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Dephosphorylation of photosystem II core proteins is light-regulated *in vivo*

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A number of photosystem II (PSII)-associated proteins, including D1, D2, CP43 and LHCII, are phosphorylated post-translationally by a membrane-bound, redox-regulated kinase activity. *In vitro* studies have demonstrated that these proteins can be dephosphorylated by membrane-bound phosphatase activity, reportedly insensitive to light or redox control. We demonstrate here that the PSII core proteins, D1, D2 and CP43, undergo light-stimulated, linear electron-transport-independent dephosphorylation *in vivo*. The *in vivo* dephosphorylation of D1 was characterized further and shown to depend upon light intensity, and to occur throughout the visible light spectrum with characteristics most consistent with light absorption by chlorophyll. PSII core protein dephosphorylation *in vivo* was stimulated by photosystem I (PSI)-specific far-red light, and inhibited by 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone, an inhibitor of plastoquinol oxidation by the cytochrome b_6f complex. Based on these findings, we propose that PSI excitation is involved in regulating dephosphorylation of PSII core proteins *in vivo*.

Key words: chloroplast/light-regulation/phosphatase/protein kinase/redox control/*Spirodela*

Introduction

Protein phosphorylation/dephosphorylation is widely employed as a regulator and initiator of cellular functions in eukaryotes. The most extensively studied protein phosphorylation system in plants involves those associated with photosystem II (PSII): the thylakoid-localized, pigment–protein complex that catalyzes the photosynthetic reactions involving the oxidation of water and reduction of plastoquinone. PSII-associated proteins that are known to be phosphorylated include members of the PSII core—D1, D2, CP43 and the *psb*H product—as well as the peripheral LHCII antennae proteins. These proteins are all phosphorylated on threonine residues located at or near their respective N-termini (Michel and Bennett, 1987; Michel *et al.*, 1988, 1991; Elich *et al.*, 1992) by a light-dependent, redox-regulated kinase(s) (for recent reviews, see Bennett, 1991; Allen, 1992). Based on mutant and inhibitor studies, it is generally believed that at least two kinases are involved in phosphorylating PSII-associated proteins (Bennett, 1991; Allen, 1992). While reduction of plastoquinone has been implicated as a key regulatory step for both activities, the LHCII kinase has an additional requirement for an active cytochrome b_6f complex (Gal *et al.*, 1990).

Photosynthetic electron transport, which results in net production of ATP coupled to the oxidation of water and the reduction of NADP, is carried out by two photosystems, photosystem I (PSI) and PSII, acting in series. Phosphorylation of the LHCII proteins is widely accepted to be involved in regulating short-term processes, called state transitions, that compensate for imbalances between the two photosystems in order to maximize photosynthetic efficiency (see Bennett, 1991; Allen, 1992). While the function of PSII core protein phosphorylation is less well established, a recent report has implicated these modifications in providing protection against photoinhibition (Harrison and Allen, 1991). Possible mechanisms for this action could involve the regulation of D1 degradation by phosphorylation as a means to prevent the disassembly of PSII (Aro *et al.*, 1992) or the promotion of PsbH protein dissociation from the core by phosphorylation (Giardi, 1993) under photoinhibitory conditions.

The vast majority of studies in this field have employed *in vitro* systems. In particular, the contention that thylakoid protein phosphatase activity is constitutive, or at least not regulated by light or redox potential, is based on experiments performed with isolated thylakoids (Bennett, 1980) and chloroplasts (Michel *et al.*, 1987). Since it has become increasingly clear that phosphatases can be as highly regulated as their kinase counterparts (see Cyert and Thorner, 1989; Hunter, 1989), we sought to re-examine potential regulation of thylakoid protein dephosphorylation, assessing such regulation *in vivo* in order to eliminate artifacts due to tissue disruption.

Previously, we developed a system to identify and characterize the *in vivo* phosphorylated form of D1 and to address the regulation of D1 protein phosphorylation *in vivo* (Elich *et al.*, 1992). This system is now used to demonstrate that PSII core phosphoproteins are subject to light-stimulated, linear electron-transport-independent dephosphorylation *in vivo*. We show that this dephosphorylation was driven by far-red light and inhibited by 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB). Since far-red light is specifically absorbed by PSI, we suggest that phosphorylation/dephosphorylation of the PSII core proteins is involved in regulating energy distribution between the two photosystems, with PSI-regulated dephosphorylation providing the perfect complement to PSII-mediated kinase activity.

Results and discussion

Incubation of *Spirodela* plants with [^{32}P]orthophosphate results in the radiolabeling of a number of thylakoid proteins

(Figure 1, time 0). The band labeled D1-P in Figure 1 was previously shown to be the phosphorylated form of the PSII reaction center protein, D1 (Elich *et al.*, 1992). The migration positions of D2, CP43, the *psb*H product and the LHCII proteins are also indicated. We tentatively identify the corresponding ^{32}P-labeled bands as the phosphorylated forms of these proteins based on the following criteria: (i) these *in vivo* ^{32}P-labeled proteins are present in the expected PSII core and antennae fractions resolved from highly purified grana by flat-bed isoelectric focusing (T.D.Elich, M.T.Giardi and A.K.Mattoo, unpublished results); (ii) co-migrating proteins are phosphorylated when isolated *Spirodela* thylakoids are incubated with [γ-^{32}P]ATP (Elich *et al.*, 1992); and (iii) *in vitro* phosphorylation of these proteins by endogenous thylakoid kinase activity has been demonstrated by protein sequencing (Michel and Bennett, 1987; Michel *et al.*, 1988, 1991).

Dephosphorylation of PSII core proteins is light-stimulated

We previously showed that phosphorylation of D1 *in vivo* is light-dependent (Callahan *et al.*, 1990; Elich *et al.*, 1992) and that 3-(3,4-dichlorophenyl-1,1-dimethylurea) (DCMU), which binds to the Q_B site on the D1 protein and prevents reduction of plastoquinone (Trebst, 1987; Gardner, 1989), effectively inhibits thylakoid protein phosphorylation *in vivo* (Callahan *et al.*, 1990; Elich *et al.*, 1992; also see Figure 2) as it does *in vitro* (Allen *et al.*, 1981; Elich *et al.*, 1992). To examine potential light regulation of dephosphorylation *in vivo*, it was necessary to inhibit the responsible kinase(s). Thus, plants labeled with [^{32}P]orthophosphate were washed and further incubated in the light or dark in the presence of DCMU. Under these conditions, the rate of phosphoprotein turnover was significantly faster in light-irradiated plants than in dark-incubated plants (Figure 1). Phosphorylated *psb*H product was least affected by light. We note that *in vivo* phosphorylation of this protein is not effectively inhibited by DCMU (Elich *et al.*, 1992; also see Figure 2).

Strictly speaking, the observed phosphoprotein turnover could result from either protein dephosphorylation or protein degradation. The fact that multiple phosphoproteins all turned over on a time-scale of hours, although they are known to vary widely in their half-lives (Edelman and Reisfeld, 1978; Edelman *et al.*, 1984; Mattoo *et al.*, 1984, 1989), argues against the involvement of protein degradation. We, therefore, tested the effect of two known phosphatase inhibitors, okadaic acid and NaF, on this process. Okadaic acid had no effect on phosphoprotein turnover *in vivo* (Figure 1, +OA lane) or *in vitro* (data not shown). In contrast, NaF (Figure 1, +NaF lane), but not NaCl (not shown), completely inhibited light-dependent phosphoprotein turnover. At the concentrations used, NaF is known to inhibit thylakoid-bound phosphatase activity *in vitro* (Bennett, 1980; Elich *et al.*, 1992) and state transitions *in vivo* (attributed to inhibition of thylakoid phosphatase activity) (Canaani *et al.*, 1984). We conclude that the phosphoprotein turnover

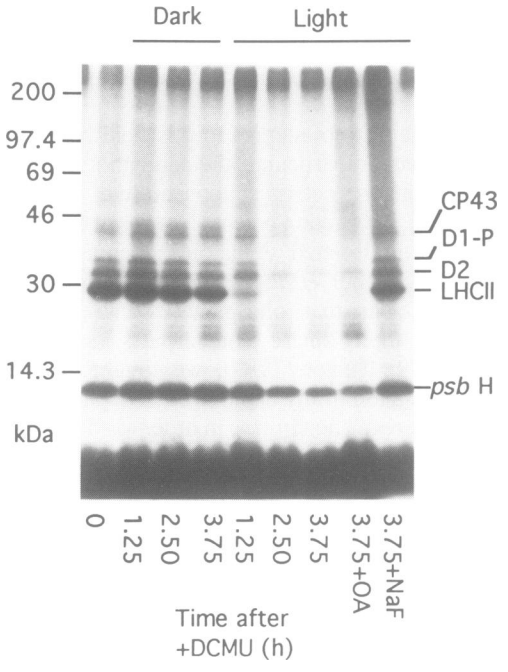

Fig. 1. Light-dependent dephosphorylation of thylakoid phosphoproteins *in vivo*. *Spirodela* plants were incubated with 0.5 mCi/ml [^{32}P]orthophosphate for 3 h under 90 μmol/m^2/s white light and washed. DCMU (10 μM) was then added to inhibit further redox-regulated kinase activity and incubation continued in the light or dark for the times indicated. Ten micromolar okadaic acid (+OA) or 125 mM NaF (+NaF) was added to some plants along with the DCMU as indicated. Thylakoids were isolated and analyzed by SDS–PAGE and autoradiography (Elich *et al.*, 1992). The autoradiograph is shown with positions of molecular weight markers indicated on the left, and positions of various PSII-associated proteins indicated on the right. The results of the experiment shown are representative of those observed in six separate experiments. Coomassie Blue staining of the gel (not shown) verified the equivalence of protein loads, and revealed no changes in steady-state protein patterns.

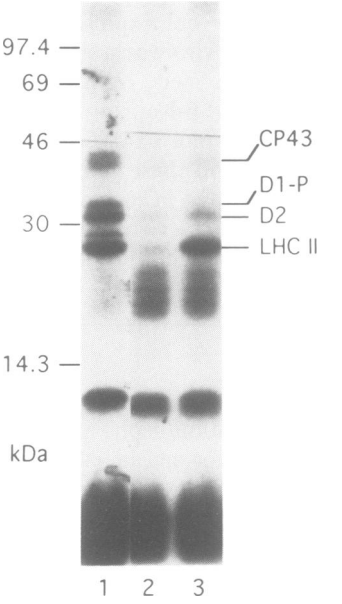

Fig. 2. Phosphorylation of LHCII occurs in darkness. Plants were labeled in the light with [^{32}P]orthophosphate as in Figure 1, in the presence or absence of 10 μM DCMU. The plants were then washed and either harvested immediately or further incubated in the dark in the presence of DCMU for 2 h. Thylakoids were isolated and analyzed by SDS–PAGE and autoradiography. Lane 1, thylakoids from plants phosphorylated in the light in the absence of DCMU; lane 2, thylakoids from plants phosphorylated in the light in the presence of DCMU; lane 3, thylakoids from plants phosphorylated in the light in the presence of DCMU, washed, and further incubated for 2 h in the dark.

observed in Figure 1 is due to protein dephosphorylation catalyzed by an NaF-sensitive phosphatase.

While the data in Figure 1 strongly suggest that the dephosphorylation was light-stimulated, they could also be explained by a constitutive phosphatase activity if protein kinase activity *in vivo* is greater in darkness than in the light in the presence of DCMU. To assess this, plants were labeled for 3 h in the presence of DCMU (as in Figure 1), then washed and further incubated in darkness for 2 h. Virtually no radiolabel was incorporated into the PSII-associated proteins in the presence of DCMU prior to the dark incubation (Figure 2, compare lanes 1 and 2). Likewise, during dark incubation, little or no phosphorylation of D1, D2 or CP43 occurred under these conditions (Figure 2, lane 3). Therefore, we can conclude that the *in vivo* dephosphorylation of these proteins is truly a light-stimulated process.

Surprisingly, however, significant LHCII phosphorylation was observed in the dark-incubated plants (Figure 2, lane 3) and similar phosphorylation was observed when the radiolabeling itself was performed in the dark (data not shown). The phosphorylation of LHCII, but not PSII core proteins, in darkness provides further circumstantial evidence for the previously proposed presence of multiple thylakoid kinases (see Bennettt, 1991; Allen, 1992), but is seemingly at odds with existing models of kinase redox-regulation. Conversely, inhibition by DCMU of light-dependent LHCII phosphorylation *in vivo* is consistent with redox-regulation via plastoquinone. One possible explanation for this paradox is a dark-specific process capable of activating the LHCII kinase. Such a process would presumably involve activation through the cytochrome b_6f complex, rather than through reduction of plastoquinone. In this regard, the recent report that LHCII is phosphorylated to a greater extent in the dark than in the light in guard cell protoplasts may be relevant (Kinoshita *et al.*, 1993). Alternatively, the report of thylakoid-bound, serine kinase activity capable of phosphorylating a 25 kDa thylakoid protein in the dark *in vitro*, albeit at significantly lower levels than the light-dependent threonine phosphorylation of LHCII (Vescovi and Lucero, 1990), might also be relevant. While the nature of the dark-phosphorylation of LHCII is beyond the scope of the present study, its existence precludes us, with the evidence at hand, from determining whether LHCII dephosphorylation is light regulated.

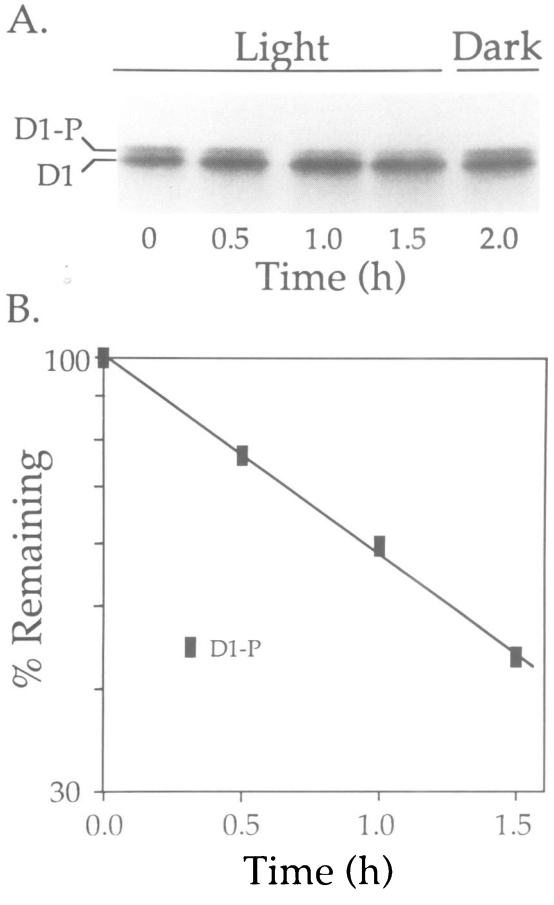

Fig. 3. Light-dependent dephosphorylation of D1 *in vivo*. *Spirodela* plants were incubated with 50 μCi/ml [^{35}S]methionine for 30 min in the light, washed and then incubated with 1 mM non-radioactive methionine for 1 h under 200 μmol/m^2/s white light to achieve significant radiolabeling of D1-P as described in the text. Plants were washed again, DCMU was added (final concentration, 10 μM), and further incubated in the light or darkness for the times indicated. Thylakoids were isolated and analyzed by SDS−PAGE and autoradiography. (**A**) Autoradiograph with positions of the phosphorylated (D1-P) and non-phosphorylated (D1) forms of the D1 PSII reaction center protein indicated. (**B**) Densitometric quantification of the D1-P bands from the light-irradiated samples. The results shown are similar to those observed in 11 separate experiments with minor variations in conditions.

Characterization of the light requirements for D1 dephosphorylation

To characterize further the light-stimulated PSII core protein dephosphorylation *in vivo*, D1 dephosphorylation was followed in intact *Spirodela* plants using [^{35}S]methionine pulse−chase experiments. An initial 1 h chase results in all of the pulse-labeled D1 protein being processed to its mature

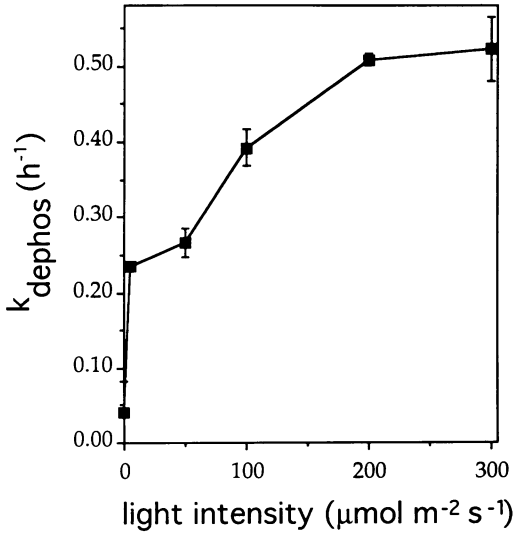

Fig. 4. Light-intensity dependence of D1 dephosphorylation. Experiments were performed as in Figure 3 except that after addition of DCMU, plants were incubated under various intensities (PPFD) of white light as indicated. Thylakoids were isolated and analyzed by SDS−PAGE and autoradiography. The D1-P bands on the autoradiographs were quantified by densitometry and apparent first-order rate constants (k_{dephos}, h^{-1}) were determined. The indicated values are the average from two rate determinations (range indicated by error bars), with each determination resulting from three time points.

size and translocated from the stroma lamellae (the site of synthesis and proteolytic processing) to the grana stacks (the site of PSII function) (Mattoo and Edelman, 1987; Mattoo *et al.*, 1989). Additionally, a significant percentage of grana-localized D1 shifts in mobility due to its light-dependent phosphorylation (Callahan *et al.*, 1990; Elich *et al.*, 1992; also see Figure 3A, time 0). In the representative experiment shown in Figure 3, following the initial 1 h chase, DCMU was added and the plants further incubated in light or darkness for the times indicated. Consistent with the results of Figure 1, phosphorylated D1 was stable in the dark but rapidly dephosphorylated upon light irradiation (Figure 3A, D1-P band) with apparent first-order kinetics (Figure 3B).

We examined the effects of light intensity and light quality on D1 dephosphorylation. As little as 5 μmol/m^2/s white light stimulated dephosphorylation ~5-fold more than the dark control (Figure 4). Thereafter, higher intensities of light increased the dephosphorylation rate more slowly, and the response appeared to approach saturation around 300 μmol/m^2/s (Figure 4), where the rate corresponds to a half-life of ~1.3 h. The dependence of D1 dephosphorylation on light intensity provides further evidence that this process is truly light-stimulated. To gain insight into the photoreceptor(s) involved, D1 dephosphorylation was assessed upon irradiation with equal quantum intensities of narrow bandpass light. Dephosphorylation of D1 was stimulated by light throughout the visible spectrum and appeared to be more sensitive to blue (430 nm) and red (660 nm) light than to green (560 nm) light (Figure 5). These results are most consistent with light absorption by chlorophyll.

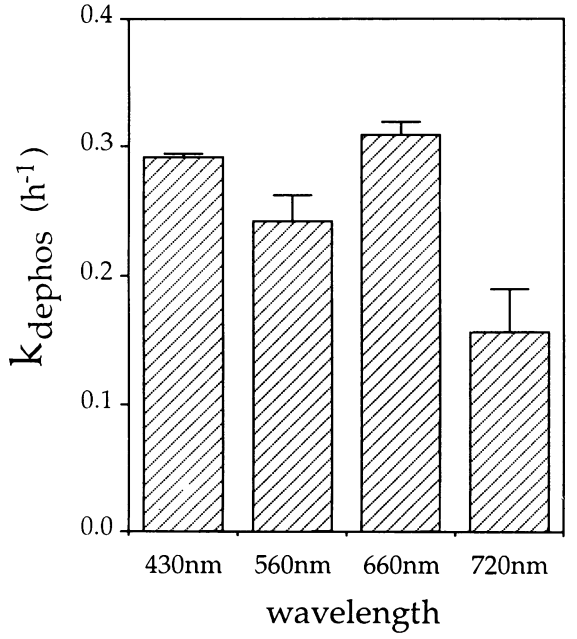

Fig. 5. Light quality dependence of D1 dephosphorylation. Experiments were performed and analyzed as in Figures 3 and 4 except that dephosphorylation was monitored in plants irradiated with narrow spectrum light provided by 10 nm bandpass interference filters of the indicated peak transmission wavelengths. The light intensity was 1.6 μmol/m^2/s in all cases except for the 720 nm light which was 2.4 μmol/m^2/s. The indicated values are the average from two rate determinations (range indicated by error bars), with each determination resulting from four time points.

Evidence for the involvement of PSI excitation in regulating PSII core protein dephosphorylation

Interestingly, dephosphorylation of D1 was also significantly stimulated by far-red (720 nm) light (Figure 5), which in the chloroplast is absorbed almost exclusively by PSI. Indeed, the involvement of PSII-driven linear electron transport could be ruled out due to the presence of DCMU in all experiments. However, in the presence of this inhibitor, cyclic PSI electron transport still occurs in *Spirodela* plants under far-red light (Jansen *et al.*, 1992). The hypothesis that PSI excitation is involved in regulating D1 dephosphorylation was tested by introducing DBMIB, a plastoquinone antagonist (Trebst, 1980) and *in vivo* inhibitor of PSI-driven cyclic electron transport (Herbert *et al.*, 1990). DBMIB inhibited white-light-stimulated D1 dephosphorylation in a concentration-dependent manner with an I_{50} of ~5–10 μM and complete inhibition at 50–100 μM (Figure 6A and B). These concentrations are in accordance with those shown to be effective at inhibiting PSI cyclic electron transport *in vivo* (Herbert *et al.*, 1990).

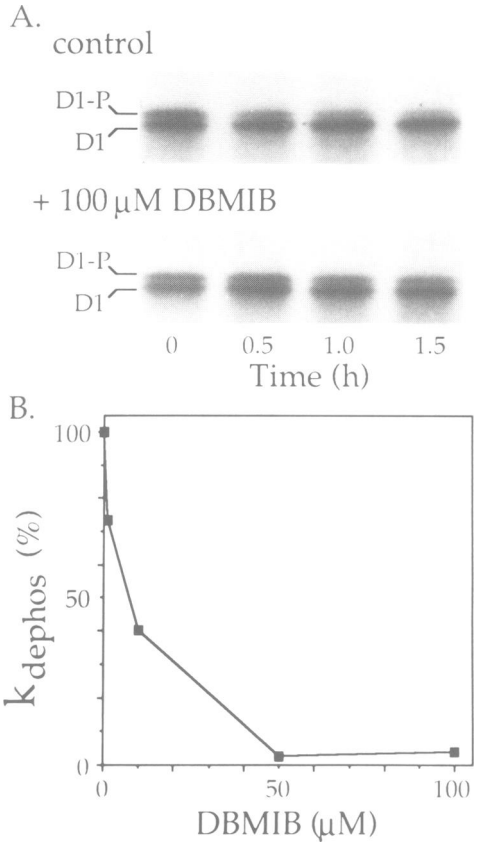

Fig. 6. Inhibition of D1 dephosphorylation by DBMIB. Experiments were performed and analyzed as in Figures 3 and 4 except that various concentrations of DBMIB were added to the plants along with DCMU. (**A**) Autoradiograph showing the phosphorylated (D1-P) and non-phosphorylated (D1) forms of the D1 protein from plants incubated in the absence (control) or presence of 100 μM DBMIB for the times indicated. (**B**) Apparent first-order rate constants for D1 dephosphorylation were determined in plants incubated with the indicated concentrations of DBMIB and expressed as a percentage of the control with no DBMIB. Data from a single (typical) experiment are shown. Complete inhibition at 100 μM DBMIB was reproduced in five separate experiments.

The effect of far-red light and DBMIB on the dephosphorylation of the other PSII-associated proteins was examined in [^{32}P]orthophosphate labeling experiments (Figure 7). Dephosphorylation of D1, D2, CP43 and LHCII occurred under far-red illumination (Figure 7, compare lanes 4 and 5). DBMIB completely inhibited far-red light-stimulated dephosphorylation of PSII core proteins, but had little or no effect on LHCII dephosphorylation (Figure 7, compare lanes 5 and 6). Inhibition by DBMIB of PSII core protein dephosphorylation was also observed when similar experiments were performed under white light (data not shown). Staining of the gel verified the equivalence of protein loads, and demonstrated no obvious changes in steady-state protein profiles between the samples (Figure 7).

Reduced DBMIB is known to activate kinase activity towards PSII core proteins, but not LHCII proteins, in isolated spinach thylakoids in the presence of DCMU, presumably by plastoquinone reduction (Bennett *et al.*, 1987). While we would expect the DBMIB to be oxidized under our conditions, it was important to verify that this compound was not activating DCMU-inhibited kinase activity. To this end, control experiments were performed whereby thylakoid protein phosphorylation was assessed in plants incubated with [^{32}P]orthophosphate in the presence of 10 μM DCMU plus or minus 100 μM DBMIB. Under these conditions, the presence of DBMIB had no effect on DCMU-inhibited kinase activity (data not shown).

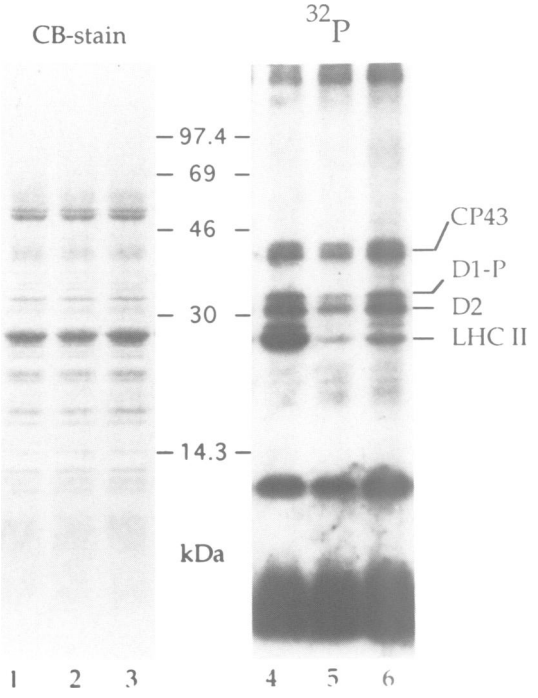

Fig. 7. Effects of far-red light and DBMIB on the dephosphorylation of PSII-associated thylakoid proteins *in vivo*. Plants were labeled with [^{32}P]orthophosphate as in Figure 1, washed and either harvested immediately or further incubated in the presence of 10 μM DCMU, plus or minus 100 μM DBMIB, for 2 h under 720 nm light (3.5 μmol/m^2/s). Thylakoids were then isolated and analyzed by SDS–PAGE and autoradiography. **Right panel**: autoradiograph labeled as in Figure 1; **left panel**: Coomassie Blue stained gel; lanes 1 and 4, thylakoids from plants immediately after phosphorylation; lanes 2 and 5, thylakoids from plants incubated with DCMU for 2 h under 720 nm light after phosphorylation; lanes 3 and 6, thylakoids from plants incubated with DCMU plus DBMIB for 2 h under 720 nm light after phosphorylation.

The fact that DBMIB inhibited dephosphorylation of multiple proteins suggests that this inhibition was not exerted through interactions with the substrates. A direct interaction of DBMIB with phosphatase has not been reported, nor does DBMIB affect thylakoid phosphatase activity *in vitro* (data not shown). The possibility of multiple phosphatases has previously been suggested (Allen, 1992) and indeed two distinct phosphatases have been partially purified from thylakoids (Sun *et al.*, 1989; Kieleczawa *et al.*, 1992). We take the differential inhibition exerted by DBMIB on LHCII and PSII core proteins as further evidence for multiple phosphatases involved in thylakoid protein dephosphorylation *in vivo*. We propose that dephosphorylation of PSII core proteins *in vivo* is regulated by PSI excitation. PSI-regulated dephosphorylation would provide the perfect complement to PSII-regulated phosphorylation, and would render the phosphorylation state of PSII core proteins exquisitely sensitive to the relative energy distribution between the two photosystems.

Materials and methods

Reagents

[^{35}S]methionine (>1000 Ci/mmol) and [^{32}P]orthophosphoric acid (8500–9120 Ci/mmol) were from DuPont/NEN; DBMIB, DCMU and propyl gallate were from Sigma; all SDS–PAGE reagents were from Bio-Rad. All other chemicals were analytical grade.

Experimental procedures

Axenic cultures of *Spirodela oligorrhiza*, an aquatic angiosperm, were grown as previously described (Elich *et al.*, 1992). *In vivo* phosphorylation of thylakoid proteins with [^{32}P]orthophosphate was performed according to published procedures (Elich *et al.*, 1992) except that radiolabeling was for 3 h, under 90 μmol/m^2/s white light (PPFD). Plants were then washed with medium containing unlabeled phosphate and treated as described in the text and figure legends. *In vivo* radiolabeling of the D1 protein with [^{35}S]methionine (Elich *et al.*, 1992) was carried out at a light intensity of 200 μmol/m^2/s. After 30 min radiolabeling, plants were washed three times and then incubated, under the same light intensity, for 1 h in the presence of 1 mM non-radioactive methionine. Further treatments were as described in the text and figure legends. In all cases, dark-incubated samples were harvested under dim (usually green) light. DBMIB and DCMU were freshly prepared as 100-fold concentrated stock solutions in methanol. Control experiments confirmed that 2% (v/v) methanol had no effect on phosphorylation or dephosphorylation. Isolation of thylakoids, SDS–PAGE procedures and autoradiography were performed according to published protocols (Elich *et al.*, 1992). Quantification of D1-P bands on autoradiographs was performed using a two-dimensional laser densitometer (Molecular Dynamics, Sunnyvale, CA). A dilution series of an appropriate extract containing radiolabeled D1-P, encompassing the range of radiolabeled D1-P in the samples to be analyzed, was routinely included on the gels to ensure analysis in the linear range. Rate constants for D1 dephosphorylation were determined by fitting first-order exponentials to time-course data by non-linear regression using MINSQ software (Micromath Scientific Software, Salt Lake City, UT).

Light sources

White light was provided by cool white fluorescent bulbs. Different intensities were achieved by varying the distance of the plants from the bulbs. For the experiments depicted in Figure 3, narrow spectrum visible and far-red light were isolated from a xenon arc lamp beam passed through a 3 inch water filter and then a 10 nm bandpass interference filter (Ditric Optics, Inc., Hudson, MA) of the indicated peak transmission wavelength. White and narrow spectrum visible light intensities were measured with a portable radiometer equipped with a quantum sensor (Skye-Probetech, Perkasie, PA). Far-red light was measured with a portable spectroradiometer equipped with a remote cosine detector (model Li-1800, Li-Cor Inc., Lincoln, NE). The accuracy of the portable radiometer in measuring low levels of narrow spectrum visible light was verified by calibration against the spectroradiometer.

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