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Phosphorylation of Photosystem II Components, CP43 Apoprotein, D1, D2, and 10 to 11 Kilodalton Protein in Chloroplast Thylakoids of Higher Plants[1]

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MASAHIKO IKEUCHI*, F. GERALD PLUMLEY, YORINAO INOUE, AND GREGORY W. SCHMIDT
Solar Energy Research Group, The Institute of Physical and Chemical Research (RIKEN), Wako, Saitama 351-01, Japan (M.I., Y.I.); *and Botany Department, The University of Georgia, Athens, Georgia 30602* (F.G.P., G.W.S.)

ABSTRACT

Phosphorylated thylakoid proteins of spinach (*Spinacia oleracea* L.) and pea (*Pisum sativum* L.) were solubilized, fractionated by sucrose density gradient centrifugation, and analyzed by gel electrophoresis and crossed immunoelectrophoresis to identify the phosphoproteins. It was found that in addition to intense phosphorylation of light-harvesting chlorophyll complex II, four photosystem II components, CP43 apoprotein, D1, D2, and a 10 to 11 kilodalton protein, are substantially phosphorylated in the light. Furthermore, the CP43 apoprotein, D1 and D2 can be resolved into two electrophoretic subspecies, only one of which is phosphorylated. This indicates that only a fraction of the PSII polypeptides is phosphorylated. Finally, analysis of detergent procedures suggests that the 10 to 11 kilodalton phosphoprotein is a peripheral component of the O_2-evolving PSII reaction center complex.

In studies of the substrates of thylakoid protein kinases, Steinback *et al.* (22) suggested that some of the PSII reaction center components are phosphorylated in pea photosynthetic membranes in the light. Although the PSII reaction center has been thought to be composed of five integral proteins (CP47[2] apoprotein, CP43 apoprotein, D1 (herbicide-binding protein), D2, and cytochrome b_{559} apoprotein) (6, 21) and one extrinsic protein of 33 kD (15), neither the identity nor the physiological role of the phosphorylated components has been established. Recently, a new phosphorylated protein of about 10 kD was specifically found in spinach PSII-enriched membranes containing the reaction center complex, LHCII and some other proteins (9, 24). On the other hand, in a green alga, *Chlamydomonas reinhardtii*, it was shown that several PSII components including CP43 apoprotein and D2 (but not D1) are phosphorylated, although a phosphoprotein homologous to the 10 kD protein of higher plants was not identified (7, 14). Here, we unambiguously identify phosphoproteins of thylakoid membranes of higher plants and report that not only CP43 apoprotein and D2, but also D1, are phosphorylated in higher plants.

MATERIALS AND METHODS

Intact chloroplasts were isolated from spinach (*Spinacia oleracea* L.) and pea (*Pisum sativum* L.) and used for protein phosphorylation as described previously (1). Intact chloroplasts were incubated with [^{32}P]orthophosphate (0.17 mCi/ml) in 0.33 M sucrose, 2 mM EDTA, 2 mM Na-isoascorbate, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 5 mM $Na_4P_2O_7$, 10 mM $NaHCO_3$, 1 mM ATP, 50 mM Hepes-NaOH (pH 7.5), and 0.1 mg Chl/ml in the light (2.3 mW/cm^2) for 10 min. After illumination, NaF was added to a final concentration of 20 mM. The chloroplasts were collected by centrifugation at 13,000*g* for 1 min and were ruptured by suspending in 10 mM EDTA, 5 mM NaF, and 10 mM Hepes-NaOH (pH 7.5). Thylakoid membranes were purified by a stepwise sucrose density gradient centrifugation (3) in the presence of 5 mM NaF.

Phosphorylated thylakoid membranes (0.5 mg Chl/ml) were solubilized with 1% (w/v) dodecylmaltoside, 10 mM NaCl, 5 mM NaF, 5 mM ϵ-aminocaproic acid, 1 mM benzamidine, and 20 mM Mes-NaOH (pH 6.0), layered onto 10 to 30% (w/v) sucrose density gradient containing 0.05% (w/v) dodecylmaltoside in the same medium, then centrifuged at 165,000*g* for 5 h. This method gave very small amount of free Chl and yielded four discrete green bands corresponding to LHCII, PSII, PSI-LHCI, and PSII-LHCII complex (from top to bottom).

Thylakoids were also solubilized with a mixture of 1% (w/v) Triton X-100 and 40 mM β-octylglucoside in the same medium as described above. The extracts were layered onto 10 to 30% (w/v) sucrose density gradients containing 0.03% (w/v) Triton X-100 instead of dodecylmaltoside, then centrifuged at 165,000*g* for 10 h. This method partially dissociated PSII complexes and yielded rather large amounts of free pigment (see "Results").

For SDS-PAGE in the presence of urea, composite separating gels were cast with 12.5% (w/v) and 14.5% (w/v) acrylamide in the upper two-thirds and lower one-third, respectively. The acrylamide solutions contained 5.5 M urea and Neville's buffer (423 mM Tris-HCl [pH 9.18]). Electrophoresis was done at 25°C according to (15). In this gel system D1 migrates faster than D2 (13). For PAGE in the absence of urea, a separating gel with 10 to 20% (w/v) acrylamide linear gradient containing Laemmli's buffer was used at 4°C according to Ikeuchi *et al.* (14). In this gel system, D2 migrates faster than D1 (5). Proteins were stained

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[2] Abbreviations: CP, Chl protein complex of photosystem I and II; LHCI and II, light-harvesting Chl complex associated with photosystem I and II, respectively.

with silver according to Heukeshoven and Dernick (12) and subjected to autoradiography.

Crossed immunoelectrophoresis was performed according to Plumley and Schmidt (20). Phosphorylated thylakoid proteins of spinach were resolved by SDS-PAGE with urea and then electrophoresed transversely into an agarose gel containing one of the antisera (×12–×25 dilution) raised against CP47, CP43, D1, or D2. To enhance electroelution of proteins from the first-dimension acrylamide gel strip, 1% sodium deoxycholate was included in the anodal agarose in which the acrylamide gel strip was embedded. Deoxycholate was also effective to reduce the background staining due to abundant IgG, although it caused broadening of precipitin arcs (20). Immunoelectrophoresis in the second dimension was done with a constant voltage at 15 V for 48 h without cooling. After electrophoresis, the agarose gel was dried, stained with Coomassie brilliant blue R-250, and then subjected to autoradiography.

For preparation of rabbit antisera, CP47 and CP43 were isolated from spinach PSII-enriched membranes by isoelectric focussing in the presence of 60 mM β-octylglucoside (M Ikeuchi, Y Inoue, unpublished data). D1 and D2 were isolated by preparative SDS-PAGE with urea (13).

RESULTS

With intact chloroplasts from spinach, light-induced phosphorylation of several thylakoid proteins, in addition to LHCII polypeptides, could be detected (Fig. 1, lane T). Although the labeling pattern was similar to those reported earlier (1, 22), it was difficult to confidently identify the phosphorylated polypeptides resolved by one-dimensional SDS-PAGE.

Following detergent solubilization, we fractionated thylakoid proteins by sucrose density gradient centrifugation and strived to precisely compare the stained and labeled polypeptides with respect to their migration in SDS-PAGE and distribution in sucrose gradient. With the thylakoids solubilized with dodecylmaltoside, all Chl protein complexes could be separated into four discrete green bands: the LHCII fraction, a PSII fraction almost devoid of LHCII, a PSI-LHCI fraction, and PSII-LHCII fraction (Fig. 1, lanes 2–5, respectively). Notably, the PSII fraction contained the extrinsic 33 kD protein (*cf.* Fig. 2, lane 9) which is involved in O_2 evolution (19). This suggests that a presumed O_2-evolving PSII reaction center complex can be simply isolated by dodecylmaltoside-solubilization of thylakoids followed by centrifugation, although the O_2 evolution activity of this fraction has not been checked because of low recovery.

Apart from the strongly labeled LHCII polypeptides, the most prominent phosphoproteins were recovered with the PSII and PSII-LHCII fractions, both of which contained CP47 apoprotein, CP43 apoprotein, 33 kD extrinsic protein, D1, D2, and cytochrome b_{559} (Fig. 1, lanes 3 and 5). The phosphorylated band of about 43 kD corresponded exactly with the upper, but not lower, band of the silver-stained doublet which is a variant of CP43 of PSII (15). The correspondence was confirmed by bimodal co-sedimentation of this labeled band and the upper stained band with both the PSII and PSII-LHCII fractions of sucrose gradient. Similarly, a heavily labeled band of about 32 kD corresponded to the faintly stained upper band of the doublet designated as D2. The identity of D2 was confirmed by western blotting with the antiserum against D2 (13). Another weakly labeled band of about 30 kD appeared to correspond to the upper band of the stained doublet denoted as D1 which had been characterized by photoaffinity labeling with [^{14}C]azidoatrazine (13). Co-sedimentation of these labeled bands and corresponding stained bands in the PSII and PSII-LHCII fractions of sucrose density gradient also substantiated their respective identification. Heterogeneity in stained doublets, which has already been reported in purified reaction center complexes (15), suggests that some but not all of CP43 apoprotein, D1 and D2 are phosphorylated and that phosphorylation results in slight retardation of phosphorylated subspecies during SDS-PAGE as suggested earlier in *Chlamydomonas* (6). Related to this observation the isoelectric point of 'phosphorylated' CP43 upper subspecies (pI = 5.4) is about 0.3 pH unit more acidic than that of the 'nonphosphorylated' lower subspecies (pI = 5.7) (Ikeuchi and Inoue, unpublished).

A labeled band of about 10 kD was also found in both the PSII and PSII-LHCII fraction (Fig. 1, lanes 9 and 11), indicative

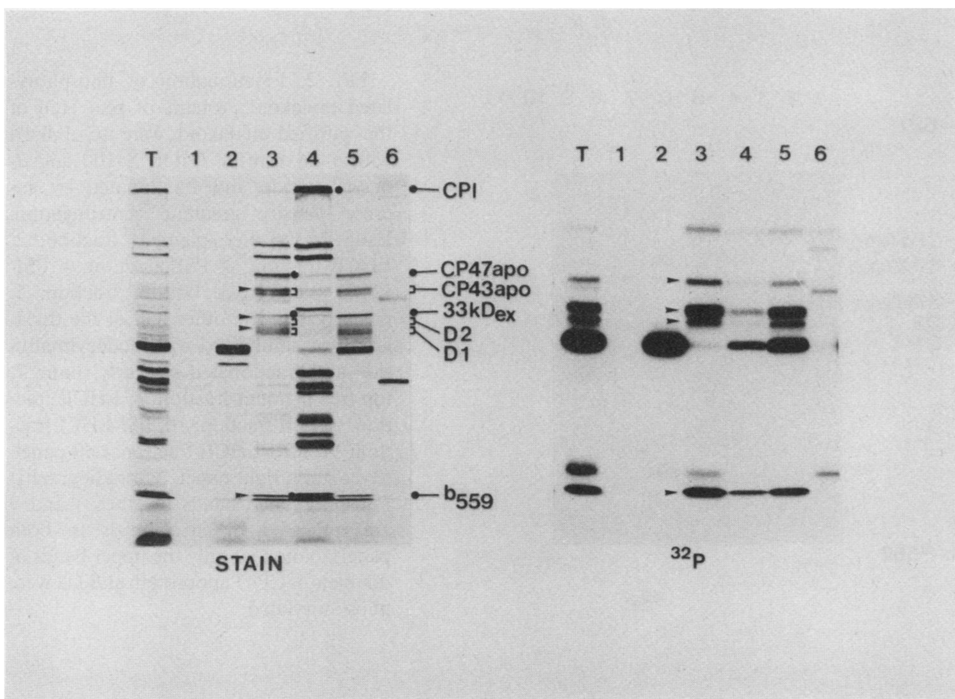

FIG. 1. Fractionation of phosphorylated thylakoid proteins of spinach. From intact chloroplasts incubated with [^{32}P] orthophosphate, thylakoid membranes (T) were isolated, solubilized with dodecylmaltoside, and fractionated by sucrose density gradient centrifugation. The fractions were subjected to SDS-PAGE in the presence of 5.5 M urea which dissociates Chl-protein complexes except CPI. Lane 1, top colorless fraction; 2, LHCII fraction; 3, PSII fraction; 4, PSI-LHCI fraction; 5, PSII-LHCII fraction; 6, pellet fraction. Left panel, silver stain; right panel, autoradiography. Phosphorylated bands in lane 3 are indicated by arrowheads in both panels. Note that upper bands of doublets of CP43 apoprotein, D2, and D1 were phosphorylated. ATP synthase is present mostly in the PSI-LHCI fraction (lane 4) but also with PSII (lane 3).

of this polypeptide's intimate association with the reaction center complex. Although this labeled band co-migrated with a heavily stained band corresponding, in part, to the Cyt b_{559} apoprotein, the co-migration proved to be fortuitous (Fig. 2). A phosphorylated band of about 70 kD was also found in PSII-enriched fractions but did not correspond to any stained band. This may not be a new PSII component but an aggregated form of phosphorylated D1 and/or D2 (11). Alternatively, the high mol wt phosphoprotein might be the thylakoid protein kinase which Coughlan and Hind (4) have found to be 64 kD and to undergo autophosphorylation. Several other phosphoproteins were found in non-PSII fractions but none of them corresponded to PSI or LHCI components; in contrast, phosphorylation of the PSI reaction center apoprotein and some LHCI components occurs in *Chlamydomonas reinhardtii* (14).

Similar results were obtained with chloroplasts isolated from pea (Fig. 2, lanes 7–11): LHCII and PSII components (CP43, D1, D2, and a 11 kD protein) were phosphorylated during illumination. Silver-stained bands of CP43 apoprotein and D2 were resolved as respective doublets both of which co-sedimented with PSII and the PSII-LHCII complexes: each upper band comigrated with the corresponding phosphorylated band in SDS-PAGE. D1 was also labeled although the phosphorylated and presumed non-phosphorylated subspecies were not well resolved. D2 was most heavily labeled among the three PSII polypeptides, yielding a labeling profile very similar to that of spinach (Fig. 1). A phosphoprotein of about 11 kD, probably equivalent to the spinach 10 kD phosphoprotein, was found at the position of heavily stained Cyt b_{559} apoprotein in each of the PSII enriched fractions.

As documented in spinach, proteins in the pea PSII-LHCII complex as well as the free PSII complex were phosphorylated (Fig. 2, lanes 9 and 11). In pea, however, two phosphorylated LHCII polypeptides were resolved by SDS-PAGE; of these, the one of higher mol wt was more prominently associated with the PSII complex, indicative of preferential protein-protein interaction. This is consistent with observations that a low mol wt species of LHCII more readily migrates from grana to stroma lamellae than does the one of high mol wt when they are phosphorylated (16).

When the pea thylakoids were solubilized with a mixture of two strong detergents, Triton X-100 and β-octylglucoside, CP43 and the 11 kD phosphoprotein were dissociated from the PSII core composed of CP47 apoprotein, D2, D1, and Cyt b_{559} (Fig. 2, lane 3). Therefore, it appears that both the phosphorylated CP43 and phosphorylated 11 kD polypeptide are rather loosely associated with other components of the reaction center complex. Again, the distribution profiles in the sucrose gradient of the phosphorylated bands assigned to CP43 apoprotein, D1 and D2 were nearly identical with those of respective stained bands. In contrast, the 11 kD phosphoprotein was recovered in the top free pigment fraction (Fig. 2, lane 1) while the co-migrating, heavily stained band of Cyt b_{559} was retained in the lower PSII fraction. This analysis indicates that two distinct polypeptides comigrate fortuitously during PAGE of the PSII fraction prepared by dodecylmaltoside treatment (Fig. 2, lanes 5, 9, 11). It is of interest that with Triton/β-octylglucoside, the CP43 doublet is recovered in the green pellet which also contains some LHCII and LHCI. This may reflect a specific interaction between the connecting LHCII complex and CP43 which is presumed to be a peripheral antenna component of PSII reaction center (18).

To eliminate the possibility that unrelated phosphoproteins fortuitously co-migrate with D1 or D2, the phosphorylated PSII fraction was analyzed in a different gel system lacking urea. Under these conditions, D2 was shown to migrate faster than D1 (5, 15). As expected, the more labeled band co-migrating with stained D2 moved faster than the less labeled one comigrating with stained D1 (Fig. 3) which was, again, located by photoaffinity labeling with [^{14}C]azidoatrazine (not shown).

Crossed immunoelectrophoresis of spinach thylakoids was performed to further confirm the identification of phosphoproteins. The antibodies raised against CP47, CP43, D1, and D2 reacted specifically with respective thylakoids proteins and formed each single precipitin peak (Fig. 4, stain). Among these, the precipitin

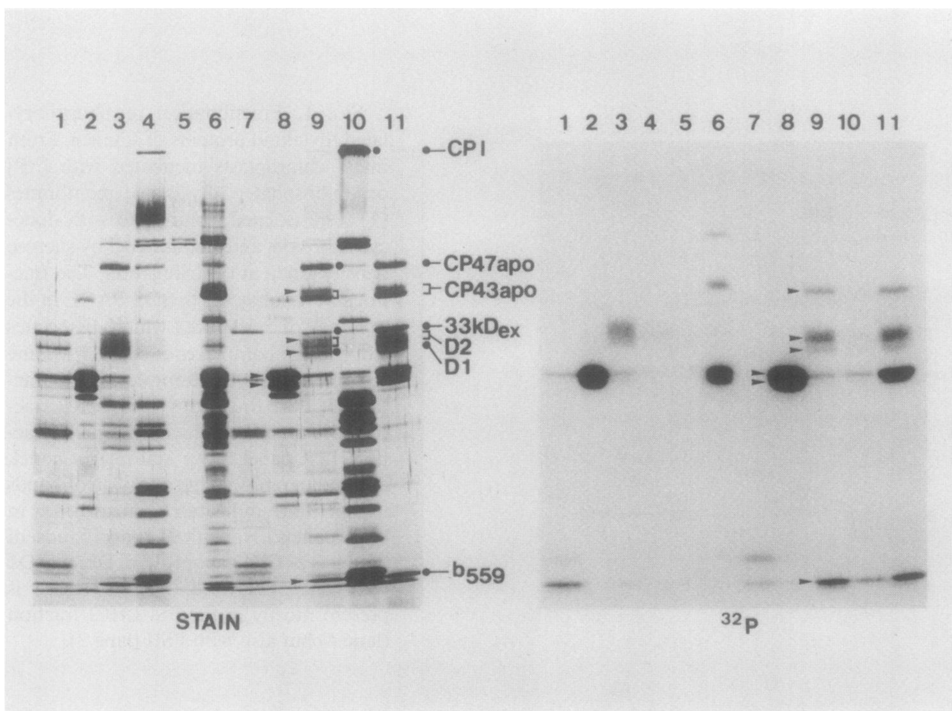

FIG. 2. Fractionation of phosphorylated thylakoid proteins of pea. Half of the purified thylakoid were solubilized with a mixture of Triton X-100 and β-octylglucoside and fractionated by sucrose density gradient centrifugation. Lane 1, top free pigment fraction; 2, LHCII fraction; 3, PSII fraction; 4, PSI-LHCI fraction; 5, bottom fraction; 6, green pellet. The other half of the thylakoid was solubilized with dodecylmaltoside and fractionated similarly. Lane 7, top free pigment fraction; 8, LHCII fraction; 9, PSII fraction; 10, PSI-LHCI fraction; 11, PSII-LHCII fraction. Left panel, silver stain; right panel, autoradiography. Phosphorylated bands in lanes 8 and 9 are indicated by arrowheads in both panels. Note that only the upper bands of doublets of CP43 apoprotein and D2 were phosphorylated.

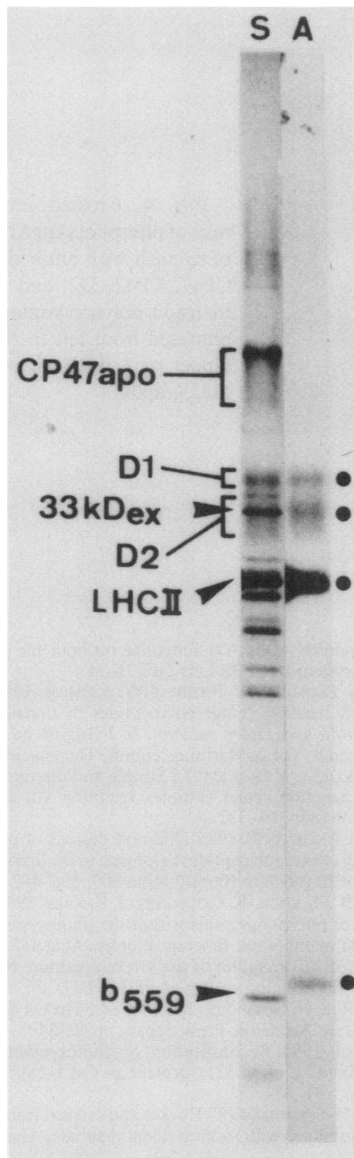

FIG. 3. Phosphorylated PSII polypeptides of pea chloroplasts analyzed by SDS-PAGE without urea. Phosphorylated thylakoids were solubilized by a mixture of Triton X-100 and β-octylglucoside and fractionated as in Figure 2. The band containing PSII complexes was collected and analyzed by SDS-PAGE without urea. Samples were heated at 100°C for 0.5 min before electrophoresis. Proteins were stained with silver (S) then subjected to autoradiography (A). Phosphoproteins corresponding to D1 and D2 are indicated by dots in addition to contaminating LHCII and 11 kD protein. CP43 is dissociated from the PSII core complex with these solubilization conditions.

arcs of CP43, D1, and D2 were found to be phosphorylated while that of CP47 was not at all (Fig. 4, autoradiogram). Radioactivity tended to be condensed in the left part of the arcs. This is consistent with the fact that the low mobility parts of the respective doublet bands in SDS-PAGE are the phosphorylated subspecies while the higher mobility parts are the nonphosphorylated subspecies (Fig. 1). All these data clearly indicate that CP43 apoprotein, D1, and D2 are phosphorylated. Weak labeling of the extrinsic 33 kD was sometimes observed in a precipitin arc with antibody against 33 kD (not shown), but its radioactivity could not be resolved from the radioactivity of heavily labeled D2 by one-dimensional SDS-PAGE (*e.g.* Fig. 1).

DISCUSSION

Our results demonstrate phosphorylation of PSII components, CP43 apoprotein, D1, D2, and 10 to 11 kD protein but not CP47 in spinach and pea. Similar results have been obtained for wheat (not shown). Again, the distribution of radioactivity among wheat phosphoproteins is similar to that of spinach and pea. In contrast to vascular plants, in *Chlamydomonas reinhardtii*, D1 is not phosphorylated although D2 and the CP43 apoprotein are and the CP43 apoprotein is more heavily labeled than D2. The apoprotein of CP47 is not phosphorylated in *Chlamydomonas* nor in vascular plants (7, 14). Since the phosphorylation sites of the membrane proteins seem to be restricted to extramembranous peptide regions (7, 10), the absence of D1 phosphorylation in *Chlamydomonas* may reflect a variant amino acid sequence in this alga either in N-terminal or C-terminal region which protrudes into stroma or lumen space (8).

In contrast to our finding that D1 is weakly phosphorylated, Millner *et al.* (17) recently reported that the prominently phosphorylated protein of 30 kD in pea thylakoid is D2, whereas the weakly labeled band of 33 kD is not D1. Their evidence was based on sensitivity of the phosphoproteins to digestion with the lysine-specific protease which cannot attack lysine-deficient proteins such as D1 and Cyt b_{559}. However, the weakly labeled 33 kD phosphoprotein is difficult to be monitored because it is not resolved well from the heavily labeled D2 and labeled proteolytic fragments originating from the higher mol wt proteins which assume positions in the D1 region. Also, it appears that the minor labeled band of 33 kD remains even after treatment with the highest concentration of the protease (Fig. 4, Ref. 17). As we have demonstrated, the assumption of Millner *et al.* that the weakly labeled protein is a different subspecies of phosphorylated D2 can be eliminated by monitoring D2's characteristic behavior in SDS-PAGE with and without urea and its specific reaction with antibodies.

A 10 kD phosphoprotein (equivalent to psbH gene product [23]) has been found in LHCII-containing PSII membranes capable of O_2 evolution (24), but the protein is reported to be absent in non-O_2-evolving PSII reaction center core complex (17). In this study, we have found that a PSII reaction center fraction devoid of LHCII but retaining the extrinsic 33kD protein, a putative minimal unit for O_2 evolution (15), contains this phosphoprotein. This suggests a possible correlation of this protein with O_2 evolution activity. Light-induced phosphorylation of the 10 kD protein and other PSII components may regulate the structure and/or function of PSII *in vivo*. Verification of this possibility by investigating the properties of isolated LHCII-free PSII reaction center complex under phosphorylated and nonphosphorylated conditions may be a subject of future work.

Allen and Holmes (2) have proposed a general model for explaining regulation of photosynthetic unit function by protein phosphorylation. The postulated phosphorylation of both peripheral and intermediate light-harvesting complexes, the latter of which is rather tightly associated with reaction center. Thus, it is expected that electrostatic repulsion between the two light-harvesting complexes results in decreased optical cross-section of the photosynthetic unit. In PSII, they supposed that the 10 kD phosphoprotein binds Chl and serves as the intermediate light-harvesting complex while LHCII serves as the peripheral antennae (2). However, our data indicate that CP43 is the more probable candidate for the intermediate antennae complex since it displays light-harvesting properties (18). With Triton/β-octylglucoside, we demonstrate that CP43 can be partially dissociated from PSII as a phosphoprotein that is subsequently recovered with LHCII. Phosphorylation of the other PSII components, documented here, may also be accommodated by the general model of regulation postulated by Allen and Holmes (2).

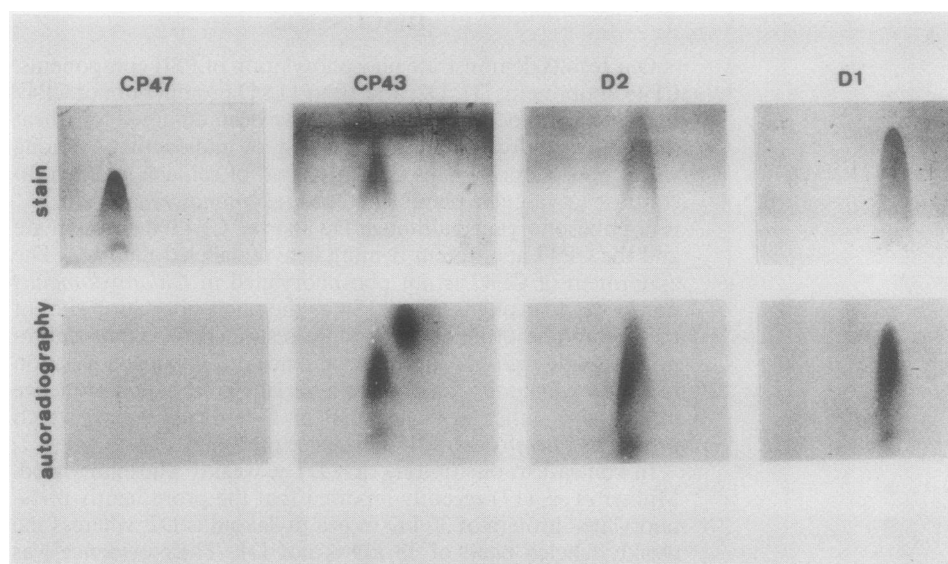

FIG. 4. Crossed immunoelectrophoresis of phosphorylated thylakoid proteins of spinach with antibodies raised against CP47, CP43, D2, and D1. The first-dimension polyacrylamide gel was electrophoresed from left to right (not shown). Upper part, dye stain; lower part, autoradiography.

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