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Phosphorylation of Thylakoid Proteins of *Oryza sativa*

IN VITRO CHARACTERIZATION AND EFFECTS OF CHILLING TEMPERATURES

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ABSTRACT

The phosphorylation of thylakoid proteins of rice (*Oryza sativa* L.) was studied *in vitro* using [γ-^{32}P]ATP. Several thylakoid proteins are labeled, including the light-harvesting complex of photosystem II. Protein phosphorylation is sensitive to temperature, pH, and ADP, ATP, and divalent cation concentrations. In the range pH 7 to 8.2, phosphorylation of the light-harvesting polypeptides declines above pH 7.5, whereas labeling of several other thylakoid polypeptides increases. Increasing divalent cation concentration from 3 to 20 millimolar results in a decrease in phosphorylation of the 26 kilodalton light-harvesting complex polypeptide and increased phosphorylation of several other polypeptides. ADP has an inhibitory effect on the phosphorylation of the light-harvesting complex polypeptides. Phosphorylation of the 26 kilodalton light-harvesting polypeptide requires 0.45 millimolar ATP for half-maximal phosphorylation, compared to 0.3 millimolar for the 32 kilodalton phosphoprotein. Low temperature inhibits the phosphorylation of thylakoid proteins in chilling-sensitive rice. However, phosphorylation of histones by thylakoid-bound kinase(s) is independent of temperature in the range of 25 to 5°C, suggesting that the effect of low temperature is on accessibility of the substrate, rather than on the activity of the kinase.

Previous studies on the effects of chilling stress have shown that thylakoid protein phosphorylation is inhibited by low temperatures (0–15°C) in chilling-sensitive rice (*Oryza sativa* L.) but not in cold-tolerant barley (*Hordeum vulgare* L.) (21). A failure to undergo state I-state II transitions (the alteration of the relative absorption cross section of PSI and PSII, or a change in spillover) accompanies the impaired protein phosphorylation and has been correlated with a tendency to undergo photoinhibition at chilling temperatures (21).

The reversible phosphorylation of polypeptides of the light-harvesting pigment protein complex of PSII has been shown to correlate with reversible state I-state II transitions. Phosphorylation of the LHCII polypeptides results in reduction of the relative absorption cross section of PSII relative to PSI (7, 24). This is thought to regulate the balance of activity between the two photosystems *in vivo* during transitions in environmental conditions such as between sun and shade where light quality and intensity change dramatically (6). When LHCII[1] polypeptides are phosphorylated donation of light excitation energy to the PSII reaction centers is decreased and a slight enhancement of excitation energy donation to the PSI reaction centers is observed (9, 22). Changes in fluorescence properties and PSII electron transport rate at limiting light are also associated with thylakoid protein phosphorylation (11, 12, 22).

Thylakoid protein phosphorylation has been studied *in vitro*, using thylakoid preparations and [γ-^{32}P]ATP. It has been shown that phosphorylation is due to the action of one or more membrane bound kinases (5). Kinase activity is sensitive to redox potential in a range that suggests it is responsive to the redox state of the plastoquinone pool (2). The kinase activities have been characterized to varying extents with respect to the effects of pH, Mg^{2+}, ATP, ADP, Pi, and cAMP in spinach, tobacco, and pea (1, 8, 17–20). The reported responses to various treatments suggests that there may be more than one kinase in the membrane. This conclusion has been based on the differential response to ATP concentration at various pH values and Mg^{2+} concentrations, differential thermal denaturation of putative kinases with different ATP affinities (19), and differential effects of inhibitors on the phosphorylation of several thylakoid-bound polypeptides (8, 20).

The inhibition of thylakoid protein phosphorylation by low temperatures in chilling-sensitive rice also blocks the normal occurrence of state I-state II transitions both *in vivo* and *in vitro* (21). In the absence of this regulatory process the plastoquinone pool may become highly reduced. Photoinhibition resulting from turnover of the 32-kD herbicide binding protein has been attributed to reaction center binding of reduced plastoquinone (13).

The effect of chilling on thylakoid protein phosphorylation could be due entirely to the effect of low temperatures on the phosphorylation reaction, or it may have a component that is an indirect effect mediated through a change in, for example, pH of the stroma. In this study, the phosphorylation of thylakoid proteins in rice was examined to determine whether variation in pH or cation, ATP or ADP concentrations have large enough effects that they should be considered as possible mediators of a component of the chilling-induced inhibition of phosphorylation seen *in vivo*.

To investigate possible causes of the low temperature effect of *in vitro* phosphorylation, the effects of low temperature on kinetics of thylakoid protein phosphorylation and on phosphorylation of histones, an exogenous substrate for thylakoid kinases (1, 19), were studied.

MATERIALS AND METHODS

Plant Material. Seeds of *Oryza sativa* L. cv IR8 (a chilling-sensitive Indica strain of rice) were germinated under water in ambient light for 2 weeks; seedlings were transplanted to 10 cm pots containing soil mix (Supersoil, Rod McLellan Co., South San Francisco, CA). Plants were grown in a greenhouse with day/night temperatures of 25/20 ± 4°C and RH 50/80 ± 10% and no supplemental lighting.

Thylakoid Isolation. Leaf tissue (0.2–0.5 g) was homogenized in a 50-ml Waring Blendor at high speed for 7 s in 20 ml of 20 mM Tricine-NaOH, pH 7.8 buffer containing 5 mM $MgCl_2$, 0.4 M sorbitol and 0.01% (w/v) DTT. The resulting homogenate was

[1] Abbreviation: LHCII, light-harvesting Chl *a/b* binding protein complex of PSII.

filtered through two layers of Miracloth (Calbiochem) and centrifuged at 2000*g* for 5 min. The pellet was resuspended in 20 mM Tricine-NaOH buffer (pH 7.8) containing 5 mM $MgCl_2$ and 0.01% (w/v) DTT, and centrifuged at 2000*g* for 5 min. The pellet, containing stroma free thylakoids, was resuspended in the this buffer including 0.1 M sorbitol. Chl concentration was determined by the method of MacKinney (16).

Phosphorylation of Thylakoid Proteins. Isolated thylakoids (0.1 mg Chl/ml) were incubated in resuspension medium (pH 7.8) supplemented with [γ-^{32}P]ATP (15 μCi/ml, Amersham, Arlington Heights, IL), 0.5 mM ATP and 20 mM NaF, unless otherwise specified. Incubation was carried out with illumination from a Kodak 2000 projector at 250 μE $m^{-2}s^{-1}$ for 15 min. The thylakoid suspension was centrifuged for 1 min in a microfuge and the pellet solubilized for SDS-PAGE. *In vivo* labeling was done as previously described (21) using ^{32}Pi. Histones (II-A, Sigma), when used as an artificial substrate, were added to a final concentration of 3 mg/ml and isolated by the following procedure. After incubation, the thylakoid suspensions were centrifuged in a microfuge for 1 min and the supernatants were removed and combined 1:1 with concentrated NH_4OH. The resultant histone precipitate was pelleted by centrifugation in a microfuge for 10 min, dried in a Speedvac (Savant), and solubilized for SDS-PAGE. Time courses were initiated by addition of thylakoids and terminated by a tenfold increase in unlabeled ATP concentration, chilling, and centrifugation within 15 s.

Gel Electrophoresis and Autoradiography. SDS-PAGE of thylakoid proteins was carried out as previously described (23) using 10 to 17% (w/v) polyacrylamide gradient gels and the buffer system of Laemmli (15). Samples were solubilized in solubilization buffer with 6% SDS for 5 min at 55°C. Mol wt standards (Bio-Rad) were used to estimate mol wt. Gels were stained and destained as described (23), and dried. Autoradiography was carried out at room temperature using Kodak XAR-5 x-ray film.

Gel Scanning. For comparative analysis of the degree of radiolabeling, autoradiographs were scanned using an LKB model 2202 gel scanner using the peak integration function. Films for densitometry were preflashed and multiple exposures were made where necessary to assure that exposure response was in the linear range.

RESULTS AND DISCUSSION

In isolated thylakoids of rice (cv IR8) several polypeptides are phosphorylated *in vitro*. These include two mol wt species of the LHCII (26 and 27 kD), four polypeptides with lower electrophoretic mobility (30, 32, 34, and 43 kD), and a polypeptide of 10 kD. This pattern is shown in Figure 1, *e.g.* pH 7.8, which is an autoradiograph from a typical experiment. In the SDS-PAGE system used, the LHCII contains three well resolved polypeptides. The relative degree of phosphorylation observed *in vitro* varies, however, the two higher mol wt polypeptides are the major labeled polypeptide species of this complex.

Alteration of environmental conditions results in changes in the pattern of thylakoid protein phosphorylation observed *in vivo*. Labeling studies with ^{32}Pi carried out *in vivo* at 25°C, followed by SDS-PAGE and autoradiography, indicate changes in protein phosphorylation with increased light intensity, as shown in Figure 2. The greatest change in labeling occurs between 1000 and 2000 μE $m^{-2}s^{-1}$. Labeling of the 26 kD polypeptide decreases by about 35%, with no significant changes in the labeling of the 27 kD polypeptide and increases of about 100, 70, and 30% in the 30, 32, and 34 kD polypeptides, respectively. This may be attributable, at least in part, to pH and cation effects.

As light intensity increases, stromal pH is expected to rise by about 1 pH unit (10). A range of pH values from 7.1 to 8.2 was employed to investigate effects on relative labeling of the various thylakoid phosphoproteins. Figure 1 shows that phosphorylation of the LHCII polypeptides decreases at pH values above 7.5. In contrast, the other thylakoid polypeptides are phosphorylated maximally at pH 8.2, the highest pH tested. Results of an analysis of autoradiograms by densitometry are summarized in Table I.

Because cations have a dynamic influence on chloroplast structure and function (3) the effect of divalent cation concen-

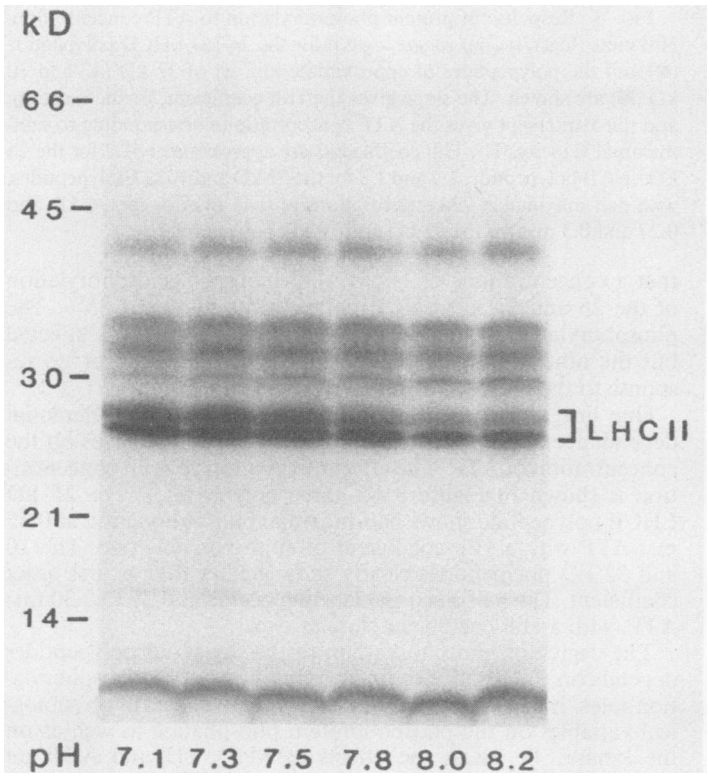

FIG. 1. Effect of pH on protein phosphorylation in isolated thylakoids of rice cv IR8. Thylakoids were isolated and phosphoproteins labeled as described in "Materials and Methods," but with the pH of the labeling reaction mix adjusted as indicated. Samples were subjected to SDS-PAGE, the gel was dried and autoradiographed. The treatment at pH 7.8 serves to illustrate the phosphorylated polypeptide species.

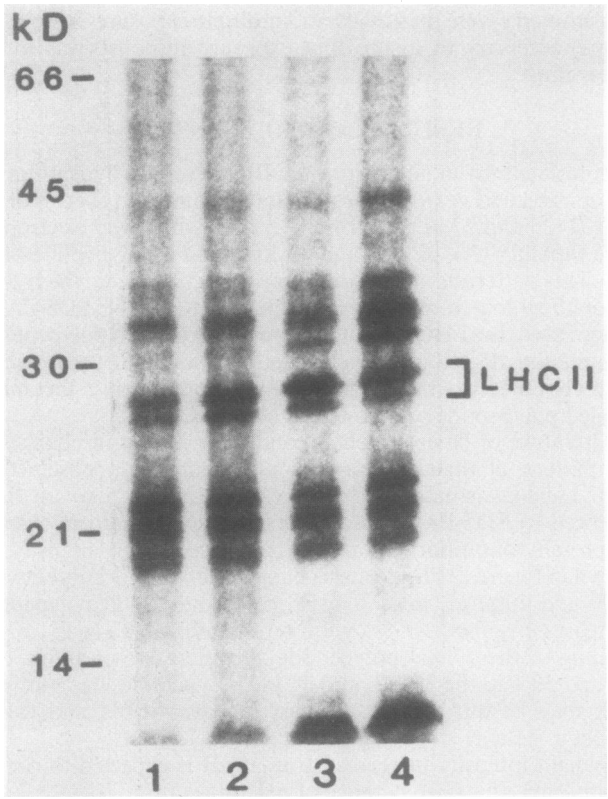

FIG. 2. Effect of light intensity on *in vivo* phosphorylation of the thylakoid proteins of rice cv IR8. Labeling with ^{32}Pi was carried out as described in Moll and Steinback (21). Light intensities were: lane 1, 50; lane 2, 250; lane 3, 1000; lane 4, 2000 $\mu E\ m^{-2}\ s^{-1}$. A Kodak 4000 slide projector was used as the light source. Thylakoids were isolated in the presence of 20 mM NaF, subjected to SDS-PAGE, the gel was dried and autoradiographed. There are three polypeptides labeled *in vivo* that are not labeled *in vitro*.

Table I. *Effect of pH on Protein Phosphorylation by Rice Thylakoids*

Labeling at the indicated pH, SDS-PAGE, autoradiography and densitometry were carried out as described in "Materials and Methods." Results of densitometry analysis are given in arbitrary units based on area measurements of densitometer scans.

Polypeptide	pH					
	7.1	7.3	7.5	7.8	8.0	8.2
kD						
10	22	26	28	31	31	34
26	77	80	77	73	56	53
27	50	52	49	39	28	29
30	14	17	23	23	24	30
32	27	29	29	29	26	29
34	22	24	26	27	28	28
43	13	14	18	18	20	21

tration on thylakoid protein phosphorylation *in vitro* was investigated. Since Mg^{2+} moves out of the inner thylakoid space during H^+ accumulation, an increase in stromal Mg^{2+} is expected to accompany an increase in light intensity. Analysis of autoradiograms by densitometry, summarized in Table II, shows that effect of varying Mg^{2+} concentration from 3 to 20 mM. In general, increased Mg^{2+} stimulated the phosphorylation of the non-LHCII polypeptides.

The balance of the adenylate pool is expected to influence kinase activity *in vivo* and ADP is known to inhibit kinase activity *in vitro* (18). Analysis of autoradiograms by densitometry showed

Table II. *Effect of Mg^{2+} Concentration on Protein Phosphorylation by Rice Thylakoids*

Labeling at the indicated concentration of Mg^{2+}, SDS-PAGE, autoradiography, and densitometry were carried out as described in "Materials and Methods." Results of densitometry analysis are given in arbitrary units based on area measurements of densitometer scans.

Polypeptide	Mg^{2+} Concentration			
	3	5	10	20
kD	*mM*			
26	72	60	42	39
27	21	30	33	42
30	21	18	18	21
32	36	45	42	54
34	18	18	21	27

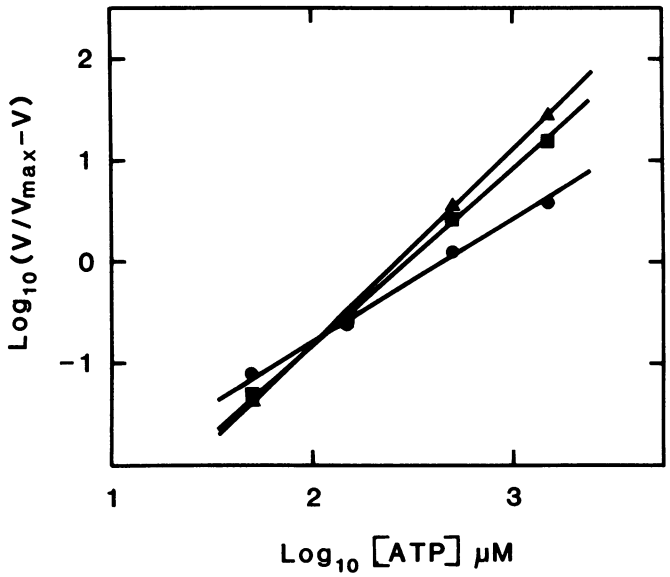

FIG. 3. Response of protein phosphorylation to ATP concentration. Hill plots ($\log(v/(v_m-v))$ *versus* $\log(S)$) for the 26-kD LHCII polypeptide (●) and the polypeptides of approximate mol wt of 32 kD (▲) and 10 kD (■) are shown. The slope gives the Hill coefficient for the reaction, and the *x*-intercept gives the ATP concentration corresponding to half-maximal velocity. The Hill coefficients are approximately 1.2 for the 26 kD LHCII polypeptide, 1.9 and 1.8 for the 32 kD and 10 kD polypeptides, with half maximal ATP concentrations of 0.45 mM for the 26 kD and 0.27 and 0.3 mM for the 32 kD and 10 kD polypeptides.

that a concentrations of 1 mM ADP inhibited phosphorylation of the 26 and 27 kD LHCII polypeptides by about 35%. The phosphorylation of the 30 kD polypeptide was similarly affected but the other non-LHCII polypeptides showed little or no response to the presence of ADP (data not shown).

One line of evidence for multiple kinases is the differential dependence of phosphorylation of different polypeptides on the concentration of ATP. The effect of variation of ATP concentration is shown in Figure 3 for three polypeptides. The 26 kD LHCII polypeptide shows half-maximal phosphorylation at 0.45 mM ATP with a Hill coefficient of approximately one. The 10 and 32 kD polypeptides clearly show greater than a first order coefficient. The half-maximal labeling occurs at 0.27 to 0.30 mM ATP, with a Hill coefficient close to two.

The degree of phosphorylation of the thylakoid polypeptides depends on the balance of phosphorylation and dephosphorylation rates. It is possible that *in vivo* there are effects of physiological variables on the phosphoprotein phosphatase as well as on the kinase. To assess the effects of Mg^{2+}, pH and ADP on

phosphatase activity *in vitro*, radiolabeling was carried out both in the presence and absence of the phosphatase inhibitor NaF. Absence of NaF results in a decrease in the net phosphorylation of the LHCII polypeptides, but no qualitative changes in the relative response to pH, Mg^{2+}, or ADP (data not shown). The effect of omission of NaF on net phosphorylation is less at lower temperatures (data not shown). Dephosphorylation of the thylakoid phosphoproteins in the absence of NaF is shown in Figure 4 for membranes phosphorylated for 5 min and then dephosphorylated for 5 min. The decrease in radiolabel in the LHCII polypeptides is over 50%. The other polypeptides undergo slower rates of dephosphorylation, with about 20% decrease in radiolabel. Lane 5 of Figure 4 shows labeling of membranes dephosphorylated in the presence of 0.5 mM ATP. Comparison of lanes 3 and 5 indicates that ATP has no large effect on phosphatase activity.

The effect of variation in pH and cation, ADP and ATP concentrations are in general similar to observations on LHCII phosphorylation in other systems (1, 8, 17–20). Since the effects of moderate changes in these parameters are much smaller than the effect of low temperature and since low temperature inhibits phosphorylation of all of the thylakoid phosphoproteins rather than a subset of them, it appears likely that temperature effects on pH, cation ATP and ADP concentrations are not primarily responsible for the observed decrease in protein phosphorylation. Reduced phosphorylation is not due to a failure of chilling sensitive thylakoids to undergo photochemistry at low temperatures. PSII activity of rice thylakoids assayed by DCPIP reduction shows a Q_{10} of about 2 at saturating light, but essentially no effect of temperature at low light (50 $\mu E\ m^{-2}\ s^{-1}$) (data not shown). A reduction in phosphorylation mediated by phosphoprotein phosphatase activity would require considerable activation of phosphatase activity. None of the factors tested, including low temperature, resulted in significant stimulation of dephosphorylation. These data indicate that the inhibition of thylakoid protein phosphorylation *in vivo* is probably due principally to the effects of temperature on protein phosphorylation observable *in vitro*.

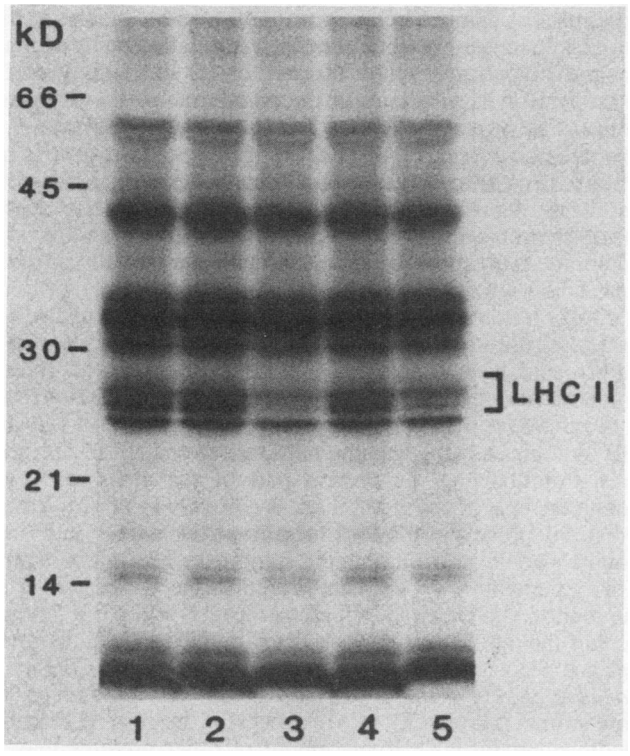

FIG. 4. Dephosphorylation of *in vitro* phosphorylated thylakoid proteins. Labeling was carried out as described in "Materials and Methods" except that the total time of labeling was 5 min. Thylakoids were centrifuged out of the labeling medium and resuspended in resuspension buffer without label and with DCPIP to act as an electron acceptor and samples were permitted to dephosphorylate for 5 min in the dark. The thylakoids were reisolated, subjected to SDS-PAGE and autoradiographed. Treatments were as follows: lane 1, no dephosphorylation control, samples were kept cold in the presence of 50 mM NaF; lane 2, 5 min in the presence of 20 mM NaF; lane 3, 5 min without NaF; lane 4, 5 min in the presence of 20 mM NaF and 0.5 mM ATP; lane 5, 5 min in the presence of 0.5 mM ATP,

We have previously shown that low temperatures inhibit *in vitro* thylakoid protein phosphorylation in a chilling sensitive rice strain but not in chilling tolerant barley (21). The effect of temperature on protein phosphorylation is further characterized in this study. A time course of protein phosphorylation at 25 and 5°C is shown in Figure 5. After an initial increase within the first 5 min, there is no further incorporation of radiolabel at 5°C, but label incorporation is approximately linear with time at 25°C from 2 to 20 min. Treatment with low temperature does not result in irreversible changes to the kinases or their phosphoprotein substrates, since all thylakoid isolations were performed at approximately 5°C, and subsequent phosphorylation at 25°C results in a phosphorylation pattern comparable to that seen *in vivo*. The effect of incubation temperature on *in vitro* protein phosphorylation by rice thylakoids is shown in Figure 6. From 35 to 20°C there is almost no effect of temperature, but from 15 to 0°C there is a strong inhibitory effect of low temperature on protein phosphorylation rate.

Histones, which can be used as an artificial substrate for thylakoid protein kinases (1, 19), were used to separate temperature effects on the kinases and temperature effects on the protein substrates or the kinase-substrate interactions involved in the phosphorylation process. Figure 7 shows phosphorylation of thylakoid proteins of IR8 and of added histones at 25 and 5°C. The effect of temperature on phosphorylation of thylakoid pro-

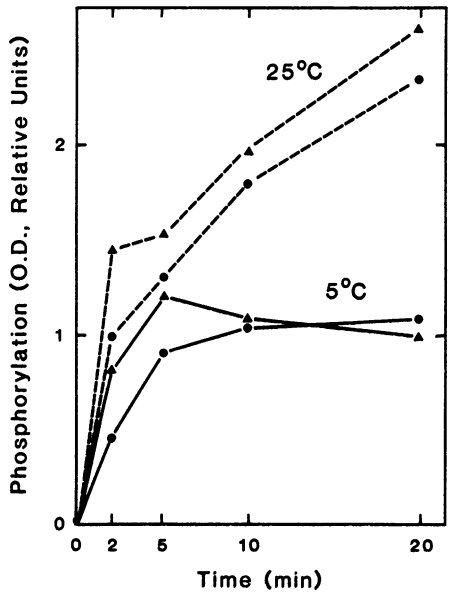

FIG. 5. Time course of *in vitro* phosphorylation of the 26 kD LHCII (▲) and 32 kD polypeptides (●) at 5 (——) and 25°C (-----). Labeling, SDS-PAGE and autoradiography were carried out as described in "Materials and Methods," but incubated for the time and temperature indicated. Temperature was established before the addition of label. After 5 min, rice thylakoids continue to show protein phosphorylation at 25°C, but not at 5°C.

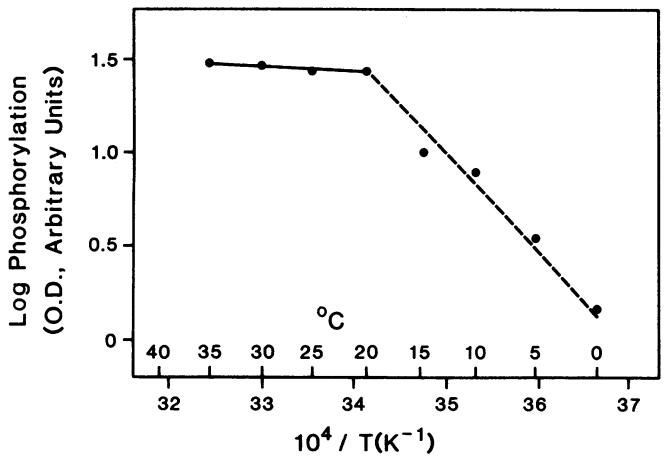

FIG. 6. Effect of temperature on protein phosphorylation in thylakoids of rice cv IR8. Labeling, SDS-PAGE, autoradiography and densitomitry were carried out as described in "Materials and Methods." Incubation was at the temperatures indicated. Results are plotted as log phosphorylation *versus* $1/T$. A constant activation energy will result in a straight line. These results indicate a break point between 15 and 20°C.

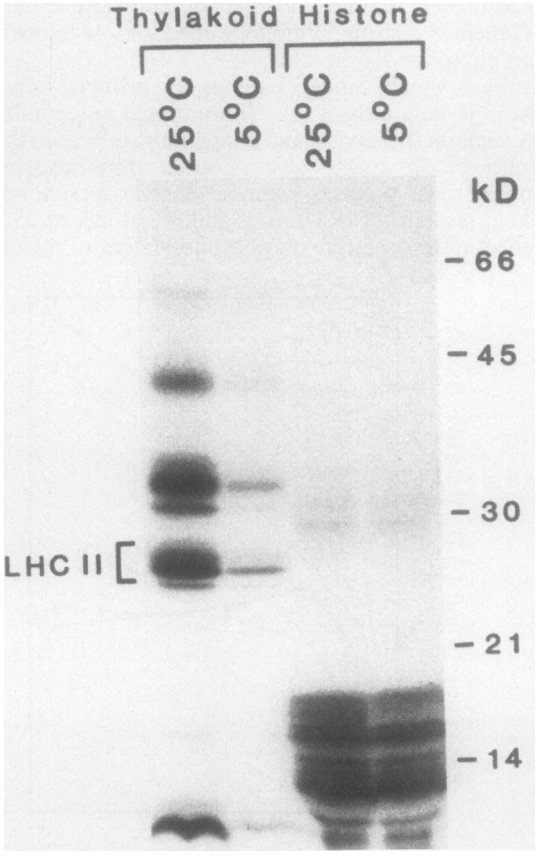

FIG. 7. Effect of temperature on phosphorylation of histones and thylakoid proteins by isolated thylakoids of rice cv IR8. Labeling, SDS-PAGE and autoradiography were carried out as described in "Materials and Methods," except that histones (3 mg/ml) were added to two samples and subsequently reisolated, and two samples were incubated at 5 instead of 25°C. Lanes 1 and 2, thylakoid proteins. No histones were added to these samples. Lanes 3 and 4, histones. Samples for lanes 1 and 3 were incubated at 25°C, lanes 2 and 4 at 5°C.

teins is quite pronounced. In the experiment shown, densitometry analyses of autoradiograms demonstrate that phosphorylation of thylakoid proteins at 5°C is about 10% of the 25°C level. The phosphorylation of histones by thylakoid protein kinases is not inhibited by low temperature. In some instances histone phosphorylation terminates within 1 min (20). This phenomenon may correspond to the initial rapid thylakoid protein phosphorylation that shows little sensitivity to temperature, which would invalidate the comparison of the temperature effect on histone phosphorylation and phosphorylation of endogenous proteins. However, rice thylakoids phosphorylate histones with kinetics very similar to the phosphorylation of endogenous proteins, with a rapid initial rate for about 1 min followed by a slower rate that is approximately constant up to 15 min (the longest time for which data were taken). The rapid phase accounts for about 30% of the total phosphorylation at 15 min. It is not known what subset of the thylakoid protein kinases are active in histone phosphorylation. Since the qualitative effect of temperature on phosphorylation of all the thylakoid phosphoproteins is similar, it is possible that the origin of the low temperature inhibition is the same for all of them.

One explanation for the described data is that the decreased level of protein phosphorylation seen at low temperatures in rice is due to inaccessibility of the protein substrate to the kinase. Inaccessibility of the phosphorylation sites may be a result of (a) changes in the conformation of the LHCII polypeptides and other phosphoproteins so that phosphorylation sites are not accessible, (b), changes in the conformation of the kinases so that they can no longer phosphorylate their normal substrate proteins but can still phosphorylate histone, or (c) immobility of the kinase and/or its substrates in the membrane so that it cannot diffuse into proximity to any but a very small subset of its substrate polypeptides. The effect of chilling temperature is not confined to a particular group of phosphoproteins, which suggests that the mechanism of chilling inhibition is general in its effects. For this reason we suggest that at low temperature the thylakoid membrane proteins in the vicinity of PSII are partially immobilized. This is not due to a large scale phase transition that prevents all diffusion in the membrane, since plastoquinone must still be mobile. Protein-lipid ratios have been found to affect membrane fluidity, and have been correlated with adaptation to chilling temperatures (4). It may be that protein-protein interactions such as occur between LHCII polypeptides or between LHCII and PSII in grana stacking become relatively strong at low temperatures, immobilizing the protein part of the membrane even though the lipid phase is still fluid. A corollary to this hypothesis is that any process in the PSII membrane dependent on protein mobility would also be severely inhibited at low temperature with a temperature dependence similar to that for protein phosphorylation. An example of such a process is that of the replacement of the rapidly turned over 32 kD herbicide binding protein that has been implicated in the photoinhibition of *Chlamydomonas* at high light intensities (13). It has been shown that low temperature inhibits 32 kD replacement in chilling sensitive *Chlamydomonas* (14). Replacement of other proteins may be important in recovery from prolonged stress, and assembly of new photosynthetic membranes would also be affected.

In summary, thylakoid protein phosphorylation in rice shows effects of pH and Mg^{2+}, ADP and ATP concentrations *in vitro*. These effects are unlikely to play a major role in inhibition of protein phosphorylation at low temperatures. Experiments with histones suggest that the inhibition of protein phosphorylation by low temperatures in chilling-sensitive rice is due primarily to inaccessibility of the polypeptide substrates to the kinase(s), possibly as a result of immobilization of the proteins or localized conformational changes within the grana regions of the thylakoid membrane.

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