phosphorylation.

Chlorophyll Fluorescence. Fresh or freeze−thaw treated thylakoid membranes at 20 μg chlorophyll mL^{-1} were used to monitor chlorophyll *a* fluorescence with a PAM 101 fluorometer (Walz, Effeltrich, Germany).

Chlorophyll Determination. Chlorophyll was extracted in 80% buffered acetone and determined as described previously (*22*).

RESULTS

Activation of the LHCII Kinase by Freeze−Thaw Treatment. The LHCII proteins in intact fresh chloroplasts isolated from dark-treated pea plants were completely dephosphorylated (Figure 1). Exposure of these chloroplasts to low light irradiances induced significant LHCII protein phosphorylation, which was further enhanced by the presence of exogenous ATP. Similar enhancement of LHCII protein phosphorylation upon increasing illumination of chloroplasts (Figure 1A) was apparently also a result from increasing ATP level, which in illuminated chloroplasts directly responds to the intensity of light (*23*). When chloroplasts were supplemented with ATP in darkness, no phosphorylation was induced indicating that the LHCII kinase was deactivated. However, after freezing and thawing of the chloroplasts, a mere addition of ATP in complete darkness initiated LHCII protein phosphorylation and resulted in a similar pattern of LHCII protein phosphorylation as induced by light (using both γ-^{32}P-ATP labeling and immunodetection methods,

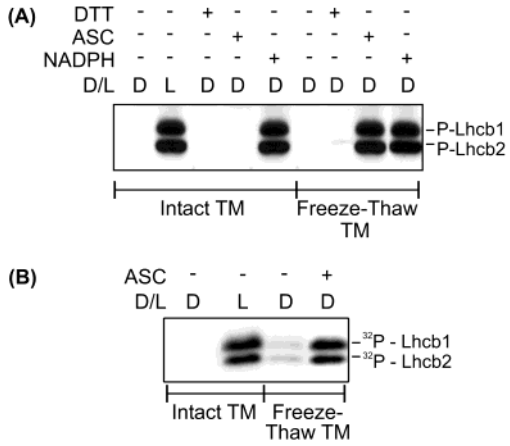

FIGURE 2: P-Thr immunoblots demonstrating LHCII protein phosphorylation in isolated thylakoid membranes (TM) in the presence of reducing chemicals. (A) Thylakoid membranes isolated from dark-acclimated pea plants were incubated in darkness for 10 min in the presence or absence of 10 mM DTT, 5 mM NADPH, or 10 mM ascorbate (ASC). Half of the samples were subsequently subjected to freeze−thaw treatment (Freeze−Thaw TM). Phosphorylation assay was performed in the presence of 0.4 mM ATP in darkness, or for control intact thylakoids in the light, for 15 min. (B) Autoradiogram of LHCII protein phosphorylation in thylakoid membranes. Isolated thylakoid membranes were incubated with or without 10 mM ascorbate for 10 min in darkness. After freeze−thaw treatment, γ-^{32}P-ATP (0.02 Ci/g chlorophyll including 0.4 mM cold ATP) was added in darkness, and incubation was continued for 15 min. Control reactions included isolated thylakoid membranes kept in darkness (D) or illuminated (L) for 15 min in the presence of ATP.

Figure 1B). LHCII protein phosphorylation in darkness by the freeze−thaw treatment of isolated chloroplasts was strongly dependent on added ATP, and the saturation level was reached at 0.4 mM ATP (Figure 1C).

To unravel the mechanism of LHCII kinase activation by the freeze−thaw treatment of intact chloroplasts, we first tested some reactants known to function either as an inhibitor or an activator of the LHCII kinase reaction. Figure 1D demonstrates that the presence of 5 mM NADPH, the reductant that initiates LHCII phosphorylation in dark-incubated thylakoid membranes (*12*), had no further inductive effect on LHCII protein phosphorylation. On the other hand, the LHCII protein phosphorylation level was markedly reduced upon the presence of 10 mM DTT, given prior to freezing. These results suggested that the LHCII kinase activation upon the freeze−thaw treatment of intact chloroplasts resulted from reduction of plastoquinone and subsequent occupation of the Q_o site of cyt b_6f complex, similar to that in the light-activation of the LHCII kinase. It is noteworthy that in isolated thylakoid membranes when subjected to similar freeze−thaw theatment, absolutely no LHCII protein phosphorylation took place when supplemented with ATP in darkness (Figure 2A). It is thus evident that some soluble compounds in the chloroplast stroma were essential for the activation of the thylakoid LHCII kinase upon the freeze−thaw treatment of intact chloroplasts.

We next searched for compounds that, when added to isolated thylakoids, can activate the phosphorylation of LHCII proteins in darkness upon the freeze−thaw treatment. As shown in Figure 2A, 5 mM NADPH was able to activate the kinase to the maximal phosphorylation of LHCII, which however was not dependent on the freeze−thaw treatment

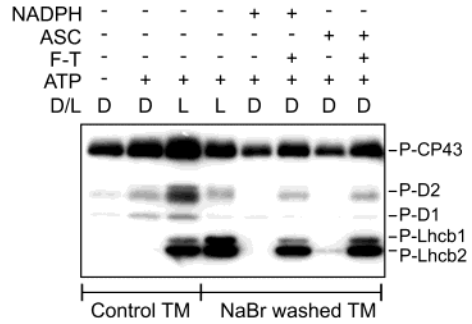

FIGURE 3: Phosphothreonine immunoblot demonstrating the phosphorylation of thylakoid proteins in NaBr-washed thylakoid membranes. Isolated thylakoid membranes (TM) from dark-acclimated pea leaves were washed with 2 M NaBr and then incubated in the presence of 5 mM NADPH or 10 mM ascorbate in darkness for 10 min. Part of the samples were subjected to freeze−thaw treatment (F−T), and subsequently the thylakoid proteins were phosphorylated in the presence of 0.4 mM ATP for 15 min in darkness. Phosphorylations of nonwashed thylakoids are presented as controls (Control TM).

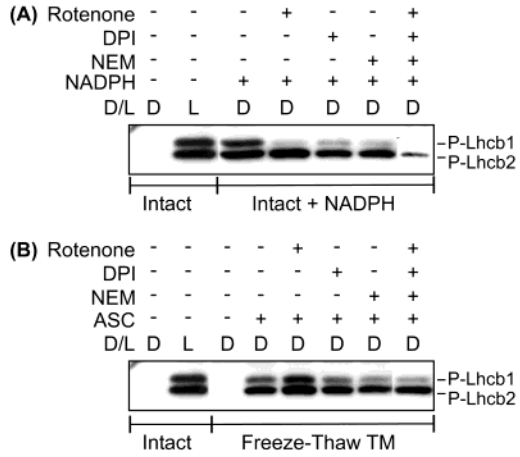

FIGURE 4: P-Thr immunoblots demonstrating LHCII protein phosphorylation in the presence of inhibitors blocking various routes of PS II independent reduction of the plastoquinone pool. Isolated thylakoid membranes from dark-acclimated pea plants were incubated with or without the indicated inhibitors in darkness for 10 min. (A) LHCII kinase activated by reduction of the plastoquinone pool by 5 mM NADPH in darkness. (B) LHCII kinase activated by a freeze−thaw-treatment of thylakoids in darkness in the presence of 10 mM ascorbate. After LHCII kinase activation, the phosphorylation was performed by incubating the samples in darkness for 15 min in the presence of 0.4 mM ATP. Appropriate controls include the phosphorylation of intact thylakoids in darkness and in light in the presence of ATP.

but occurred similarly also in intact thylakoid membranes (Figure 2A). Interestingly, addition of 10 mM ascorbate had no effect on intact thylakoids, whereas it highly activated the kinase in freeze−thaw treated thylakoids inducing the phosphorylation of LHCII proteins to a similar extent as obtained with addition of NADPH. By using γ-^{32}P-ATP labeling, the ascorbate-mediated activation of the LHCII kinase was demonstrated to phosphorylate the LHCII proteins to similar extents as the traditional light-activation mechanism (Figure 2B). The universal nature of the ascorbate-mediated LHCII kinase activation was confirmed by repeating the freeze−thaw phosphorylation experiments with tobacco, pumpkin, and rye thylakoids (data not shown).

To exclude the possibility of unspecific proteins, associated with the thylakoid membrane during isolation, being involved in the activation of the LHCII kinase via reduction of plastoquinone pool, the isolated thylakoid membranes were washed with 2 M NaBr in darkness. As shown in Figure 3, such membranes were still able to induce LHCII protein phosphorylation in light but failed to phosphorylate LHCII proteins in darkness via the NADPH-mediated pathway, apparently because of a loss of bound ferredoxin or NADPH-plastoquinone oxidoreductase (*24*). Notably, after subjecting the NaBr-washed membranes to freeze−thaw treatment, both NADPH and ascorbate were able to induce the phosphorylation of LHCII proteins in darkness in the presence of ATP. The results confirmed the existence of NADPH- and ascorbate-mediated LHCII kinase activation by the freeze−thaw treatment. It is noteworthy that the thylakoid membranes washed with NaBr had clearly reduced phosphorylation capacity for all the other thylakoid membrane-bound phosphorylatable proteins except for LHCII, both in light and in darkness.

Mechanism of Ascorbate-Mediated Activation of LHCII Phosphorylation in Darkness upon the Freeze−Thaw Treatment. It has been suggested that in isolated thylakoid membranes in darkness, NADPH activates the phosphorylation of LHCII proteins by reduction of pool plastoquinone either via ferredoxin-mediated pathways or via nonferredoxin-mediated pathways (*24*, *25*). Blocking these various pathways with 1 mM rotenone together with 50 μM DPI and 5 mM NEM, indeed, inhibited the phosphorylation of LHCII proteins in intact thylakoids in darkness in the presence of NADPH (Figure 4A). None of these inhibitors, when given alone, were able to completely block LHCII phosphorylation. In sharp contrast to NADPH-mediated LHCII phosphorylation in intact thylakoids in darkness, the ascorbate-mediated dark-activation of LHCII protein phosphorylation in freeze−thaw treated thylakoid membranes could not be inhibited with the combination of these inhibitors (Figure 4B). Taken together, the data (Figures 2A and 4) suggest that the pathway of ascorbate-mediated LHCII kinase activation does not involve a direct reduction of pool plastoquinone by ascorbate.

Another possibility for reduction of the plastoquinone pool is via the first bound quinone electron acceptor Q_A of PS II. Interestingly, blocking this pathway with 10 μM DCMU completely blocked the ascorbate-mediated activation of LHCII protein phosphorylation, while DCMU had no significant effect in darkness on NADPH-mediated LHCII protein phosphorylation in freeze−thaw treated thylakoids or in intact thylakoids (Figure 5A).

The above results with DCMU-poisoned thylakoids suggested that ascorbate might be capable of reducing Q_A during the freeze−thaw treatment of thylakoids. Therefore, the fluorescence characteristics of freeze−thaw treated thylakoid membranes were investigated in the presence and absence of ascorbate. As shown in Figure 5B,C, the freeze−thaw treatment of thylakoids did not directly alter the PS II photochemical efficiency as demonstrated by similar F_o and F_m and similar responses to addition of ascorbate or DCMU in freeze−thaw-treated and intact control thylakoid membranes. Addition of ascorbate together with DCMU prior to the freeze−thaw treatment of thylakoids, however, induced a drastic increase of F_o (Figure 5C). This demonstrates that in the freeze−thaw-treated thylakoid membranes, ascorbate is capable of slow reduction of Q_A and thereby also the

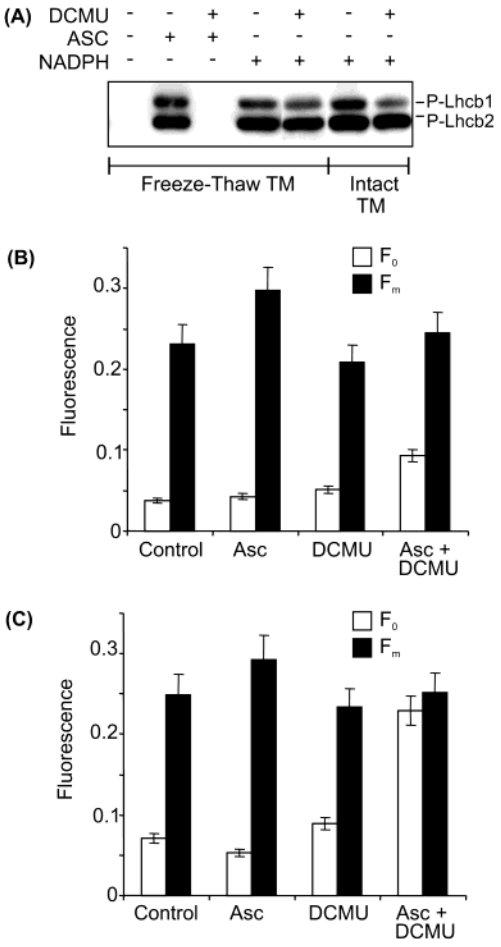

FIGURE 5: Effcet of DCMU on LHCII protein phosphorylation in darkness. (A) P-Thr immunoblots demonstrating the inhibition of ascorbate-mediated LHCII protein phosphorylation by DCMU. Isolated thylakoid membranes from dark-acclimated pea plants were incubated with 20 μM DCMU for 10 min in darkness. Ascorbate (10 mM) or 5 mM NADPH were thereafter added, and the ascorbate samples were additionally subjected to a freeze−thaw treatment. Phosphorylation was performed in darkness in the presence of ATP. (B and C) Changes of F_o and F_m induced by ascorbate and DCMU in intact (B) and in freeze−thaw treated (C) thylakoid membranes. DCMU and assorbate were added to thylakoid suspension in darkness 5 min before the measurements or prior to the freeze−thaw treatment of thylakoid membranes.

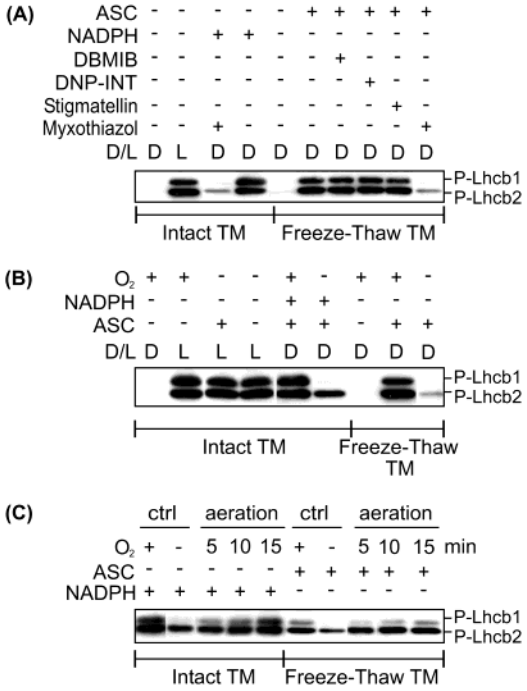

FIGURE 6: Regulation of LHCII protein phosphorylation in darkness by Qo site inhibitors of the cyt b_6f complex and by the presence and absence of oxygen. (A) Effect of cyt b_6f complex inhibitors. Isolated thylakoid membranes were incubated with 10 μM DBMIB, 10 μM DNP-INT, 10 μM stigmatellin, or 10 μM myxothiazol for 10 min in darkness. The LHCII kinase was then activated by a freeze−thaw treatment with ascorbate or by addition of NADPH and protein phosphorylation performed in darkness in the presence of ATP. (B) Effects of O_2 depletion on LHCII protein phosphorylation in isolated thylakoids. Thylakoid membranes were incubated with 10 mM ascorbate or 5 mM NADPH for 10 min in darkness, and phosphorylation assays were performed under low light conditions or in darkness via NADPH- and ascorbate-mediated activation routes as above. For O_2 depletion, thylakoid solutions were flushed with argon in darkness for 15 min prior to addition of NADPH and ATP and flushed for further 15 min in darkness. In the case of freeze−thaw treatment, samples were flushed from the beginning of thawing until the end of the assay. For the light illumination sample, glucose/glucose oxidase was added to isolated thylakoids in darkness 10 min before exposure to light illumination. Essentially similar results were obtained whether glucose/glucose oxidase trap or argon flush was used to deplete oxygen. (C) Restoration of LHCII protein phosphorylation upon shift of thylakoid samples from anaerobic to aerobic conditions. Thylakoids were depleted of oxygen, and one portion of the samples was used to directly detect the level of LHCII protein phosphorylation (ctrl − O_2), the rest of the thylakoids were shaken in air, and samples for phosphorylation assays were taken after 5, 10, and 15 min of aeration. Phosphorylation of thylakoid membranes without depletion of oxygen (ctrl + O_2) is presented as a control.

reduction of a pool plastoquinone. The fact that ascorbate could not keep Q_A fully reduced in darkness in the absence of DCMU (Figure 5B) suggests that oxidation of plastoquinol by ambient molecular oxygen took place in freeze−thaw thylakoids.

Regulation of LHCII Kinase Activation and Deactivation in the Freeze−Thaw System. Freeze−thaw-treated thylakoids were used to study the mechanism of the LHCII kinase activation/deactivation system. This ascorbate mediated LHCII kinase activation system has a benefit of functioning in a similar mechanism as the light-induced LHCII kinase activation, yet it avoids the postulated pure light-induced modifications of thylakoid components, like the LHCII proteins (*26*), the substrates of the LHCII kinase.

Plastoquinone analogues DBMIB, DNP-INT, and stigmatellin were first tested in the freeze−thaw treated thylakoid membrane system in darkness. These inhibitors are known to inhibit the cyt b_6f complex by binding to the Qo site in a position proximal to the Rieske 2Fe−2S cluster (Class I inhibitors) (see ref *27*). The presence of 20 μM DBMIB, 10 μM DNP-INT, or 10 μM stigmatellin did not, however, block the ascorbate-dependent LHCII protein phosphorylation in freeze−thaw thylakoid membranes in darkness (Figure 6A). This is not completely unexpected as it is known that the inhibitory properties of these chemicals can vary with cyt *bc* types among different species (*28*) and are dependent on the redox state of the Rieske iron−sulfur protein as well as the inhibitor itself: in reducing conditions the inhibitory effects of these chemicals are generally drastically weakened (*16*, *29*−*30*).

Myxothiazol is another type of cyt *bc* complex inhibitor (Class II inhibitor) that binds to the distal position (related

to Rieske 2Fe−2S cluster) in the Qo site, close to the low potential cyt *b* and thereby prevents semiquinone oxidation. Myxothiazol thus prevents the reduction of the Rieske iron−sulfur cluster, contrary for example to stigmatellin, which allows reduction of the cluster but locks the Rieske protein in the reduced conformation (*29*−*31*). When myxothiazol was incubated with isolated thylakoid membranes prior to the freeze−thaw treatment, the subsequent capacity for LHCII protein phosphorylation was dramatically decreased (Figure 6A). Myxothiazol decreased to the same extent also the NADPH-dependent LHCII protein phosphorylation in darkness (Figure 6A). It is important to note that the same concentration (10 μM) of myxothiazol did not inhibit light-activated LHCII protein phosphorylation in isolated intact thylakoid membranes (data not shown). Thus, the consensus about myxothiazol not being a potent inhibitor of cyt b_6f complexes (see ref *27*) agrees with our results in light, whereas the inhibition of LHCII protein phosphorylation in darkness in the presence of myxothiazol strongly suggests that myxothiazol interacts with the cyt b_6f complex in darkness similarly to that with the cyt *bc* complex.

To study the role of dynamic electron flow in the activation mechanism of the LHCII kinase, we next depleted the thylakoids of oxygen, probably the only electron acceptor in isolated thylakoid preparations in darkness. As shown in Figure 6B, the depletion of O_2 caused a strong decrease in subsequent ascorbate-mediated LHCII protein phosphorylation in darkness. Notably, similar to that in the ascorbate-mediated process, anaerobiosis caused a significant reduction of LHCII protein phosphorylation (remaining phosphorylation ca. 40% of control) also in intact thylakoid membranes when phosphorylation was induced in darkness via the NADPH pathway. Conversely, anaerobiosis had no inhibitory effect on LHCII protein phosphorylation in light when the phosphorylation assay was performed with intact thylakoid membranes (Figure 6B). Upon a shift of the anaerobically treated reaction system to aerobic conditions (removing of argon by mixing the reaction system in air), the capacity for LHCII protein phosphorylation was restored relatively rapidly in both the ascorbate- and NADPH-mediated LHCII kinase activation systems (Figure 6C).

We further performed a series of assays using the freeze−thaw system to elucidate the LHCII kinase activation/deactivation mechanisms. In the absence of DCMU, even after 45 min of thawing the thylakoid membranes, the LHCII proteins became fully phosphorylated upon addition of ATP in darkness (Figure 7A). However, addition of DCMU to block the sustained plastoquinone reduction by ascorbate (via Q_A), after the initial activation of the kinase by freeze−thaw treatment, gradually induced a deactivation of the kinase as shown by a decreased capacity with time for the phosphorylation of the LHCII proteins (Figure 7B, line Asc to DCMU). Such deactivation of the LHCII kinase showed similar kinetics to the deactivation of the light-activated kinase upon transfer of thylakoids to darkness (Figure 7B, line Light to Dark). Transfer of thylakoids to anaerobic conditions, after initial activation of the kinase under aerobic conditions, and concomitant blockage of plastoquinone reduction by DCMU, on the other hand, resulted in a more sustained capacity for phosphorylation of LHCII proteins (Figure 7B, line Asc to DCMU, $-O_2$). Thus, apart from the fact that anaerobiosis severely inhibited the kinase activation

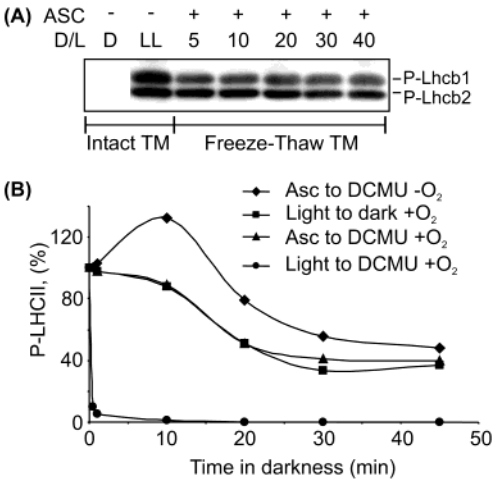

FIGURE 7: Sustenance of the capacity for LHCII protein phosphorylation in the presence and absence of electron flow. (A) P-Thr immunoblot demonstrating sustained capacity for LHCII protein phosphorylation in freeze−thaw thylakoids in the presence of (10 mM) ascorbate. Phosphorylation capacity after thawing of thylakoids in air was tested at times indicated by addition of 0.4 mM ATP. (B) Kinetics of LHCII kinase deactivation under different conditions of electron flow. Isolated thylakoids, with the LHCII kinase activated in light for 10 min, were shifted to darkness for deactivation (Light to Dark), or deactivation was induced in light by adding 20 μM DCMU to block the electron flow (Light to DCMU). Thylakoids with the LHCII kinase activated via ascorbate-involved freeze−thaw treatment in darkness were also supplemented with 20 μM DCMU to block sustained LHCII kinase activation (see panel A), and deactivation was then followed both under aerobic (Asc to DCMU, $+O_2$) and anaerobic (Asc to DCMU, $-O_2$) conditions. ATP at 0.4 mM was added to initiate phosphorylation at indicated times.

process, once activated the depletion of oxygen did not exert any detrimental effects on the catalytic phosphorylation activity of the kinase (Figure 6B). Anaerobic conditions, with no possibility for kinase reactivation, also provided a tool for estimation of the half-life of the activated LHCII kinase, which appeared to be relatively long, ca. 40 min. On the other hand, addition of DCMU to intact thylakoids in light, after initial activation of the kinase, rapidly lead to a complete loss of LHCII phosphorylation capacity, with 95% inhibition within the first 30 s (Figure 7B, line Light to DCMU). Thus, although electron flow is required for LHCII kinase activation, the lifetime of the activated kinase is strictly dependent on the reduction state of the plastoquinone pool and the cyt b_6f complex.

DISCUSSION

Ascorbate Reduces Pool Plastoquinone in Freeze−Thaw Treated Thylakoids via Electron Transfer from Q_A. Reduction of the plastoquinone pool, particularly the binding of reduced plastoquinone at the Q_o site of the reduced cyt b_6f complex, has been postulated as a prerequisite for the activation of the LHCII kinase (*9*−*10*). The fact that ascorbate induced LHCII protein phosphorylation in darkness during the freeze−thaw cycle of thylakoid membranes indicates that ascorbate can induce the reduction of free plastoquinone. Owing to the presence of high concentration of ascorbate in chloroplasts (*32*), the freeze−thaw treatment of isolated chloroplasts thus directly induced LHCII kinase activation in darkness (Figure 1). Apparently, the pathway transferring electrons from ascorbate to plastoquinone pool differs from

that of the NADPH-mediated plastoquinone reduction (Figures 3 and 5), where NADPH-plastoquinone oxidoreductases are involved (*24*). Physicochemically, ascorbate is not capable of directly reducing the plastoquinone pool because of its insufficiently low reduction potential as well as to its constrained accessibility to the pool (*33*−*35*). This was also evidenced by the failure of exogenously added ascorbate in intact thylakoid membranes (Figure 2A) or the endogenous ascorbate in isolated intact chloroplasts (Figure 1) to induce LHCII protein phosphorylation in darkness when supplemented with ATP. It is noteworthy that the ascorbate-mediated phosphorylation of LHCII proteins in darkness in freeze−thaw treated chloroplasts and thylakoids was blocked by addition of DCMU (Figure 5A). Analysis of chlorophyll *a* fluorescence parameters in the presence of DCMU (Figure 5B,C) further revealed that in freeze−thaw thylakoids the reduction of the plastoquinone pool occurs via ascorbate-mediated donation of electrons to Q_A. How ascorbate can overcome the problems in the accessibility and midpoint potential for reduction of Q_A is difficult to reconcile. Q_A has previously been shown to become accessible to exogenous redox compounds upon trypsination of the thylakoid membrane (*36*), which attaches the D1 protein. Our freeze−thaw treatment might also influence the conformation of the D1 protein, although no proteolytic cleavage was evident either in the D1 or the D2 protein, deduced from the electrophoretic mobility of these proteins (data not shown). Freeze−thaw treatment apparently also affected the midpoint potential of Q_A. Large changes in the midpoint potential of Q_A have previously been reported upon inactivation of the donor side of PSII (*37*), which might become susceptible to inactivation during a freeze−thaw cycle as well and thereby induce a sufficient increase in the redox potential of Q_A to become a target for reduction by ascorbate.

The LHCII protein phosphorylation pattern induced by the presence of ascorbate was qualitatively and quantitatively identical to that induced by the light-activated kinase (Figures 1B and 2B). This result, together with those obtained by the activation of the LHCII kinase with NADPH in darkness, argues against the requirement of light for the exposure of the N-terminal phosphorylation sites of LHCII proteins (*26*). Indeed, after the kinase has been activated, either in light or in darkness, the extent of LHCII protein phosphorylation is primarily dependent on the availability of ATP (Figure 1C). It was further shown that the LHCII kinase is tightly bound to the thylakoid membrane while other kinase(s), responsible for PSII core protein phosphorylation, are more loosely bound and released from the thylakoid membrane upon washing with 2 M NaBr (Figure 3).

Reduction of plastoquinone pool via Q_A in darkness in freeze−thaw thylakoids, similar to that in the light, provides a unique opportunity to study the LHCII kinase activation/deactivation mechanisms, which now can be studied irrespective of light-induced conformational modifications of various thylakoid components involved in the regulation of LHCII protein phosphorylation.

Dynamic Binding and Oxidation of Plastoquinol at Q_o Site of Reduced Cyt b_6f Complex Triggers LHCII Kinase Activation. It has been suggested that the interaction of reduced cyt b_6f complex with a reduced plastoquinone at Qo site acts as a kinase activator complex inducing the phosphorylation of LHCII proteins (*9*−*10*, *38*). Reduction of the plastoquinone pool via the ascorbate/freeze−thaw system or via NADPH also results in the formation of the kinase activator complex (*9*) and consequently fully activated the phosphorylation of LHCII proteins (Figure 2). However, severe inhibition of LHCII protein phosphorylation in both the ascorbate and the NADPH systems upon depletion of oxygen, the only electron acceptor in darkness, reveals that mere binding of plastoquinol at the Qo site of the cyt b_6f complex, when the electron transfer components between PS II and I are reduced, is not sufficient for induction of LHCII protein phosphorylation (Figure 6B). Indeed, only after allowing electron transfer to oxygen upon aeration of the reaction mixture did the phosphorylation of LHCII proteins become feasible. Since anaerobiosis has no direct inhibitory effect on LHCII protein phosphorylation if the LHCII kinase has been previously activated (Figure 7B, ref *9*), this behavior can only be attributed to a failure in the activation of the LHCII kinase because of the absence of electron acceptors. In line with these results, Wollman et al. have suggested, based on their results on contrasted effects of the inhibitors of the cyt b_6f complex on state transitions, that the dynamics of the cyt b_6f complex is a clue for activation of the LHCII kinase and phosphorylation of the LHCII proteins (*16*−*17*).

By using various cyt *bc* and cyt b_6f complex inhibitors, the role of the occupation of the Qo pocket and conformational changes in the Rieske protein in the activation of the LHCII kinase were further studied in darkness. We report here, for the first time, an inhibitory effect of myxothiazol on LHCII kinase activation. This inhibitor is generally regarded as inefficient in the inhibition of the cyt b_6f complex (*39*), whereas it is a potent inhibitor of the cyt *bc* complex concomitantly releasing the Rieske extrinsic domain from proximal to distal position (*40*). Importantly, the inhibition of LHCII phosphorylation by myxothiazol occurred only in darkness when all the components of the cyt b_6f complex as well as the plastoquinone pool were oxidized. On the other hand, locking of the Rieske extrinsic domain in the proximal position by binding of stigmatellin, a Class I inhibitor, has also been shown inhibitory for phosphorylation of LHCII proteins in *Chlamydomonas* (*16*). Using pea thylakoids, however, none of the Class I inhibitors, stigmatellin, DBMIB, or DNP-INT inhibited LHCII protein phosphorylation in darkness. Thus, the inhibition capacity of both the Class I and II inhibitors seems to be dependent on light. It is conceivable that sustained electron flow through the cyt b_6f complex in light modifies the conformation of the two sites in the Qo pocket, reinforcing the inhibition by Class I inhibitors and conversely preventing the inhibition by Class II inhibitor myxothiazol. Indeed, the conformation of the Qo pocket might in darkness more closely resemble that of the cyt *bc* complex, which is known to be susceptible to inhibition by myxothiazol but relatively insensitive to DBMIB (*41*). It remains to be studied whether the chlorophyll and/or carotenoid molecules of the cyt b_6f complex are involved in light-induced modification of the susceptibility of the Qo site to Class I and II inhibitors and thus possibly also in the regulation of LHCII kinase activation/deactivation processes.

Two extreme locations for the Rieske extramembrane domain in the presence of inhibitors have been demonstrated (for a review, see ref *42*), and similar locations are likely to occur also in completely oxidized (proximal position) or

completely reduced (anaerobic conditions) (distal position) cyt b_6f complexes in pea thylakoids in darkness. It is thus clear that neither of these extreme conformations of the cyt b_6f complex (Rieske extramembrane domain) as such functions as an activator of the LHCII kinase. Instead, rotatory changes in the cyt b_6f complex, possibly involving additional intermediate conformations (*43*), are apparently required, as demonstrated by only poor activation of the LHCII kinase under anaerobic but reducing conditions and subsequent successful activation upon introducing oxygen to the thylakoid sample (Figure 6).

Maintenance of the LHCII Kinase Active State. There is a general consensus that the oxidation of plastoquinol at the Qo site of the cyt b_6f complex deactivates the LHCII kinase (*9*−*10*). The question thus arises how the oxidation of the plastoquinol at the Qo site can perform such a dual function in the regulation of LHCII protein phosphorylation (being involved both in the activation and in the deactivation processes). The kinase activation process, connected to conformational changes in the cyt b_6f complex, apparently involves conformational changes in the kinase itself as well (*7*, *12*). The active state of the kinase is sustained most efficiently under PS II light (*7*) or under anaerobic and reducing conditions in darkness where plastoquinol oxidation is negligible (Figure 7). The half-life of the activated kinase, being ca. 40 min (Figure 7), was possible to estimate under anaerobic conditions where kinase reactivations are blocked. This also demonstrates that the maintenance of the active state of the LHCII kinase does not require sustained electron flow. Conversely, the kinase deactivation, occurring at its best in a time scale of only a few seconds, takes place under conditions that oxidize the plastoquinone pool, for instance, upon exposure of thylakoids to PS I light or after addition of DCMU in light (Figure 7, ref *9*). Since the kinase deactivation rapidly follows the oxidation of plastoquinol at the Qo site of the cyt b_6f complex, it is conceivable that a physical contact between the cyt b_6f complex and the LHCII kinase is a prerequisite also for kinase deactivation. Indeed, at least as long as ATP is omitted from the reaction system, and thus no catalytic turnovers of the kinase have yet taken place, the activated form of the LHCII kinase directly reads the redox conditions in the cyt b_6f complex, probably in a similar close proximity to the cyt b_6f complex as does the deactivated form of the enzyme under oxidizing conditions.

After kinase activation, a rapid reoccupation of the Qo site with reduced plastoquinone is crucial for the maintenance of the kinase active state. Under these reducing conditions, in the absence of electron flow, the Rieske protein apparently occupies a distal position close to cyt *f* (*43*). During sustained electron flow, the Rieske protein rotates probably between three different positions (*43*) that apparently also modify the LHCII kinase active state. When the plastoquinone pool and the cyt b_6f complex become fully oxidized, the Rieske protein relaxes to an intermediate position (*43*), and the kinase is directly deactivated, possibly via conformational changes in the kinase itself (*7*, *12*). In line with this conclusion, the LHCII kinase activated by a pH shift (*9*) was prone to deactivation by a single turnover flash oxidizing the bound plastoquinol. The LHCII kinase regulation system postulated above implies physical proximity of the kinase and the cyt b_6f complex both in the activated and in the deactivated states of the kinase, at least as long as ATP is not bound to the activated kinase.

Given the above conditions for regulation of the LHCII kinase activation and deactivation, the equilibrium at high light intensities should strongly favor the phosphorylation of LHCII proteins. This is indeed the case in vitro upon illumination of isolated thylakoid membranes. However, the in vivo monitoring of LHCII protein phosphorylation shows strong down regulation of LHCII protein phosphorylation at high light intensities (*6*). This inhibition results from a superimposed regulation mechanism for LHCII protein phosphorylation in vivo (i.e., a separate regulatory loop governed by the chloroplast thiol redox state (*7*, *12*) and becoming mandatory under highly reducing conditions in chloroplasts). It is intriguing that the thiol reductants are efficient inhibitors of the LHCII kinase under deactivating conditions for the kinase, whereas sustained occupation of the Qo site with plastoquinol (e.g., upon illumination at PS II light) protects the kinase from inhibition with thiol reductants (*7*, *12*). Under sustained electron flow, the LHCII kinase probably also rotates between different conformations, like the Qo site and the Rieske extramembrane domain of the cyt b_6f complex, rendering the kinase at a distinct conformation susceptible to inhibition by thiol reductants. Increasing susceptibility of the LHCII kinase to inhibition with increasing irradiance is due to light-enhanced accumulation of thiol inhibitors in chloroplasts.

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