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Redox Regulation of Thylakoid Protein Phosphorylation

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ABSTRACT

The photosystem II of chloroplast thylakoid membranes contains several proteins phosphorylated by redox-activated protein kinases. The mechanism of the reversible activation of the light-harvesting antenna complex II (LHCII) kinase(s) is one of the best understood and related to the regulation of energy transfer to photosystem II or I, thereby optimizing their relative excitation (state transition). The deactivated LHCII protein kinase(s) is associated with cytochrome b_6f and dissociates from the complex upon activation. Activation of the LHCII protein kinase occurs via dynamic conformational changes in the cytochrome b_6f complex taking place during plastoquinol oxidation. Deactivation of the kinase involves its reassociation with an oxidized cytochrome complex. A fine-tuning redox-dependent regulatory loop inhibits the activation of the kinase via reduction of protein disulfide groups, possibly involving the thioredoxin complex. Phosphorylation of LHCII is further modulated by light-induced conformational changes of the LHCII substrate. The reversible phosphorylation of LHCII and other thylakoid phosphoproteins, catalyzed by respective kinases and phosphatases, is under strict regulation in response to environmental changes. *Antioxid. Redox Signal.* 5, 55–67.

INTRODUCTION

REVERSIBLE PHOSPHORYLATION OF THYLAKOID PROTEINS is one of the yet incompletely understood phenomena related to photosynthetic redox reactions. Following the discovery of thylakoid protein phosphorylation, ascribed to redox-controlled membrane-bound protein kinase activity (7, 8), this phenomenon and its significance have been investigated at both the physiological and biochemical levels for over three decades, and the progress in this field has been periodically reviewed (31, 54, 62).

The pioneering work of Bennett and his colleagues recognized several thylakoid phosphoproteins and, later on, identified their phospho-amino acid residues by mass spectrometric approaches (7, 8). The thylakoid phosphoproteins so far identified fall into four groups, those of the photosystem II (PSII) core complex, the PSII light-harvesting proteins, the cytochrome b_6f (cyt. b_6f) complex, and finally the kinases themselves, which are, at least in some cases, prone to reversible phosphorylation (73). During the past few years, the apparent molecular weights of several protein kinases have been inferred following electrophoretic separation, and partially purified thylakoid protein kinase preparations have been obtained. One thylakoid protein kinase [thylakoid-associated kinase-1 (TAK1)] has been cloned and its activity demonstrated *in vitro* (see below). Although the exact mechanisms of activation, deactivation, and inhibition of distinct thylakoid protein kinases have not yet been solved at the molecular level, our general understanding of the above redox-mediated processes, in terms of identification of the system components and physiological implications, has greatly advanced. Phosphatases, the counterparts of reversible protein phosphorylation reactions, have been identified from both the thylakoid membrane and the chloroplast stroma. Unfortunately, the experimental efforts devoted to this field have been less extensive than those to protein kinase research.

Phosphorylation cascades are a universal regulatory mechanism of cellular processes. So far, direct evidence is missing

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for the involvement of thylakoid protein phosphorylation, or the corresponding kinases and phosphatases, in any signaling cascade inside the chloroplast or from chloroplast to the nucleus, to modulate gene expression according to environmental cues. However, similarities between animal receptors and the TAK enzyme (73), as well as the correlation between the activation of the redox system controlling the thylakoid kinase and the expression of nuclear genes (58), might suggest that regulatory networks controlled by reversible thylakoid protein phosphorylation and/or associated kinases and phosphatases are likely to be discovered in the future.

THYLAKOID PHOSPHOPROTEINS

Four intrinsic PSII core proteins in higher plants, the D1 and D2 reaction center proteins, CP43 internal antenna protein, and the 9-kDa *psbH* gene product, can be classified as phosphoproteins. Recent mass spectrometric "phosphorylome" analysis, as well as earlier studies, demonstrated that D1, D2 and CP43 are phosphorylated at their N-terminal threonine (Thr) residues located on the stromal side of the thylakoid membrane (51, 85). Moreover, a doubly phosphorylated PsbH modified at both the Thr-2 and Thr-4 was detected (85). It is intriguing that green algae, mosses, and ferns lack the reversible phosphorylation of the reaction center D1 protein, yet exhibit the reversible phosphorylation of the other three PSII core proteins (17, 57). In cyanobacteria and red algae, on the other hand, none of PSII core proteins undergo reversible phosphorylation (2, 49, 57).

The second distinct group of thylakoid phosphoproteins consists of the light-harvesting chlorophyll a/b antenna system of PSII. Two of these phosphoproteins, Lhcb1 and Lhcb2, belong to the major light-harvesting antenna of PSII, referred to as LHCII. Trimeric LHCII complexes consist of phosphorylatable Lhcb1 and Lhcb2 proteins and nonphosphorylatable Lhcb3 protein in different combinations (43, 72). In both Lhcb1 and Lhcb2, the N-terminal Thr-3 residue (or sometimes possibly a Ser residue) is posttranslationally phosphorylated (52, 85). The possibility that a yet unidentified tyrosine residue is phosphorylated in LHCII was also reported (79).

In addition, one of the minor chlorophyll a/b antenna proteins of PSII, the Lhcb4 protein of the CP29 chlorophyll–protein complex, is phosphorylated in the thylakoid membrane under particular conditions, including excessive light and low temperature (9). The phosphorylation site of this protein is not likely to reside in the immediate N-terminus. On the contrary, upon cold treatment, the Thr-83 residue of Lhcb4 was identified as a phosphorylation site in Lhcb4 *in vivo* (78).

Recently, a component of the cyt. b_6f complex, the subunit V of 15.2 kDa, was identified as a phosphoprotein (26, 35). The amino acid residue undergoing the reversible phosphorylation in this protein still remains to be identified.

An interesting group of phosphoproteins in the thylakoid membrane, yet least studied, are the protein kinases themselves. Recently, a family of proteins, called thylakoid-associated kinases (TAKs), was identified. Three TAKs form a complex, and the kinases themselves were shown to be prone to phosphorylation (73, 74).

PHOSPHORYLATION OF THYLAKOID PROTEINS UNDER *IN VIVO* CONDITIONS

There are principally three different ways to monitor the steady-state level of thylakoid protein phosphorylation in intact leaves or cells: the mass spectrometric approach (51, 52, 85), radiolabeling methods, and the use of antibodies, either phosphoserine/threonine/tyrosine-specific antibodies (11, 64) or protein-specific antibodies after separation of the nonphosphorylated and phosphorylated protein forms by specific gel-electrophoretic systems (22, 46). Mass spectrometry is probably the most accurate method, but laborious in routine use. One major difficulty of the antibody approach resides in the fact that the specificity of antibodies for different phosphoproteins differs, and thus the quantification of the phosphate/protein ratio requires calibration for each protein independently. Radiolabeling methods *in vitro* with [γ-^{32}P]-ATP or [^{32}P]orthophosphate, on the other hand, may bring contradictory results depending on the endogenous phosphorylation state of the proteins to be studied, and thus on the availability of the substrate phosphorylation sites for the phosphorylation reactions under any particular condition. The phosphorylation level of the thylakoid phosphoproteins can be accurately determined with radiolabeled phosphate, allowing prolonged growth of the organism in the presence of radioactive tracers [^{32}P]- or [^{33}P]orthophosphate (88). This method can be easily applied to cyanobacteria and microalgae, but it is more difficult to use in higher plants. A combination of immunodetection methods with radiolabeling methods, however, brings further dimensions for experimentation and allows conclusions to be made on the activation state of the protein kinases responsible for phosphorylarion of any particular protein *in vivo* or *in vitro*.

The phosphorylation level of thylakoid proteins *in vivo* is strongly affected by environmental cues, which modulate the activities of both the kinases and phosphatases. Steady-state phosphorylation of the PSII reaction center proteins D1 and D2 in intact leaves generally increases with increase in the light intensity, or lowering of the ambient temperature (68). The reduction state of the plastoquinone (PQ) pool seems to play a crucial role, the more reduced the PQ pool, the more phosphorylated are the PSII cores in the appressed thylakoid regions. Although stress conditions generally enhance D1/D2/CP43 protein phosphorylation, it was intriguing to observe that abrupt transfer of leaves to heat-shock temperatures induced a rapid dephosphorylation of all these PSII core proteins, occurring via activation of the respective phosphatase (66, 85). Recently, an interesting observation on rapid reversible hyperphosphorylation of PsbH at Thr-4 was demonstrated upon light/dark transitions of *Arabidopsis* plants (85).

The steady-state *in vivo* phosphorylation pattern of Lhcb1 and Lhcb2 proteins distinctively differs from that of the PSII core proteins (64). Maximal phosphorylation of Lhcb1 and Lhcb2 proteins in intact leaves can be found only at low irradiance levels, far below that experienced during the plant growth. Indeed, the LHCII proteins become maximally phosphorylated when plants are transferred from normal growth light conditions to much lower irradiances. Transfer of leaves

to high irradiances, or to low temperature, on the contrary completely inhibits LHCII protein phosphorylation (58). Despite the environmental light/temperature conditions, the metabolic state of the photosynthetic cells also exerts a control over LHCII protein phosphorylation. A notorious case is the observation that LHCII in *Chlamydomonas* cells grown in the presence of acetate is permanently phosphorylated *in vivo*, in darkness as well as in the light, even in the presence of inhibitors of PSII activity thus preventing the light-dependent reduction of the PQ pool (55). This phenomenon is due to the reduction of the PQ pool via metabolic supply of reducing power and not to a loss of the kinase(s) redox control. The complex redox regulation of the LHCII kinase in chloroplasts that explains these observations and the related mechanisms are discussed below in detail.

Observation of Lhcb4 (CP29) protein phosphorylation was first made with a C4 plant upon exposure to low temperature (9). Later it was found that the phosphorylation of Lhcb4 is also typical for C3 plants (10), and occurs when plants are exposed not only to low temperatures, but also at very high light intensities (58).

Subunit V of the cyt. b_6f complex was only recently found to undergo reversible phosphorylation in *Chlamydomonas* cells (26, 35). Phosphorylation occurs concomitantly with LHCII protein phosphorylation during transition of cells from state 1 to state 2. The reported phosphorylation of TAKs (73, 74) has not yet been thoroughly studied.

THYLAKOID PROTEIN KINASES

Although authors in most cases refer to an LHCII or a PSII protein kinase, the data so far published favor involvement of several protein kinases among which not all are redox-regulated. Differential substrate and site specificity, different ionic requirements, and different sensitivity to inhibitors have been reported (3, 8, 26, 31). Furthermore, it appears that the specific redox-dependent regulation of thylakoid protein phosphorylation also involves more than one protein kinase (70). A model of a kinase cascade, one kinase being activated by thylakoid redox control and subsequently activating other phosphorylation processes by phosphotransferase activity, has been suggested (73, 89). Despite efforts of many research groups toward the elucidation of thylakoid protein phosphorylation, the progress in the identification and characterization of the enzymes and their corresponding genes, as well as the elucidation of their precise specificity toward each of the different membrane intrinsic substrates, is extremely limited.

Initial attempts to purify protein kinase from isolated thylakoids indicated that protein kinase activity comigrates with the cyt. b_6f complex in sucrose gradients (29, 90). These results were in line with the concept that the light-regulated phosphorylation of LHCII is related to the reduction of the PQ pool, and cyt. b_6f may be involved in the activation of the protein kinase (see below). Perfusion chromatography of cyt. b_6f crude preparations resulted in the isolation of protein kinase-enriched fractions (30, 92). The peak activity of such fractions correlated with the presence of protein kinase active bands in the range of 33, 55, 64, and 85 kDa, identified by renaturation of the polypeptides resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The major kinase activity coincided with the 64-kDa band (92) that could be partially resolved from a 64-kDa contaminant polyphenol oxidase (40, 75).

The phosphorylation of CP29 occurs under different conditions from that of the other PSII-associated polypeptides, and it was suggested that CP29 phosphorylation is carried out by a specific kinase different from the LHCII kinases (9).

Kinase activity has also been reported to be associated with PSII core preparations (59), being capable of phosphorylating the N-terminal truncated LHCII from pea (18). Recent results, however, demonstrate that PSII core preparations exhibiting a major protein kinase of ~33–35 kDa do not phosphorylate native LHCII, but instead have significant redox-independent phosphorylation activity toward CP43 (86). The phosphorylation of the D1 protein, although light-regulated (see below), appears to be unrelated to the redox control exhibited by the LHCII protein kinase *in situ* (31).

We can conclude that to date, thylakoid protein kinases include proteins of 85, 60–64, 55, and 28–33 kDa (31, 48). The kinases of apparent molecular masses of 30, 64, and 85 kDa typically copurify with the cyt. b_6f complex (87).

Among all putative thylakoid kinases, so far the only successful identification of a pure protein kinase that phosphorylates LHCII was achieved by using the N-terminus of LHCII as a selection target in the yeast two-hybrid system (73). The corresponding gene was cloned from *Arabidopsis,* overexpressed in *E. coli,* and the translation product was proved to have kinase activity. This 55-kDa thylakoid protein kinase (TAK1) copurifies with the cyt. b_6f complex and belongs to a family of protein kinases including members localized in the plant cell wall (73, 74). The activity of purified TAK1 is enhanced by addition of dithiothreitol (DTT), indicating that vicinal dithiols may be involved in the stabilization of the active form of purified enzyme. DTT did not enhance the phosphorylation activity of the PSII core preparation, but enhanced the activity of the solubilized kinase preparation reported in Zer *et al.* (92; unpublished observations). The above effects of DTT on the isolated protein kinase are, however, only marginal as compared with the >100-fold increase in the LHCII phosphorylation activity of the protein kinase via the plastoquinol (PQH_2)/cyt. b_6f interaction *in situ.*

PHOSPHATASES INVOLVED IN DEPHOSPHORYLATION OF THYLAKOID PROTEINS

The reversibility of the phosphorylation of thylakoid proteins is achieved via the activity of protein phosphatases. As compared with our knowledge on the protein kinases, even less is presently known about the nature, substrate specificity, and identity of the thylakoid phosphoprotein phosphatases. Although several serine/threonine phosphatases, both integral and extrinsic membrane proteins as well as soluble proteins, have been partially purified from chloroplasts (36–39, 77, 84), the genes and protein sequences remain unavailable. It also appeared that the classification of chloroplast phosphatases

to conventional groups of serine/threonine phosphatases is not appropriate because of their insensitivity to mammalian type PP1 and PP2A phosphatase inhibitors, okadaic acid and microcystin (76). This opinion should, however, be revisited after a recent isolation and purification of a thylakoid-associated protein phosphatase that was prone to inhibition by okadaic acid and tautomycin, and thus could be characterized as a PP2A-type phosphatase (84). Discrepancy with previous results is possibly due to the inaccessibility of the regulatory domains in the phosphatase by the inhibitors in intact thylakoid membranes. The purified phosphatase was further shown to be under regulation by an immunophilin TLP40 (27, 84) located in the thylakoid lumen (27, 45). TLP40 completely inhibited the activity of the thylakoid-associated phosphatase when bound to the thylakoid membrane, whereas release of TLP40 to the thylakoid lumen fully activated the phosphatase. Possible relation of TLP40 binding activity to the redox state of the thylakoid membrane has not yet been elucidated.

Specific dephosphorylation of PSII core proteins with concomitant release of TLP40 from the thylakoid membrane was demonstrated to occur rapidly under both *in vivo* and *in vitro* conditions when isolated thylakoids or intact leaves were suddenly exposed to heat-shock temperatures (66, 85). LHCII dephosphorylation was, however, not affected under *in vivo* conditions. This differential effect could be ascribed to the dissociation of the TLP40 from the protein phosphatase specifically involved in the regulation of the PSII core complex phosphoproteins, a process that may be involved in the rapid turnover of the D1/D2 proteins (see below).

In accordance with a lack of strict biochemical, structural, or genetic data on chloroplast phosphatases, the information accumulated on regulation of thylakoid protein phosphatases is also scattered and fairly vague. However, accumulating evidence suggests that the phosphatases have distinct substrate specificity and, at least some of them, are highly regulated by light, possibly via the redox conditions in the chloroplasts. The possibility that the activity of the phospho-LHCII phosphatase is redox-regulated is still controversial. However, in isolated spinach thylakoids, the phospho-LHCII phosphatase activity appears to be light- and redox-independent (71). The activation of the phosphatase involved in the dephosphorylation of the phospho-D1 protein in *Spirodela oligorritza* was reported to be light-stimulated (22). In fact, there might be two different kinds of phosphatases involved in D1 protein dephosphorylation (63). Dephosphorylation of damaged D1 proteins only occurs in light, whereas dephosphorylation of nondamaged phospho-D1 proteins is independent of light.

REDOX CONTROL OF THYLAKOID PROTEIN PHOSPHORYLATION

Phosphorylation of LHCII

The components involved in the redox-dependent LHCII kinase activation have been established as being reduced PQ or quinone analogues that can serve as electron donor to the cyt. b_6f complex and a cyt. b_6f complex that can bind a PQH_2 molecule at the quinol oxidation site (Q_o site), but cannot oxidize it (26, 81–83, 95) (Fig. 1). Thus, kinase activation can be induced by light, generating reduced PQH_2 via PSII electron flow or, alternatively, by addition of reductants such as dithionite or duroquinol in darkness. Under illumination, the ratio cyt. b_6f red/ox at any steady-state level is related to the ratio PQH_2/PQ (*i.e.,* the relative concentration of the electron donor) and to the rate of oxidation of the cyt. b_6f electron acceptor, plastocyanin. The oxidation of the latter in its turn depends on photosystem I (PSI) activity, a light-driven process, as well as on the rate of electron flow from PSI to the final acceptor sink that *in vivo* is represented by the process of CO_2 fixation. As mentioned above, a reduced cyt. b_6f, interacting with a reduced quinol at its Q_o site, appears to act as the "kinase activator" complex. The relative concentration of the kinase activator, in light-exposed chloroplasts, will depend on the relative activities of the light-driven PSII reduction of PQ and thus of the cyt. b_6f and their oxidation by light-driven activity of PSI. The activity of the latter is obviously related to the carbon fixation activity. These activities can be modulated by the light quality. Excessive excitation of chlorophyll *b* (650 nm) associated mostly with LHCII, relative to excitation of chlorophyll *a* present in both PSII and PSI antennae will increase the ratio PQH_2/PQ and thus the reduced cyt. b_6f population. Therefore, light absorbed preferentially by the PSII and the LHCII antennae will promote the protein kinase activation, whereas light absorbed preferentially by PSI (710 nm), resulting in the oxidation of the PQ pool, will deactivate the kinase.

The kinase activation mechanism is fully functional in isolated thylakoids at both high and low light. Even very low light intensities reduce all electron chain carriers in isolated thylakoids, and thus the kinase is expected to be fully activated at light intensities of only 5–10% of the intensity required for the saturation of electron flow (that is sufficient to prevent the reoxidation of the PQH_2 pool by ambient oxygen). Under such conditions, the phosphorylation level of LHCII is limited only by the ratio of the LHCII kinase/phosphatase activity. The same is true if the kinase activation *in vivo* is driven in darkness by the reduction of the PQ pool via the respiratory metabolic supply of reductants, whereas the oxidation of plastocyanin is blocked in the absence of light-driven PSI activity.

In natural chloroplast redox environment *in vivo*, the LHCII protein phosphorylation is additionally subjected to regulation by the thiol-reducing activity of the stroma (Fig. 1). An increase in the thiol-reducing activity of the chloroplast stroma activates another regulatory loop that leads to inhibition of LHCII protein phosphorylation (see below, 62). The LHCII protein kinase seems to be a target for such thiol-induced inhibition mechanism. The LHCII kinase can thus be found in either the active, inactive (deactivated), or inhibited state (Fig. 1) in the ever-changing chloroplast redox environment. Gradual thiol-induced inhibition of the kinase upon increasing reduction state of the stroma is, however, not an independent regulation system, but instead is closely linked to the function of the kinase activation/deactivation mechanism (65). These two redox regulation mechanisms of the LHCII protein kinase, the cyt. b_6f-dependent activation/deactivation and the thiol-induced inhibition, are discussed below in more detail.

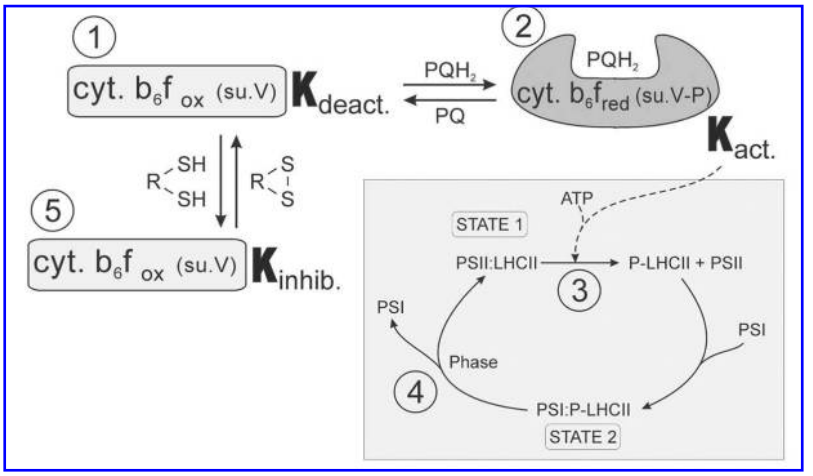

FIG. 1. Hypothetical scheme showing the reversible association of the LHCII kinase (bold K) with the cyt. b_6f complex with concomitant transition of the kinase between the activated, deactivated, and inhibited forms, and relations between the LHCII phosphorylation/dephosphorylation and the state 1 to state 2 transition in the thylakoid membrane (see, *e.g.,* 26, 34, 65, 83). (**1**) Rectangular box represents the cyt. b_6f complex in its oxidized form (ox), and outside the box the assumed associated kinase is in its deactivated form ($K_{deact.}$). (**2**) PQH_2 reduces the cyt. b_6f complex (red); PQH_2 binds at the cyt. b_6f Qo site, and in the process of its oxidation, conformational changes occur in the complex and subunit V (su.V) is phosphorylated (su.V-P). The kinase is activated and released from the cytochrome complex ($K_{act.}$). (**3**) The PSII-bound LHCII (PSII:LHCII) (state 1) is phosphorylated by the activated kinase when ATP is present. The phosphorylated LHCII complex (P-LHCII) dissociates from PSII, binds to PSI to form the PSI:P-LHCII complex, and thus reduces excitation of PSII and increases that of PSI (state 2). (**4**) The phosphorylated LHCII can be dephosphorylated due to the activity of the phosphatase (Phase). LHCII dissociates from PSI and reassociates with PSII. (**5**) The kinase in its deactivated form, while bound to the cyt. b_6f complex, can be inhibited (K_{inhib}) by reducing dithiol reagents and reactivated by thiol oxidants.

Mechanism of the redox-controlled activation/deactivation of the LHCII protein kinase via PQH_2/cyt. b_6f interaction. As mentioned above, redox activation of the thylakoid protein kinase occurs under conditions promoting the generation of reduced PQH_2 and reduced cyt. b_6f. LHCII phosphorylation is abolished in mutants lacking cyt. b_6f (28), as well as in mutants in which functional cyt. b_6f is impaired in the activity of the Q_o site (95). Inhibitors competing with the binding of PQH_2 at the cyt. b_6f Q_o site, such as 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB), prevent the activation of the LHCII protein kinase in light-exposed thylakoids. On the other hand, inhibitors of cyt. b_6f oxidation, such as 2-(*n*-heptyl)-4-hydroxyquinoline *N*-oxide (HQNO), promote the activation of the LHCII kinase (81). The extent of kinase activation in darkness by promoting the reduction of PQ via transient acidification of the medium (81, 82) can be estimated by titrating the binding of DBMIB to the cyt. b_6f complex (82). In these experiments, the LHCII protein kinase remained active as long as the cyt. b_6f complex remained reduced and a PQH_2 molecule was present and could interact with the complex at the Q_o site. However, a single turnover flash in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) causes the oxidation of the PQH_2 at the cyt. b_6f Q_o site via consecutive electron transfer via the cytochrome electron carriers and plastocyanin to reduce the oxidized PSI complex. Thus, the high-potential path electron carriers of the cyt. b_6f complex, as well as plastocyanin, maintained their reduced state after the flash, whereas the reduced quinol at the Q_o site was oxidized, resulting in the immediate deactivation of the protein kinase (82). These results demonstrate that the kinase activation is related to the occupancy of a cyt. b_6f Qo site by a reduced PQH_2.

In the above scheme, it is assumed that the protein kinase in an inactive state (deactivated kinase) is in some way associated with the oxidized cyt. b_6f complex. It was thus proposed that the occupancy of the cyt. b_6f Q_o site by PQH_2 may cause a conformational change in the complex that may result in the kinase activation (83). The above hypothesis on the possible role of conformational changes in cyt. b_6f was based on the finding that large transient conformational changes occur at the Q_o site of the cyt. bc1 complex during the quinol oxidation process. In the presence of inhibitors of quinol oxidation, the extramembrane segment of the Rieske Fe-S protein subunit of the cyt. bc1 complex is located in a position "proximal" to the Q_o site, whereas in the presence of inhibitors of cyt. c1 oxidation it is located in a "distal" position close to the heme of the cyt. c1 subunit of the complex. In the absence of inhibitors, this Rieske protein segment is in an intermediate or a distal posititon location (42, 94). As the cyt. b_6f complex is an analogue of the cyt. bc1 in terms of electron carrier composition, sensitivity to quinol oxidation inhibitors, and redox potentials, one could assume that similar changes may occur during the oxidation of PQH_2 via the cyt. b_6f complex in the thylakoid membrane (83, 95). At the time of formulation of this hypothesis, it was assumed that the movement of the Rieske protein is related to the conformational changes that cause the kinase activation (83). Site-directed mutants induced in the *Chlamydomonas* cyt. b_6f subunit IV that participates in the formation of the Q_o site, together with the Rieske Fe-S subunit of the complex, affected the binding

of PQH_2 to the Q_o site and resulted in a loss of the redox activation of the LHCII protein kinase (95), thus providing direct molecular evidence for the above hypothesis. Results obtained using electron crystallography of the cyt. b_6f complex from *Chlamydomonas* indeed indicated a similar movement of the extramembrane helix of the Rieske subunit upon binding of stigmatellin to the Q_o site of the complex, as predicted by the analogy of the cyt. b_6f to the bc1 complex (12, 13).

As opposed to the large body of experimental results demonstrating that binding of reduced PQH_2 at the Q_o site of cyt. b_6f is mandatory for the LHCII protein kinase activation, the hypothesis that the activation is related to the conformational changes in cyt. b_6f induced by the interaction of the quinol with the Q_o site remains to be further elucidated. The original proposal concerning the role of the conformational changes in the kinase activation did not specify whether a particular conformation of the cyt. b_6f complex is responsible for the activation of the kinase. One could consider that this may occur when the Rieske protein is "locked" into one of the two positions, distal or proximal, or released allowing freedom of movement between the two positions during the quinol oxidation process. This possibility was examined in a series of experiments in which phosphorylation of LHCII and the related state transition in *Clamydomonas* cells was induced in darkness by strong aeration (state 1) or oxygen depletion (state 2) in the presence or absence of the Q_o site inhibitors DBMIB or stigmatellin. The results of this work (26) indicated that "locking" of the Rieske protein in either the distal or proximal position prevents the kinase activation. However, concomitant with the kinase activation, the subunit V of the cyt. b_6f complex became also phosphorylated. Thus, it was proposed that actually the conformational changes induced by the movement of the Rieske protein extramembrane segment from the distal to the proximal position during the oxidation of PQH_2, *i.e.,* the dynamics of the process, might be the condition required for the kinase activation. Besides the movement of the Rieske extramembrane segment, electron crystallography data indicate also some conformational changes in a transmembrane helix located close to the interface of the monomers forming the cyt. b_6f complex (13). These changes and possibly the phosphorylation of subunit V may be involved in the release of the activated kinase (26).

The above conclusion on kinase activation is not at variance with the original hypothesis proposed by Vener *et al.* (83). The PQH_2 residence time at the Q_o site of a reduced cytochrome complex [occurring under the experimental conditions described by the above authors, and referred to as " occupancy" (82)] is short due to its reversible binding and release because the quinol cannot be oxidized. This may lead to an oscillation of the Rieske "hinge" between the proximal position upon binding and distal position upon relaxation due to the dissociation of the PQH_2. Thus, a dynamic state of complex conformational changes may occur that induces the protein kinase activation similar to that occurring during the sustained PQH_2 oxidation by the cytochrome complex. Gal and colleagues (31) have proposed that the protein kinase is inactivated while bound to the complex and is released in its active state following the interaction of the cyt. b_6f with a reduced PQH_2 (Fig. 1). Reassociation of the kinase with an oxidized cytochrome complex may serve as the mechanism of its inactivation. This possibility is in agreement with the observation that extraction of the cyt. b_6f complex and the kinase from thylakoids treated with detergents releases LHCII kinase in an active form that does not respond to redox control, as is the case for the membrane-bound enzyme(s) (31, 92).

The $t_{1/2}$ of the kinase active state *in situ*, in darkness following the oxidation of PQH_2, has been estimated to ~3 min (81). Assuming that in the active state the kinase is free, the inactivation may be due to the reassociation of the kinase with a cyt. b_6f complex that is oxidized by the light-driven PSI activity or by ambient oxygen in darkness. The protein kinase(s) content of thylakoids is not stoichiometric to that of the cyt. b_6f complex and represents a minute fraction of the total thylakoid protein [<0.01% (31)]. The chances of an activated "free" kinase encountering in its lateral migration within the membrane plane an LHCII complex that has an open phosphorylation site may be competing with the chances of encountering an oxidized cyt. b_6f complex and rebinding, thus being inactivated. For a more detailed discussion of the dynamics of the activation/deactivation of the LHCII kinase see references 31 and 54.

Thiol redox regulation of the LHCII protein kinase. The question arises whether conformational changes may occur also in the LHCII kinase upon activation, thereby affecting the position and availability of active-site residues to the substrates, ATP and LHCII protein. Due to limited knowledge on the molecular identity of the LHCII kinase(s), this question is more difficult to answer. Accumulating biochemical data, however, allow us to draw some conclusions about the modification of the kinase itself when changing from the inactive to active form. Rintamäki *et al.* (65) probed the susceptibility of LHCII phosphorylation to thiol-modifying agents by applying a thiol alkylating agent, thiol reductants, and oxidants to thylakoid membranes under conditions when the LHCII kinase was deactivated (Q_o site empty) or activated (Q_o site occupied with reduced PQH_2). Phosphorylation capacity was then tested in a traditional way by exposing the thylakoids to white light in the presence of ATP. These experiments revealed that LHCII phosphorylation is susceptible to modifications by externally added thiol reactant only if it is added under conditions when the kinase is deactivated (*i.e.,* the Q_o site of cyt. b_6f complex is oxidized either by incubation of thylakoids in darkness or via illumination by PSI light). Treatment of thylakoid membranes with thiol reductants (or alkylating agents) under conditions that keep the kinase active (Q_o site occupied by PQH_2 via illumination with PSII light or via metabolic reduction of PQ), on the contrary, allowed full phosphorylation of LHCII upon addition of ATP. As the inhibition of LHCII protein phosphorylation by thiol reductants is strictly dependent on the activation state of the kinase, and occurs efficiently also in darkness, it is likely that the kinase rather than LHCII substrate is a target for inhibition by thiol reductants. It is thus conceivable that the deactivated kinase, or some vicinal protein, has a disulfide bridge exposed on the surface of the protein. Reduction of this bond to a dithiol would exert a conformational change in the kinase rendering it inhibited in the phosphorylation of LHCII proteins. Oxidation of the dithiol rapidly restores the capability of the enzyme to phosphorylate LHCII proteins, most likely by

reestablishing the disulfide bridge. Deactivated kinase apparently has a regulatory disulfide bridge exposed on the surface of the protein, being thus easily prone to reduction by dithiols. Activation of the kinase, on the other hand, seems to induce a conformational change in the protein, thus occluding the regulatory disulfide bridge inside the protein molecule and making it inaccessible to reduction by dithiols. It remains to be elucidated whether the regulatory disulfide bridge is located directly in the active center of the kinase, in a close vicinity to the active center, or even in a different protein, which, however, upon oxidation/reduction would induce a distinct conformational change in the kinase active site.

Does the disulfide bridge in the LHCII protein kinase have any relevance for LHCII protein phosphorylation in the chloroplast redox environment? It is well known that apart from the activation and deactivation of the LHCII kinase by reversible binding of reduced PQH_2 to the Q_o site of the cyt. b_6f complex, the LHCII phosphorylation is also prone to inhibition. Inhibition of LHCII protein phosphorylation was first observed *in vivo* at high photoinhibitory light intensities (69), and more recently the mechanism was found to become operational in the chloroplast redox environment already at moderate light intensities (19, 64). Inhibition of LHCII protein phosphorylation following preillumination of isolated thylakoids before the activation of the protein kinase has been reported (54). The thiol-induced inhibition of LHCII protein phosphorylation *in vitro*, on the other hand, can be induced in darkness as well as in the light, and the inhibition is dependent on the concentration of the thiol reductant (65). The down-regulation of LHCII protein phosphorylation at increasing light intensity *in vivo* can thus be ascribed to (a) inactivation of the kinase by a thiol redox-dependent mechanism and (b) inaccessibility of the LHCII phosphorylation site(s) due to light-induced conformational changes of the LHCII N-terminal domain (92) (see below). These two phenomena are not mutually exclusive.

The down-regulation of LHCII protein kinase in intact leaves functions in close cooperation with the activation/deactivation of the LHCII protein kinase by occupation/release of PQ at the Q_o site of the cyt. b_6f complex. Under the thiol-oxidizing environment of the chloroplast stroma (low light, darkness), the activity of the kinase is solely dependent on the occupation of the Q_o site at the cyt. b_6f complex by PQH_2, and on the availability of ATP for phosphorylation. At increasing irradiance (or decreasing temperature), the production of reducing equivalents by photosynthetic light reactions exceeds the capacity for their utilization in carbon fixation and other metabolic reactions in the chloroplast stroma. Under such conditions, the reduced thiol compounds accumulating in the stroma convert the disulfide bridge exposed on the surface of the LHCII kinase to dithiol, thus inhibiting the kinase activity. Such inhibition only occurs when the kinase in its deactivated form is transiently associated with oxidized cyt. b_6f complex. In the chloroplast redox environment in the light, the kinase undergoes constant activation/deactivation cycles, and it is the concentration of thiol reductants in the stroma that is decisive for inhibition of the kinase. The phosphorylation of LHCII is maintained at a certain constant level in plants acclimated to either low or high light intensity. Shifting the plants to light of higher intensity than that experienced during the acclimation strongly inhibits the LHCII phosphorylation. However, exposure of the plants to a light intensity lower than that experienced during the acclimation process increases the level of LHCII phosphorylation. The same mechanism, a thiol-induced inhibition of the LHCII kinase, is likely to operate also at low temperatures where inhibition of LHCII protein phosphorylation occurs in nonacclimated plants (57, 58).

Thioredoxin is a likely mediator of LHCII kinase inactivation in chloroplasts (65). It is interesting to note that an increasing number of kinases, in both plant and animal cells (14, 67), have recently been found as targets for inhibition by thioredoxin. As to the LHCII kinase, a tight coregulation of kinase activity by the two partially interdependent redox mechanisms, together with the phosphatase activity, determines the state of LHCII protein phosphorylation in changing chloroplast redox environment.

Control of PSII, D1/D2, and CP43 protein phosphorylation

The phosphorylation extent of the PSII core phosphoproteins increases in light-exposed chloroplasts or intact leaves. However, the level of their phosphorylation does not seem to be solely under redox control. Mutants impaired in the activity of the cyt. b_6f complex, in which the LHCII phosphorylation is completely inactivated, continue to phosphorylate the PSII core complex proteins (31, 81). Dephosphorylation under preferential excitation of PSI is more pronounced for the LHCII than for the PSII core proteins, indicating a more rapid inactivation of the LHCII kinases as compared with that of the PSII core protein kinases (15). Differences also include the extent of protein phosphorylation with respect to light intensity. The phosphorylation of the D1/D2 proteins increases with increasing illumination, whereas that of the LHCII decreases significantly at high irradiance (63, 64). Although it is possible that different protein kinases responding to redox conditions may be involved in the phosphorylation of PSII core proteins, it is also possible that illumination may affect the exposure of various membrane-integrated phosphoproteins to the protein kinase/phosphatases in different ways (see below).

Light-induced substrate activation/deactivation

The light-induced phosphorylation of the LHCII and PSII proteins has been considered so far in relation to the process of the protein kinase activation/deactivation and inhibition. The reversible phosphorylation of LHCII plays an important role in the regulation of energy transfer from LHCII to PSII or PSI (state transition), whereas the phosphorylation of the PSII core complex proteins D1/D2 is considered to regulate their proteolysis during the light-induced turnover process (20, 46, 63). So far, no specific role has been ascribed to phosphorylation of CP43. The above substrates of the protein kinases are assembled with chlorophyll and carotene into functional complexes. It is well established that illumination of LHCII induces changes in the macroorganization of isolated LHCII as well as *in situ* (6, 16). Changes in the carotene composition of the LHCII following the activation of the xanthophyll cycle affects the ratio of energy transfer/

dissipation (24, 32, 53) and possibly the structure of the LHCII. The question arises if conformational changes may not be light-induced in LHCII or PSII core preparations that may affect the exposure of the phosphorylation site to the protein kinase. Using an *in vitro* reconstituted system containing isolated LHCII and a solubilized protein kinase preparation obtained from spinach thylakoids, it was demonstrated that preillumination of LHCII increases significantly the subsequent rate and extent of LHCII phosphorylation in darkness as compared with a nonilluminated control (92). This effect is reversible and is ascribed to a light-induced exposure of the N-terminal domain of LHCII to the enzyme as indicated also by an increase in the exposure of this domain to trypsin cleavage. A similar effect was observed also in isolated thylakoids, which were briefly preilluminated in the absence of ATP and with addition of DCMU, thus preventing the activation of the redox-controlled kinase. Following the illumination, phosphorylation was carried out in darkness, activating the kinase by addition of duroquinol. However, prolonged preillumination of thylakoids (20–40 min) under the above conditions drastically inhibited the phosphorylation of the membrane-bound LHCII, but not that of exogenously added, solubilized complex. These results indicate that the exposure of membrane-bound LHCII to illumination, while preventing its phosphorylation, induces changes in the organization of LHCII, rendering its N-terminal domain inaccessible not only to the membrane-bound kinase, but to tryptic cleavage as well (54).

This phenomenon could be ascribed to light-induced aggregation of the nonphosphorylated LHCII that is only slowly reversible. Based on these results, it appears that phosphorylation of LHCII is required not only to induce the dissociation of LHCII from PSII, but also to maintain the complex in a free state during the process of its lateral migration and association with PSI. Light induced exposure of the phosphorylation sites of the CP43 chlorophyll-protein antenna of the PSII core complex has also been demonstrated (54, 86). However, in this case, the exposure increases with light intensity and does not result in occlusion of the sites to the kinase. The above phenomena may contribute, at least partially, to the observed phenomenon that under high light excitation the phosphorylation level of LHCII decreases, whereas that of the PSII core complex proteins increases.

PHYSIOLOGICAL IMPLICATIONS OF THYLAKOID PROTEIN PHOSPHORYLATION

Role of PSII core protein phosphorylation in electron transfer, susceptibility of PSII to photoinhibition, and the repair of PSII

Strong phosphorylation of PSII core proteins, particularly that of the D1 and D2 proteins, was initially suggested to regulate PSII electron transfer. However, these earlier experiments failed to demonstrate any causal relationships between the phosphorylation level of PSII core proteins and the activity of PSII. More recently, an elaborate EPR and fluorescence study was accomplished to elucidate possible relationships between various partial electron transfer reactions and the phosphorylation state of the PSII reaction center proteins, but, no causal relationships were identified (F. Mamedov, personal communication). In another set of experiments, the role of PSII core protein phosphorylation in the susceptibility of PSII to photoinhibition was addressed (46). It is notable that both phosphorylated and nonphosphorylated PSII centers are equally susceptible to light-induced inactivation. However, the degradation of damaged D1 protein was prevented in phosphorylated PSII centers, and indeed dephosphorylation was shown to be a prerequisite for proteolytic degradation of the damaged D1 protein (63).

Phosphorylation of the D1 protein is a typical feature of seed plants only (57). These plants also have clearly defined organization of the thylakoid membrane network into grana structures with connecting stroma thylakoids. Functional PSII, as well as the photodamaged PSII, are located in the appressed grana membranes, whereas repair of PSII centers, with cotranslational assembly of *de novo* synthesized new D1 copy into partially disassembled PSII (93), takes place on stroma-exposed thylakoid domains accessible to polyribosome binding. It is conceivable that D1 protein phosphorylation in appressed thylakoid domains serves to maintain the integrity of damaged PSII before migration to stroma-exposed membranes where dephosphorylation and degradation take place with concomitant insertion of the new D1 copy into PSII. Indeed, the seed plants have a capacity to accumulate photodamaged PSII centers in grana appressions under conditions where the repair capacity in stroma thylakoids has been exceeded (5). Photosynthetic organisms with no D1 protein phosphorylation either completely lack the grana structures (cyanobacteria) or have a looser thylakoid organization (green algae, mosses). In the above organisms, the D2 protein forming together with the D1 counterpart the photochemical reaction center (60, 61, 96) is phosphorylated and possibly plays a similar role. The process of migration of photoinactivated PSII core to the stroma membranes occurs also in these organisms exhibiting a less stringent grana organization, and the degradation and replacement of the damaged D1 protein follows the same rules (1). The PSII core protein phosphorylation has also been implicated in stabilization of the dimeric structure of the PSII complexes in the grana (47).

Thylakoid protein phosphorylation and state 1/state 2 transitions

The physiological role of the redox-controlled LHCII phosphorylation is to balance the transfer of absorbed light energy between the two photosystems, thus ensuring maximal efficiency of electron flow. In *Chlamydomonas*, the state transition process is accompanied by an increase in the cyclic electron flow via PSI concomitant with translocation of cyt. b_6f from the appressed grana membranes to the stromal membranes, thus supporting the increase in cyclic electron flow (25, 80). The major role ascribed to the redox-controlled LHCII phosphorylation is that of balancing the distribution of the absorbed light energy between PSII and PSI under moderate light intensities. In the metabolic state of the chloroplast whereby the PQ pool is oxidized, the "mobile" LHCII com-

plex formed by the *lhcb1* and *lhcb2* gene products is dephosphorylated and associated with PSII (state 1). Shifting the metabolism to conditions promoting reduction of the PQ pool and cyt. b_6f, thus inducing the phosphorylation of LHCII, coincides with the dissociation of the mobile LHCII antenna from PSII and its association with PSI (state 2) (Fig. 1).

The state 1/state 2 transition and its relation to the redox-controlled reversible phosphorylation of LHCII have been recently reviewed (4, 34, 44, 54). Thus, transition from state 1 to state 2, preferentially occurring at low light intensities, increases the supply of ATP required for regeneration of RuBP to match with the supply of reduced NADP thereby optimizing carbon fixation.

Until recently, it was assumed that the binding of LHCII to PSII, and its release, are the steps controlling the state transition, whereas energy transfer to PSI by phospho-LHCII was supposed not to necessitate binding of the antenna to the complex. Recent results indicate that this may not be the case. In *Arabidopsis* mutants, an inhibition of *psaH* gene expression, encoding the PsaH subunit of PSI, lowered the energy transfer to PSI under conditions promoting state 2, and apparently resulted in a state 1-type of energy transfer to PSII despite LHCII being phosphorylated (34). PsaH subunit is located on the stromal side of the PSII complex and seems to be essential for physical association of mobile phospho-LHCII with PSI. It was thus proposed that the association of LHCII with PSI or PSII is the result of competition on the LHCII/phospho-LHCII between PSII and PSI, the latter apparently having a higher affinity for phospho-LHCII than PSII.

Does the plastid redox state and thylakoid kinase activation play any role in chloroplast to nucleus signaling?

Although the phenomenon of chloroplast to nucleus signaling is well established (33), the mechanisms of signal perception, as well as the downstream events that convey the signal to the nucleus, are not understood. There is a considerable body of evidence implicating the chloroplast electron transfer and thus the photosynthetic redox reactions in the signaling process. Particular emphasis has been paid on the regulation of *lhcb* gene expression with respect to chloroplast signals in adjusting the efficiency of the light-harvesting capacity of the thylakoid membranes according to environmental cues. Short-term acclimation is achieved by state-1/state-2 transitions related to the activation/deactivation of the LHCII protein kinase(s), whereas the long-term effects would require an adjustment of the expression level of nuclear *lhcb* genes. Strong down-regulation of the *lhcb* gene expression at high light has been linked to a high reduction state of the PQ pool (23, 41, 56, 91). Signals transduced from chloroplasts under such conditions were suggested to activate in the nucleus a repressor protein, which binds to the 5´ regulatory region of the *lhcb* gene (23). In line with this hypothesis, a *Lemna* mutant lacking cyt. b_6f complex, and thus exhibiting a constant high PQH_2/PQ ratio, was shown to be locked to a high-light acclimation state with respect to accumulation of LHCII proteins (91). In the experimental conditions described above, the LHCII protein kinase could not be activated in the absence of the cyt. b_6f complex, nor were the plants able to accumulate LHCII proteins when shifted to low-light conditions. The question thus remains whether the active state of LHCII protein kinase possibly has a role in signal perception or transduction from chloroplasts to the nucleus to accelerate the expression of *lhcb* genes.

Extensive studies with rye plants revealed a distinct correlation between the active state of the LHCII protein kinase and the accumulation of *lhcb* mRNA (58) upon short-term (up to 2 days) changes in light and temperature conditions. Moreover, it has been shown by amino acid sequence analysis that the TAK kinases, active in LHCII phosphorylation, share common domain characteristics with transforming growth factor-β receptors (73, 74), which in turn are known to elicit a diversity of physiologically important signals in vertebrates and *Drosophila* (50). It is thus conceivable to consider that the redox state of the plastid and the activation of thylakoid LHCII kinase(s) are possibly involved in the initiation of signaling cascades from chloroplasts to the nucleus. So far, however, there is no evidence indicating that the LHCII kinase or other thylakoid protein kinases would phosphorylate any downstream signal-transduction components in chloroplasts. Thus, the molecular mechanisms possibly linking the activation of the thylakoid-associated LHCII kinase to the accumulation of *lhcb* mRNA remain to be elucidated.

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ABBREVIATIONS

CP43, chlorophyll a binding protein of photosystem II; cyt. b_6f, cytochrome b_6f complex; D1 and D2, reaction center II proteins; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DTT, dithiothreitol; LHCII, light-harvesting chlorophyll a/b binding protein complex II; PQ, plastoquinone; PQH_2, plastoquinol; PSI and PSII, photosystem I and II, respectively; Q_o site, quinol oxidation site of the cytochrome b_6f complex; TAK, thylakoid-associated kinase; Thr, threonine residue.

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