Thylakoid Protein Phosphorylation and the Thiol Redox State†

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ABSTRACT: Illumination of thylakoid membranes leads to the phosphorylation of a number of photosystem II-related proteins, including the reaction center proteins D1 and D2 as well as the light-harvesting complex (LHCII). Regulation of light-activated thylakoid protein phosphorylation has mainly been ascribed to the redox state of the electron carrier plastoquinone. In this work, we show that this phosphorylation in vitro is also strongly influenced by the thiol disulfide redox state. Phosphorylation of the light-harvesting complex of photosystem II was found to be favored by thiol-oxidizing conditions and strongly downregulated at moderately thiol-reducing conditions. In contrast, phosphorylation of the photosystem II reaction center proteins D1 and D2 as well as that of other photosystem II subunits was found to be stimulated up to 2-fold by moderately thiol-reducing conditions and kept at a high level also at highly reducing conditions. These responses of the level of thylakoid protein phosphorylation to changes in the thiol disulfide redox state are reminiscent of those observed in vivo in response to changes in the light intensity and point to the possibility of a second loop of redox regulation of thylakoid protein phosphorylation via the ferredoxin−thioredoxin system.

Protein phosphorylation plays a major role in gene expression, cellular signaling, and metabolic regulation in living cells. In the thylakoid membranes of plant chloroplasts, at least 13 different proteins, most of which belong to photosystem II (PSII)[1] and its light-harvesting antenna (LHCII), are phosphorylated on threonine residues, in a light-dependent manner (*1*−*4*). Among those most strongly phosphorylated are the two major polypeptides of LHCII, the PSII reaction center proteins D1 and D2, the chlorophyll *a* binding protein CP43, the 9 kDa psbH gene product, and a 12 kDa phosphoprotein. The phosphorylation of LHCII has generally been implicated in the regulation of excitation energy distribution between photosystems I and II (*1*, *2*) and also in optimizing the production of ATP and NADPH in relation to the metabolic requirements of the cell (*5*, *6*). Furthermore, LHCII phosphorylation has been proposed to have a role in the long-term acclimation of the PSII antenna to different levels of light (*7*). Phosphorylation of the D1 protein, on the other hand, has been proposed to coordinate degradation and resynthesis during turnover of this reaction center protein (*8*−*12*) and therefore has been considered vitally important for the integrity of damaged PSII centers before migration to the stroma-exposed membranes for dephosphorylation and repair.

Light-dependent thylakoid protein phosphorylation has generally been assumed to be controlled by the redox state of the plastoquinone pool (*13*−*15*). However, recently it was shown that the presence of reduced plastoquinol at the quinol oxidizing (Q_o) site in the cytochrome *bf* complex is sufficient to obtain phosphorylation of the phosphoproteins also when the quinone pool is oxidized (*16*, *17*). Redox titrations have indicated that phosphorylation of all of the major phosphoproteins in the thylakoid membrane are controlled by the same endogeneous agent (*18*), and antibodies toward a fraction containing a putative kinase were found to inhibit protein phosphorylation of all thylakoid phosphoproteins (*19*). On the other hand, studies on cytochrome *bf*-less mutants as well as experiments with specific inhibitors of this cytochrome complex (*20*−*24*) suggest that, in contrast to PSII, only phosphorylation of LHCII requires the presence of the cytochrome *bf* complex for redox-dependent kinase activity. These latter observations suggest the involvement of at least two different redox-controlled protein-phosphorylating processes in the thylakoid membrane. Other significant differences in the phosphorylation of LHCII and the PSII-associated proteins include differences in the sensitivity to nucleotide affinity labels (*25*) and NEM (*26*, *27*).

A major protein kinase activity is found to be closely associated with the thylakoid membrane and has been ascribed to a protein in the 64 kDa range (*28*−*30*). Subfractionation studies have suggested a close physical association of this kinase with the cytochrome *bf* complex, in line with the phosphorylation activation studies (*29*). More recently, isolated PSII core particles have also been reported to display protein kinase activity (*31*). In general, however, little is known about the identity and characteristics of the thylakoid

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[1] Abbreviations: CF_1, catalytic subunit of the chloroplast ATP synthase; chl, chlorophyll; CP43, chlorophyll *a* binding protein of photosystem II; DTT_{ox}, oxidized dithiothreitol; DTT_{red}, reduced dithiothreitol; LHCII, light-harvesting complex of photosystem II; NEM, *N*-ethylmaleimide; PFD, photon flux density; PQ, plastoquinone; PSII, photosystem II; Q_o site, quinol-oxidizing site of cytochrome *bf*.

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kinase(s). Consequently, the knowledge about the mechanism of signal transfer to the kinase(s) and the possible interrelationship between protein phosphorylating activities is limited.

In this paper, we show that the protein phosphorylation in vitro in the thylakoid membrane is strongly influenced by the thiol disulfide redox state and that the phosphorylation of LHCII and PSII proteins appears to respond not only differently but also oppositely to changes in the thiol redox state. Possible structural and metabolic implications for the influence of the thiol redox state on the thylakoid protein phosphorylation, including the possible involvement of thioredoxin, are discussed.

MATERIALS AND METHODS

Spinach thylakoids were prepared as described earlier (*32*) and resuspended in 25 mM Tricine (pH 7.8), 100 mM sorbitol, 20 mM NaCl, and 5 mM $MgCl_2$ (incubation medium). DTT_{red} and DTT_{ox} were purchased from Sigma Chemical Co. DTT_{ox} was purified as described previously (*33*). Thioredoxin from *Escherichia coli* was a generous gift from A. Holmgren.

Thylakoid membranes were phosphorylated in the incubation medium, at a chlorophyll concentration of 0.2−0.4 mg/mL in the presence of 0.25 mM ATP containing [γ-^{32}P]ATP (0.02 mCi/mg of chl) and 10 mM NaF, by illumination at 120−200 μmol of photons m^{-2} s^{-1} at room temperature for 5 min. Before phosphorylation, thylakoids were incubated in the dark for at least 30 min to allow dephosphorylation.

The ^{32}P content of the various proteins was analyzed by SDS−PAGE and autoradiography. Proteins were resolved according to the methods described in ref *34* using a 12 to 22% polyacrylamide gradient in the separation gel. Membranes were solubilized by addition of sample buffer essentially according to the methods described in ref *34* to a final concentration of 4% SDS and 5% β-mercaptoethanol and incubated at 37 °C for 25 min. The gels were dried and analyzed by phosphorimaging or alternatively exposed to film and analyzed by laser densitometry. The D1 and D2 phosphoproteins are not well resolved in our system and were analyzed as one band.

Thiol redox titrations were performed by incubating thylakoids at 0.2−0.4 mg of chl/mL with the indicated ratios of DTT_{red} and DTT_{ox} at a total DTT concentration of 25 mM under nitrogen for 3 h in darkness in the cold room with mild agitation. After this treatment, the samples were phosphorylated and analyzed as described above. The concentration of DTT_{red} in the samples was determined with Ellman's reagent (*35*) as described in ref *36*.

Treatments with DTT_{red} or DTT_{ox} alone were also performed by incubating thylakoid membranes at 0.2−0.4 mg of chl/mL at indicated concentrations for 20−30 min in darkness at room temperature.

Treatment with iodosobensoate was performed by incubating thylakoid membranes for 10 min at room temperature at the concentrations indicated, whereafter the membranes were quickly spun down in an Eppendorf centrifuge, washed once, and finally resuspended in incubation medium.

Treatment of whole spinach leaves with DTT_{ox} or diamide (*37*) was performed by immersing the petioles of dark-adapted leaves in 10 mM Hepes/KOH (pH 7.5) containing 10 mM DTT_{ox} or 5 mM diamide for 3 h in darkness. Leaf disks punched from the leaves were transferred to float on thiol reagent containing buffer solution and illuminated at various light intensities for 1 h at 23 °C. After illumination, the leaf disks were rapidly frozen in liquid nitrogen and the thylakoid membranes isolated. In vivo phosphorylation of thylakoid proteins was detected by Western blotting using a phosphothreonine antibody (Zymed Laboratories Inc.) as described by Rintamäki et al. (*38*).

Chlorophyll was determined according to the methods described in ref *39*.

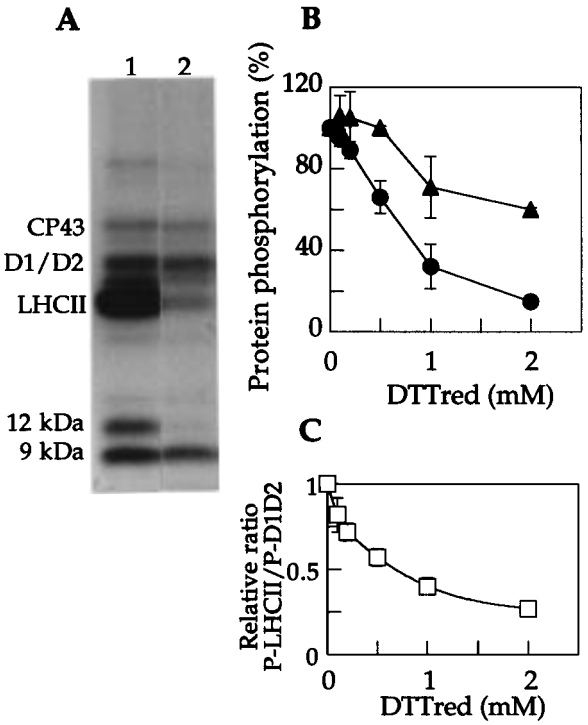

FIGURE 1: Effect of the thiol reductant DTT_{red} on the level of thylakoid protein phosphorylation. (A) Autoradiogram showing thylakoid protein phosphorylation in the absence (lane 1) or presence (lane 2) of 1 mM DTT_{red}. (B) Relative level of phosphorylation of LHCII (●) and the D1 and D2 proteins (▲) at different DTT_{red} concentrations. (C) Relative ratio between phospho-LHCII and phospho-D1 and -D2 at different DTT_{red} concentrations. The actual ratio in the absence of DTT_{red} was around 7. Thylakoid membranes were preincubated in darkness for 15 min at room temperature in the absence or presence of DTT_{red}, whereafter [γ-^{32}P]-ATP was added and the samples were illuminated for 5 min. Each point gives the mean of three independent experiments ± SD.

RESULTS

Illumination of isolated thylakoid membranes in the presence of radioactive ATP leads to the typical labeling of several polypeptides as illustrated in Figure 1A. When thylakoid membranes were phosphorylated in light in the presence of the thiol reducing agent dithiothreitol (DTT_{red}), a dramatic change in the protein phosphorylation pattern was observed (Figure 1A,B). The phosphorylation level of LHCII was found to be considerably lower compared to that of control thylakoids phosphorylated in the absence of DTT_{red}. The decrease was primarily due to a lowering of the initial rate of LHCII phosphorylation. Phosphorylation of the D1 and D2 reaction center proteins and the other PSII subunits CP43 and 9 kDa phosphoprotein, on the other hand, was much less sensitive to the presence of DTT_{red} (Figure 1A,B).

At 0.5 mM DTT_{red}, the level of D1 and D2 protein phosphorylation was not decreased at all, while the level of phosphorylation of LHCII was reduced to 65%. At the lower concentrations, even a slight stimulation of D1 and D2 phosphorylation could be observed. The difference in response is clearly seen when the ratio between phospho-LHCII and phospho-D1 and -D2 is plotted as a function of the DTT_{red} concentration (Figure 1C). The poorly characterized 12 kDa phosphoprotein (*40*) was the only protein among the more strongly phosphorylated ones that was affected in a manner similar to that of LHCII (Figure 1A).

The response to DTT_{red} was the same whether protein phosphorylation was activated by light or if plastoquinone was reduced in the dark by addition of NADPH and ferredoxin. The LHCII phosphorylating capacity was fully restored when DTT_{red} was removed and the thylakoid suspension left to reoxidize in air for 10−20 min.

The thiol redox state of the thylakoid membrane was also varied by incubation with an excess of total DTT (25 mM) at different ratios of oxidized and reduced DTT (*41*−*43*) before the addition of radioactive ATP and the onset of illumination. In these experiments, it could clearly be seen that, in complete contrast to that of LHCII, phosphorylation of the PSII proteins (D1, D2, 9 kDa phosphoprotein, and CP43) was stimulated by thiol-reducing conditions (Figure 2A). In Figure 2B, the response of the level of protein phosphorylation to an increasing ratio between reduced and oxidized DTT is shown. At a DTT_{red}/DTT_{ox} ratio as low as 0.005, the level of phosphorylation of LHCII was decreased to around 50% of that in the presence of DTT_{ox} alone. The level of phosphorylation of the D1 and D2 proteins, on the other hand, was increased around 1.4-fold under the same conditions. As the ratios between DTT_{red} and DTT_{ox} are increased, the level of LHCII phosphorylation continues to decrease, to below 10% at the highest ratio. Due to the complexity and the instability of the isolated thylakoid membrane, we were unable to obtain equilibrium data and could consequently not determine a midpoint redox potential for the decrease in the level of LHCII phosphorylation.

After the initial increase at low DTT_{red}/DTT_{ox} ratios, the level of D1 and D2 phosphorylation was also found to decrease at the higher ratios (Figure 2B). However, the level of phosphorylation of the D1 and D2 proteins was never found to decrease below 40−50%, even at very high DTT_{red}/DTT_{ox} ratios (up to 1 was tested). This dual effect of changes in the DTT_{red}/DTT_{ox} ratio on the level of PSII phosphorylation could indicate two overlapping events with opposite responses to the thiol disulfide redox state (one that increases and another one that decreases the level of PSII protein phosphorylation) and that at each specific DTT_{red}/DTT_{ox} ratio we observe the sum of these two events.

Figure 2B also depicts the responses to variations in the DTT_{red}/DTT_{ox} ratio, of the level of phosphorylation of the 12 kDa phosphoprotein and that of the 9 kDa phosphoprotein, and it can be seen that they follow the change in the level of LHCII and D1 and D2 phosphorylation, respectively, albeit to a somewhat lesser extent.

Since the ATPase activity of CF_1 is known to be strongly activated by DTT_{red} (*44*, *45*), a trivial explanation for a decrease in the level of phosphorylation could be a decrease in the level of ATP. Several control experiments were therefore performed to rule out this possibility; e.g., the

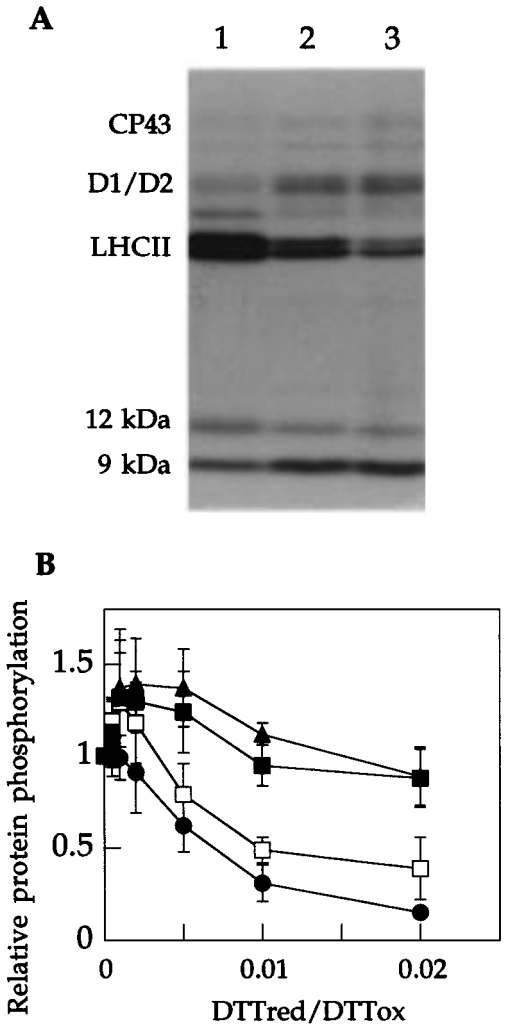

FIGURE 2: Thiol redox titration of thylakoid protein phosphorylation. (A) Autoradiogram showing thylakoid protein phosporylation in the presence of different ratios of DTT_{red} and DTT_{ox} at a total concentration of 25 mM. Thylakoid membranes were preincubated in darkness under nitrogen at 5 °C for 3 h before phosphorylation in light: (1) DTT_{ox} only, (2) ratio of 0.005, and (3) ratio of 0.02. (B) Relative level of phosphorylation of LHCII (●), D1 and D2 (▲), 12 kDa (□), and 9 kDa (■) at diffent ratios of DTT_{red} and DTT_{ox} at a total concentration of 25 mM. The ratios shown on the *x*-axis are the starting ratios at the onset of incubation. The determinations of the actual ratios at the end of incubation show an average decrease of around 50%. A ratio of zero represents DTT_{ox} only. Each point gives the mean of four independent experiments ± SD.

presence of an ATP-regenerating system (creatine phosphate and creatine kinase) or of the ATPase inhibitor bathophenanthroline did not affect the inhibition of LHCII protein phosphorylation by DTT_{red}. Furthermore, no significant variation was found upon direct determinations of the ATP levels in the protein phosphorylation assay with or without added DTT_{red}.

Upon addition of DTT_{ox} alone to the thylakoid membranes, a varying but significant, up to 5-fold, concentration-dependent increase in the level of phosphorylation of LHCII was observed (Figure 3A). Surprisingly however, in most experiments, the level of phosphorylation of the D1 and D2 proteins was also found to increase upon incubation with DTT_{ox} (Figure 3B). Panels A and B of Figure 3 also show the effect of addition of DTT_{red} to a ratio of 0.005 at the different DTT_{ox} concentrations, verifying the opposing effects

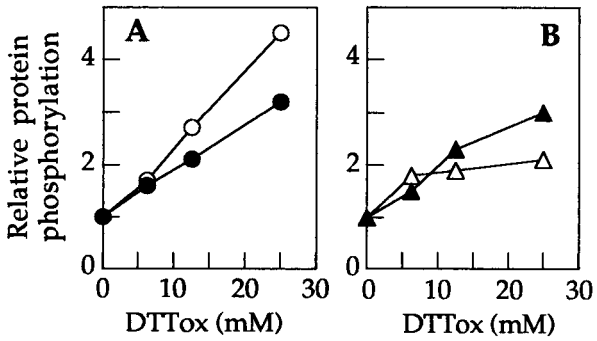

FIGURE 3: Effect of the thiol oxidant DTT_{ox} on thylakoid protein phosphorylation. Thylakoid membranes were preincubated, as described in the legend of Figure 2, in the presence of different concentrations of DTT_{ox}, in the absence (white symbols) or in the presence (black symbols) of DTT_{red} at a ratio of 0.005: (A) phosphorylation of LHCII and (B) phosphorylation of the D1 and D2 protein.

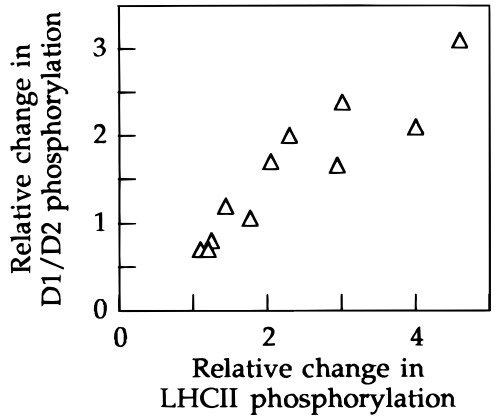

FIGURE 4: Correlation between the relative effect of preincubation with DTT_{ox} on the levels of LHCII and D1 and D2 phosphorylation. The relative effect of 25 mM DTT_{ox} on the level of D1 and D2 phosphorylation, in 11 individual experiments, is plotted against the relative effect of DTT_{ox} on the level for LHCII in the same experiment.

of thiol-reducing conditions on the level of phosphorylation of LHCII and the D1 and D2 proteins, respectively. In Figure 4, the relative effect of addition of 25 mM DTT_{ox} on the level of phosphorylation of LHCII and D1 and D2 from a number of individual experiments is shown. It can be seen that there is a strong correlation ($R = 0.92$) between the relative effects on the level of LHCII phosphorylation and D1 and D2 phosphorylation, indicating that the effects of DTT_{ox} on the two phosphorylating activities are related. However, the level of D1 and D2 protein phosphorylation was consistently less affected than the level of LHCII phosphorylation. Also, in a number of experiments, addition of DTT_{ox} indeed inhibited D1 and D2 phosphorylation (Figure 4, points below 1 on the *y*-axis). Due to the very low DTT_{red}/DTT_{ox} ratios needed to observe an increase in the level of phosphorylation of the D1 and D2 proteins (Figure 2), we cannot exclude at present the possibility that the increase under thiol-oxidizing conditions is due to a limited formation of DTT_{red} in the incubation mixtures. However, we have indications of a large difference in the kinetics of the effects produced by DTT_{ox} ($t_{1/2}$ of 1−2 min at 25 mM) and those produced by DTT_{red} ($t_{1/2}$ of at least 30−60 min at a ratio of 0.005 at 25 mM DTT_{ox}) which does not support this possibility.

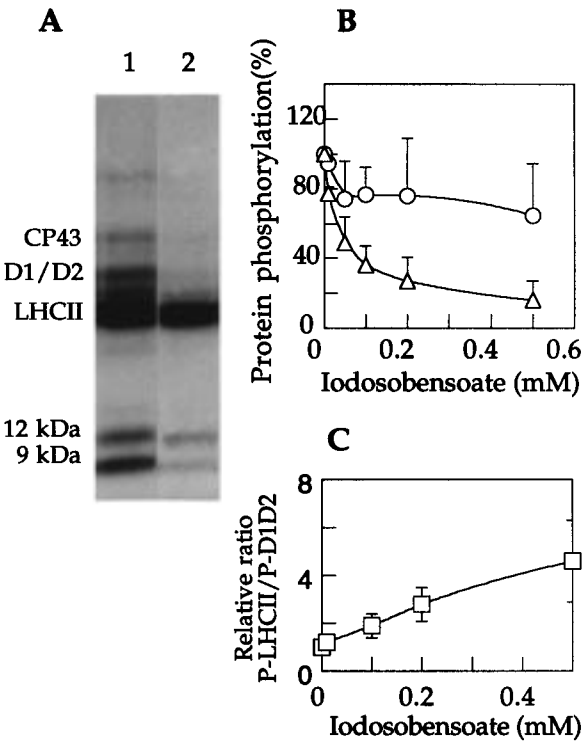

FIGURE 5: Effect of the thiol oxidant iodosobensoate on thylakoid protein phosphorylation. (A) Autoradiogram showing the thylakoid phosphorylation pattern in control thylakoids (1) or in thylakoids after pretreatment with 1 mM iodosobensoate (2). (B) Relative level of phosphorylation of LHCII (○) and D1 and D2 (△) after pretreatment at different iodosobensoate concentrations. (C) Relative ratio between phospho-LHCII and phospho-D1 and -D2 after pretreatment with different iodosobensoate concentrations. Each point gives the mean of three independent experiments ± SD.

Alternatively, the observed response of the level of phosphorylation of the D1 and D2 proteins to DTT_{ox} could be explained by the existence of two events with opposite responses to changes in the thiol redox state, as is clearly indicated in the case of the effect of thiol-reducing conditions on the level of D1 and D2 protein phosphorylation (Figure 2).

It should be emphasized that irrespective of the net effect of adding DTT_{ox} in a particular experiment, moderately thiol-reducing conditions consistently increased the level of phosphorylation of the D1 and D2 proteins further.

Experiments were also carried out using the stronger thiol oxidant iodosobensoate, a reagent which preferentially oxidizes vicinal dithiols to disulfides (*46*). When thylakoid membranes were preincubated with iodosobensoate, we did not observe any increase in the phosphorylating capacity of either the LHCII or the PSII proteins (Figure 5), as was the case with the weaker oxidant DTT_{ox} (Figure 4). However, the differential effect on the level of LHCII and PSII phosphorylation was again evident. As can be seen in Figure 5A,B, iodosobensoate strongly inhibits D1 and D2 phosphorylation at sub-millimolar concentrations, while leaving the phosphorylation of LHCII much less affected. This is also depicted as an increase in the ratio between phospho-LHCII and phospho-D1 and -D2 with increasing concentrations of iodosobensoate (Figure 5C). High concentrations of iodosobensoate tended to produce an overall inhibition of phosphorylation; however, the effect was consistently much more pronounced toward the D1 and D2 proteins than toward

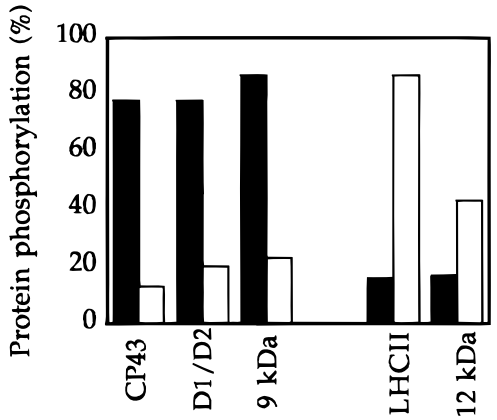

FIGURE 6: Comparison of the effects of 1 mM DTT_{red} and 0.5 mM iodosobensoate on thylakoid protein phosphorylation. Effects of DTT_{red} (black) and effects of iodosobensoate (white).

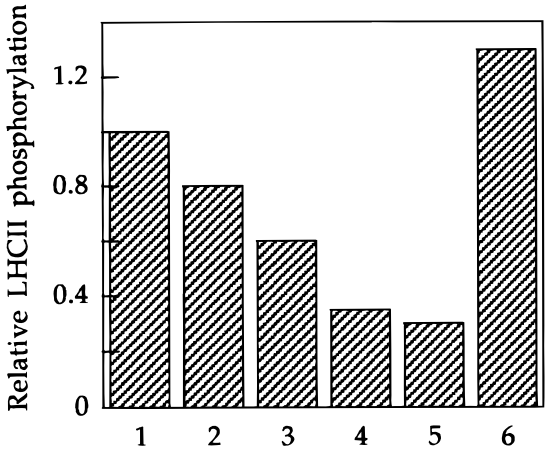

FIGURE 7: Thioredoxin as a mediator of thiol redox effects on LHCII protein phosphorylation. Thylakoid membranes were preincubated for 20 min without additions (1) or with 0.2 mM DTT_{red} (2), 0.2 mM DTT_{red} and 20 μM *E. coli* thioredoxin (3), 1 mM DTT_{red} (4), 1 mM DTT_{red} and 20 μM *E. coli* thioredoxin (5), or 20 μM *E. coli* thioredoxin alone (6), before protein phosphorylation in light.

LHCII. The inhibition by iodosobensoate was not easily reversed by incubation with DTT_{red} and subsequent washings, which could indicate that a more complex change has occurred in this case.

The phosphorylation of the two other PSII subunits (the CP43 and the 9 kDa phosphoprotein) is affected qualitatively in the same manner as that of the D1 and D2 proteins; i.e., they are more strongly inhibited by iodosobensoate than by 1 mM DTT_{red} (Figure 6). The 12 kDa phosphoprotein, on the other hand, follows the response of LHCII in being much more sensitive to 1 mM DTT_{red} than to iodosobensoate (Figure 6). These data again (cf. Figure 2) clearly reveal two separate classes of phosphoproteins, one LHCII-related and one PSII-related, with respect to the response of the level of protein phosphorylation to thiol reagents with different redox characters.

The possible involvement of thioredoxin (*47*−*49*) as a physiological mediator for the observed thiol redox effects on thylakoid protein phosphorylation was investigated. In Figure 7, it can be seen that the presence of 20 μM thioredoxin from *E. coli* enhances the negative effect of DTT_{red} on the level of LHCII phosphorylation, indicating that reduced thioredoxin is more efficient than DTT_{red} in

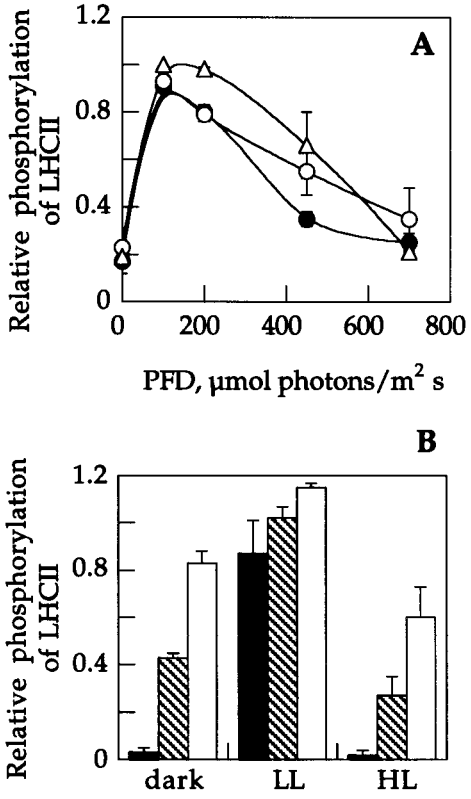

FIGURE 8: Effect of thiol-oxidizing agents on LHCII phosphorylation in vivo in leaves exposed to different levels of light (A) and in vitro in thylakoids isolated from differently preilluminated leaves (B). (A) Dark-adapted leaves were preincubated in the absence (●) or in the presence of 10 mM DTT_{ox} (○) or 5 mM diamide (△), whereafter they were exposed to increasing levels of light. (B) The endogenous level of phospho-LHCII (black) and the level of phospho-LHCII after in vitro phosphorylation in the light for 20 min without any pretreatment (striped) and after pretreatment with 10 mM DTT_{ox} for 30 min (white) in thylakoids isolated from dark-adapted leaves (dark), low-light (LL; 100 μmol of photons m^{-2} s^{-1}) treated leaves, or high-light (HL; 1300 μmol of photons m^{-2} s^{-1}) treated leaves. The level of phospho-LHCII was analyzed with a polyclonal phosphothreonine antibody. The presented data are means ± SD of two independent experiments.

producing the observed lowering of the level of phosphorylation. Also in these experiments, controls were made to ascertain that the observed effects were not the result of an increased level of ATP hydrolysis due to a possible activation of CF_1 ATPase activity. In the case of D1 and D2 protein phosphorylation, the presence of *E. coli* thioredoxin did not enhance the stimulation by DTT_{red}. On the contrary, reduced thioredoxin was found to decrease also the level of phosphorylation of the D1 and D2 proteins (not shown). Attempts to use a reconstituted *E. coli* thioredoxin−thioredoxin reductase system (a generous gift from A. Holmgren) have so far been unsuccessful.

In vivo it has previously been shown that high light treatment, in *Chlamydomonas* (*50*) as well as in spinach and pumpkin leaves (*38*) or in isolated intact spinach chloroplasts (*51*), causes a decrease in the level of LHCII protein phosphorylation. The level of D1 protein phosphorylation on the other hand remains constantly high at elevated light intensities (*38*, *50*, *51*). In Figure 8A, it can be seen that pretreatment of spinach leaves with DTT_{ox} or another thiol-oxidizing agent, diamide (*37*), partially counteracted the decrease in the steady-state level of LHCII phosphorylation occurring at high light intensities.

Figure 8B shows the in vitro capacity for LHCII phosphorylation in thylakoid membranes isolated from spinach leaves pretreated at different light levels, and its stimulation by DTT_{ox}. Thylakoids from dark-adapted and high-light (1300 μmol of photons m^{-2} s^{-1}) illuminated leaves both have negligible amounts of endogeneous phospho-LHCII in agreement with Figure 7A. Furthermore, compared to thylakoids from low-light (100 μmol of photons m^{-2} s^{-1}) illuminated leaves, they both showed a limited capacity for in vitro phosphorylation. Preincubation with DTT_{ox} raised the level of LHCII phosphorylation not only in thylakoids from high-light illuminated leaves but also in thylakoids from dark-adapted leaves, suggesting that both of these are in a relatively thiol-reduced state. On the other hand, in accordance with Figure 8A, thylakoids isolated from low-light illuminated leaves, which have a high endogenous level of phospho-LHCII, were only slightly affected by treatment with DTT_{ox}. These observations connect the in vitro and in vivo effects of DTT, giving credit to the possibility that, physiologically, protein phosphorylation is regulated by the thiol redox state.

DISCUSSION

The regulation of thylakoid protein phosphorylation has mainly been ascribed to the redox state of plastoquinone (*1*−*3*). In this work, we provide evidence, based upon experiments in vitro and in vivo, for the possibility of an additional regulatory mechanism for thylakoid protein phosphorylation, involving the thiol disulfide redox state.

LHCII and PSII phosphorylation are generally considered to respond similarly to conditions that reduce plastquinone, such as light or NADPH/ferredoxin (*1*−*4*). In contrast to this, they appear to respond oppositely to changes in the thiol disulfide redox state. While moderately thiol-reducing conditions are favorable for PSII phosphorylation, thiol-oxidizing conditions are found to stimulate and thiol-reducing conditions to inhibit LHCII phosphorylation. This latter observation is in striking contrast to the well-documented (*1*−*4*) activation of thylakoid protein phosphorylation under PQ-reducing conditions and gives credit to the possibility of multiple redox regulation of the process.

A closer inspection of PSII phosphorylation reveals a dual response to thiol redox changes. The data indicate that, in vitro, part of the PSII phosphorylation might be due to a process with "LHCII characteristics", while a second process is clearly stimulated by thiol-reducing conditions.

At the molecular level, changes in the thylakoid protein phosphorylation pattern due to changes in the thiol disulfide redox state could be explained in a number of principal ways. The catalytic activity or the activation of the participating kinase(s) could be directly or indirectly influenced, but the availability or accessibility of the protein substrates could also be affected.

The degree of homology between protein kinase catalytic subunits, irrespective of their amino acid specificity, is generally considered very high (*52*), and so far, Cys residues have not been implicated in their catalytic mechanisms. However, modification of Cys residues in the EGF receptor tyrosine kinase alters its affinity for ATP (*53*), and disulfide-containing compounds are potent tyrosine kinase inhibitors presumably via thiol−disulfide interchange (*54*). Also, it has been shown that ATP could protect thylakoid protein phosphorylation against inactivation by the sulfhydryl reagent NEM, an effect that was more pronounced toward LHCII than toward the 9 kDa phosphoprotein of PSII (*26*). Consequently, the opposing effects of changes in the thiol disulfide redox state could be due to the participation of two thiol redox sensitive kinases responding oppositely to these changes.

Cytochrome *bf* has been postulated to play a central role in the regulation of the LHCII protein phosphorylation, but not in that of PSII (*16*, *17*, *20*, *22*−*24*). Effectors of cytochrome *bf* as well as cytochrome *bf*-less mutants have been shown to produce phosphorylation patterns with only low levels of phospho-LHCII (*20*−*22*, *24*) that largely resemble those we obtain in the presence of reduced DTT. It could be speculated that reduction of a possible disulfide in the cytochrome *bf* complex could interfere with the interaction between the cytochrome and the LHCII kinase. The positive effect on PSII phosphorylation could be a secondary result of this change in interaction, or represent a second unrelated event.

A direct change in the thiol redox state of the protein substrates themselves seems less likely, due to the large number of different proteins whose phosphorylation is affected in a similar manner.

Irrespective of the nature of the specific thiol-redox target-(s) and the difference in the response between LHCII and PSII, it appears clear that a correct thiol redox state is critical for in vitro thylakoid protein phosphorylation, and that the identification of thiol redox sensitive components in the thylakoid membrane should be instrumental in the elucidation of thylakoid protein phosphorylation and its regulation.

In vivo regulation via changes in the thiol disulfide redox state has been strongly implicated in the light-dependent modulation of several chloroplast enzyme activities, including a number of Calvin cycle enzymes (*48*) and the chloroplast ATP synthase (CF_1) (*45*) as well as certain translation factors (*55*, *56*).

Previous in vivo studies have shown that LHCII phosphorylation is downregulated in response to increases in the light intensity (*51*) and to photoinhibitory light (*50*) in contrast to the PSII phosphorylation. Recently, these types of studies were extended by the use of phosphothreonine antibodies to determine the in vivo level of phosphorylation in plant leaves upon exposure to increasing light intensities (*38*). It was shown that as the light intensity is raised, the level of LHCII phosphorylation responds and increases rapidly to reach its maximal level already at light intensities below the growth light. At higher light levels, the level of LHCII phosphorylation decreases, and at high light levels, LHCII is largely in its unphosphorylated form. The phosphorylation of the D1 protein and other PSII proteins, on the other hand, responds more slowly to increasing light intensities, and the levels reach maximal values around the growth light, whereafter the phosphorylation level remains high. Interestingly, these in vivo data reveal changes in the relative level of phosphorylation of LHCII and PSII in response to increased light intensities that are reminiscent of those obtained in the study described here by changing the thiol redox state in vitro. Our observation that addition of DTT_{ox} or diamide counteracts the downregulating effect of increasing light intensities on the level of LHCII phos-

phorylation in leaves supports the possibility that oxidation and reduction of thiol groups might play a role in in vivo regulation of protein phosphorylation.

A high phosphorylation level of the D1 protein at elevated light intensities appears to be logical since phosphorylation is vital during repair of PSII upon photoinhibitory damage (*8*−*11*). LHCII phosphorylation, on the other hand, is mostly connected to the balancing of excitation energy between the two photosystems via the state transitions (*1*, *2*). At high light levels where the two photosystems approach saturation, it can be assumed that this regulation is of less importance (*6*). However, in high light, energy dissipation through nonphotochemical quenching is an important mechanism for the protection against photodamage (*57*, *58*). This process is believed to involve LHCII (*59*), and it could be speculated that protein phosphorylation is downregulated at high light intensities to favor LHCII aggregation and energy dissipation.

The ferredoxin−thioredoxin system in plant chloroplasts serves to maintain and modulate the thiol disulfide redox state of specific proteins in response to light (*48*, *49*). In light, thioredoxin is reduced in a ferredoxin-dependent reaction with the subsequent activation of a number of chloroplast enzymes. Our present observations, including experiments with heterologous thioredoxin, indicate that this system might also function in the downregulation of LHCII phosphorylation at high light intensities. Redox potentials of −0.30 (*41*) and −0.21 V (*60*) have been estimated for chloroplast thioredoxin. The reduction level of thioredoxin has been reported to vary from 0 to 20% in darkness to around 90% in light (*61*−*63*). If we take the higher and more recent value of −0.21 V and assume that thioredoxin is reduced around 10% in darkness, a dark−light transition would correspond to a variation in the effective redox potential from around −0.18 V to around −0.24 V (*42*). The ratios of DTT_{red} and DTT_{ox} used in our study, causing a 10-fold variation in the level of LHCII phosphorylation, correspond to redox potentials varying between around −0.21 and −0.25 V. The calculation is based on a redox potential for DTT of −0.31 V (*43*). According to these values thioredoxin would, from a thermodynamic point of view, indeed be able to modulate the level of LHCII protein phosphorylation in a light-dependent manner.

In the case of the PSII specific phosphorylation, *E. coli* thioredoxin did not give any clear and conclusive effects. However, it must be emphasized that a heterologous reconstitution system has its limitations. Plant thioredoxins have generally been found to show a high level of specificity (*48*, *49*). Recently, it has been shown that both thioredoxins *f* and *m* are encoded by multigene families in *Arabidopsis thaliana* (*64*). Also, a protein disulfide isomerase (PDI), which is proposed to be reduced by the ferredoxin thioredoxin reductase and to participate in translational regulation of D1 protein synthesis, has been found in *Chlamydomonas reinhardtii* (*56*). These findings significantly increase the spectrum of possible thiol redox mediators.

The question of an involvement of thioredoxin in the light-dependent activation of thylakoid protein phosphorylation has been addressed earlier (*65*). In that work, no effects on thylakoid protein phosphorylation, of a reconstituted ferredoxin−thioredoxin system, were however observed.

In conclusion, it is clear that thylakoid protein phosphorylation is strongly influenced by the thiol disulfide redox state, in such a manner that LHCII and PSII protein phosphorylations show opposite response patterns. The possible regulation of protein phosphorylation via thiol groups, most likely through the ferredoxin−thioredoxin system, could represent a second feedback loop of redox control generated by the electron flow, in addition to the redox control via plastoquinone.

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