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In Vitro Phosphorylation of Maize Leaf Phosphoenolpyruvate Carboxylase[1]

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ABSTRACT

Autoradiography of total soluble maize (*Zea mays*) leaf proteins incubated with ^{32}P-labeled adenylates and separated by denaturing electrophoresis revealed that many polypeptides were phosphorylated *in vitro* by endogenous protein kinase(s). The most intense band was at 94 to 100 kilodaltons and was observed when using either [γ-^{32}P]ATP or [β-^{32}P]ADP as the phosphate donor. This band was comprised of the subunits of both pyruvate, Pi dikinase (PPDK) and phosphoenolpyruvate carboxylase (PEPCase). PPDK activity was previously shown to be dark/light-regulated via a novel ADP-dependent phosphorylation/Pi-dependent dephosphorylation of a threonyl residue. The identity of the acid-stable 94 to 100 kilodalton band phosphorylated by ATP was established unequivocally as PEPCase by two-dimensional gel electrophoresis and immunoblotting. The phosphorylated amino acid was a serine residue, as determined by two-dimensional thin-layer electrophoresis. While the *in vitro* phosphorylation of PEPCase from illuminated maize leaves by an endogenous protein kinase resulted in a partial inactivation (~25%) of the enzyme when assayed at pH 7 and subsaturating levels of PEP, effector modulation by L-malate and glucose-6-phosphate was relatively unaffected. Changes in the aggregation state of maize PEPCase (homotetrameric native structure) were studied by nondenaturing electrophoresis and immunoblotting. Enzyme from leaves of illuminated plants dissociated upon dilution, whereas the protein from darkened tissue did not dissociate, thus indicating a physical difference between the enzyme from light- *versus* dark-adapted maize plants.

Numerous polypeptides in a leaf have been shown to undergo covalent modification by phosphorylation. Most of these phosphoproteins are thought to be associated with the thylakoid membrane (3, 19), and only a few have been identified to date. To our knowledge, only three specific soluble leaf proteins have been identified as being phosphorylated: pyruvate dehydrogenase complex (PDC[3]; [24]), PPDK (1, 9), and the large and small subunits of Rubisco (12). Phosphorylation of the mitochondrial PDC results in inactivation (24), while chloroplastic PDC is apparently not phosphorylated (8). The C_4 mesophyll chloroplast enzyme PPDK exhibits a novel ADP-dependent phosphorylation which causes inactivation (1, 6, 9). Phosphorylation/inactivation of PDC and PPDK has also been established *in situ* (6, 24). In contrast, phosphorylation of Rubisco has unknown effects on the enzyme (12). Data on hydroxymethyl glutaryl CoA reductase have indicated that it may also be phosphorylated (25). While quinate:NAD oxidoreductase has been shown to be activated by phosphorylation (23), it is not known if this enzyme occurs in leaves.

Protein phosphorylation is generally analyzed by autoradiography of SDS-PAGE gels from which the phosphorylated polypeptides are identified immunologically and/or by their mol wt. Due to the similar mol wt of the subunits of PPDK (94 kD) and PEPCase (99 kD) there have been problems of misidentification or inconclusive identification. The results described herein establish unequivocally an ATP-dependent seryl phosphorylation of maize leaf PEPCase in comparison to the previously demonstrated ADP-dependent threonyl phosphorylation of maize leaf PPDK. Both of these C_4 mesophyll-cell enzymes are considered to catalyze key reactions during C_4 photosynthesis.

MATERIALS AND METHODS

Plant Material. Maize (*Zea mays* L., cv Golden Cross Bantam) plants were grown from seed in an illuminated growth room (16-h photoperiod, 25°C day/16°C night, ~300 $\mu E \cdot m^{-2} \cdot s^{-1}$, PAR). Laminar tissue from 3- to 5-week-old plants was used for sample preparation.

Sample Preparation. Ten to 15 g (wet weight) of illuminated laminar tissue were homogenized with a Waring Blendor in 50 ml of 0.1 M Tris-HCl (pH 8.0, 20°C) buffer (chilled to 4°C) containing 10 mM $MgCl_2$, 1% (w/v) insoluble PVP, 2 mM Na_2HPO_4, 2 mM Na ascorbate and 7 mM β-mercaptoethanol. The homogenate was filtered through cheesecloth and then centrifuged for 5 min (48,000*g*, 4°C). The supernatant fluid was brought quickly to 60% saturation with ultrapure $(NH_4)_2SO_4$ (0.361 g/ml, 4°C) and the protein precipitate collected by centrifugation for 5 min. The protein pellet was dissolved in a minimal volume of 50 mM Tris-HCl (pH 8.0) containing 5 mM $MgCl_2$, 1 mM Na_2HPO_4, and 7 mM β-mercaptoethanol prior to desalting on a Sephadex G-25 (fine) column (1 × 8 cm) equilibrated with the same buffer. The first 2-ml of the protein peak was collected and clarified by centrifugation. This yielded a pale-green, concentrated soluble leaf protein sample.

Phosphorylation Assays. The desalted soluble leaf protein sample was incubated (30°C) with 0.25 mM AP_5A (to inhibit adenylate kinase activity [6, 11]), 0.75 mM [γ-^{32}P]ATP, and a phosphocreatine (4 mM)/creatine phosphokinase (10 units) ADP-scavenging system for studies of ATP-dependent phosphorylation, or with 0.25 mM AP_5A, 1 mM [β-^{32}P]ADP, and 0.2 mM

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[3] Abbreviations: PDC, pyruvate dehydrogenase complex; PPDK, pyruvate, Pi dikinase; PEP, phosphoenolpyruvate; PEPCase, PEP carboxylase; AP_5A, P^1P^5-di(adenosine-5′)pentaphosphate; IEF, isoelectric focusing; Rubisco, ribulosebisphosphate carboxylase/oxygenase.

ATP for ADP-dependent phosphorylation of PPDK. After 15 min the reaction was quenched with either an equal volume of acidic denaturing medium (0.2% [w/v] SDS, 1 M acetic acid, a trace of bromophenol blue, and 1 M β-mercaptoethanol) and neutralized with 2 N NaOH prior to one-dimensional SDS-PAGE as described by Budde *et al.* (6) or quenched in two-parts of O'Farrell's (22) lysis buffer for two-dimensional electrophoresis (20). Autoradiographs were made on Kodak X-Omat AR film utilizing two Lightning-Plus intensifying screens (Du Pont) at −80°C.

Aggregation State of Native PEPCase. Plants were placed for at least 4 h in the dark or light prior to homogenization of 2 to 5 g of laminar tissue in 50 ml of cold 0.1 M Tris-HCl (pH 8.0) buffer containing 10 mM $MgCl_2$, 2% (w/v) insoluble PVP, 25% (w/v) glycerol, and 7 mM β-mercaptoethanol. The homogenate was filtered through cheesecloth and then centrifuged for 10 min (48,000*g*, 4°C). A trace of bromophenol blue in 50% glycerol was added to the supernatant fluid and aliquots immediately electrophoresed at 4°C on a nondenaturing 5 to 10% polyacrylamide gradient gel (37.5 mM Tris-HCl [pH 8.9]) with a 4% stacking gel (62.5 mM Tris-HCl [pH 6.7]). The upper electrode buffer was comprised of 37.6 mM Tris/40 mM glycine (pH 8.9), 5 mM $MgCl_2$, and 10 mM Na thioglycolate. The lower electrode buffer contained 63 mM Tris-HCl (pH 8.9), 5 mM $MgCl_2$, and 10 mM Na thioglycolate.

Immunoblots. Western transfers and immunolocalization from SDS-PAGE gels were performed as described previously (10). For transfers following native PAGE, the gel was first incubated (25°C) for 30 min in transfer buffer (25 mM Tris/192 mM glycine [pH 8.3]) in which methanol was omitted and 0.25% (w/v) SDS plus 0.5 M β-mercaptoethanol were added. The gel was then equilibrated for 30 min in transfer buffer containing 20% (v/v) methanol and transferred to nitrocellulose. This prior denaturation of native proteins was performed since our antibodies are more sensitive and have a higher titer to the denatured antigen (*i.e.* PEPCase, PPDK) from which they were raised in rabbits. The antibodies against maize leaf PPDK and PEPCase were evaluated for any cross-reaction with purified PEPCase and PPDK, respectively, by immunoblotting following two-dimensional (urea-IEF/SDS-PAGE) electrophoresis and were found to be specific for their respective antigens.

PEPCase and Protein Assays. PEPCase activity was assayed spectrophotometrically at 340 nm and 30°C in an assay medium (1 ml, final volume) containing 50 mM Tris-HCl [pH 8.0], 5 mM $MgCl_2$, 7 mM β-mercaptoethanol, 10 units of NADH-malate dehydrogenase, 0.25 mM NADH, and 5 mM $NaHCO_3$. The reaction was initiated with 6 mM PEP unless stated otherwise. PEPCase activity was also assayed radiometrically at pH 8.0 and pH 7.0 (in 50 mM Mops) as described above except for 1 mM NADH and 20 mM $NaH^{14}CO_3$ (0.3 Ci/mol). The ^{14}C-assays were terminated after 3 min at 30°C by the addition of 0.2 ml 6 M acetic acid, and PEP-dependent acid-stable ^{14}C-dpm fixed determined by liquid scintillation spectroscopy.

Protein content was determined by a sensitive dye-binding assay (6) using crystalline BSA as the standard.

Radiochemicals. [^{14}C]$NaHCO_3$ and [γ-^{32}P]ATP were purchased from Amersham Corp. [β-^{32}P]ADP was prepared from labeled ATP as described previously (6).

RESULTS AND DISCUSSION

***In Vitro* Phosphorylation of PPDK *versus* PEPCase.** When the soluble maize leaf proteins are analyzed by one-dimensional SDS-PAGE an intense band is visualized at 94 to 100 kD by Coomassie blue staining (Fig. 1, lane B). This band is actually a doublet (Fig. 1, inset to lane B) composed of the subunits of

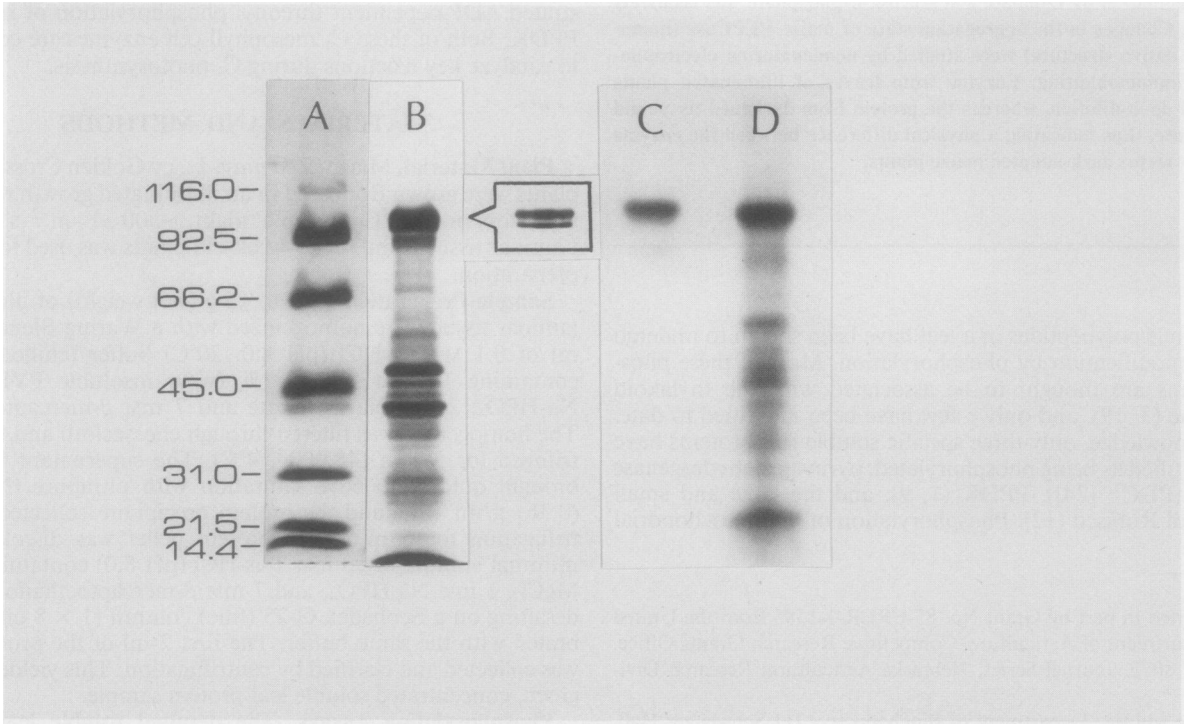

FIG. 1. ADP- *versus* ATP-dependent phosphorylation of maize leaf soluble proteins. The soluble leaf protein samples were phosphorylated *in vitro* and separated by one-dimensional SDS-PAGE (6). Lanes A and B are from gels stained with Coomassie blue R-250. Lanes C and D are autoradiographs following a 24-h exposure at −80°C. Lane A, mol wt markers (values in kD); lane B, soluble leaf proteins (inset shows the doublet at 94 to 100 kD at one-fourth the protein load); lane C, protein sample phosphorylated with [β-^{32}P]ADP (1×10^8 dpm/μmol); lane D, protein sample phosphorylated with [γ-^{32}P]ATP (1×10^8 dpm/μmol). Sample load = 40 μg protein except for the inset to lane B.

PPDK and PEPCase (see Fig. 2, A–C). Native PPDK is generally referred to as being composed of four "identical" 94-kD subunits (28), whereas PEPCase is composed of four identical 99-kD protomers (29). Low sample loads on SDS-PAGE will generally resolve the doublet at 94 to 100 kD, but these polypeptides are not separated at higher loads (>30 μg total protein).

When the soluble leaf protein sample was incubated with either [β-^{32}P]ADP plus ATP or [γ-^{32}P]ATP alone, acid-stable labeling of the 94 to 100 kD band resulted (Fig. 1, lanes C and D, respectively). The phosphorylation of PPDK by ADP is well established (1, 2, 6). The ADP-dependent regulatory phosphorylation of PPDK is absolutely dependent upon its prior phosphorylation by ATP to form the E-HIS-P catalytic intermediate (7). However, the phosphohistidine linkage is an acid-labile bond and, thus, the acid-stable ATP-dependent phosphorylation of the 94 to 100 kD band (Fig. 1, lane D) cannot be assigned to the E-HIS-P form of PPDK. Close inspection of the autoradiograph reveals that the polypeptide phosphorylated by ADP has a slightly larger relative mol wt than the polypeptide phosphorylated by ATP. This acid-stable ADP- or ATP-dependent phosphorylation of the 94 to 100 kD band was also observed with crude leaf extracts of the C_4 plant *Saccharum officinarum* (data not shown).

Maize leaves contain high levels of adenylate kinase activity which will rapidly randomize the labeled phosphate between ADP and ATP. Thus, the adenylate kinase inhibitor AP_5A (6, 11) was added to the phosphorylation reaction mixtures. For the ATP-phosphorylation reactions an ADP-scavenging system composed of phosphocreatine/creatine phosphokinase was also included. When the phosphorylation reactions were quenched for SDS-PAGE, aliquots were also analyzed by poly(ethyleneimine)cellulose-TLC (6) to assess the stability of the ^{32}P-label. These analyses indicated that approximately 7% of the label was randomized between ADP and ATP.

When the soluble leaf protein sample was incubated with ^{32}P-labeled ADP plus ATP for 15 min this resulted in a 35% inactivation of PPDK and the exclusive labeling of the 94 to 100 kD band (Fig. 1, lane C). In contrast, incubation with ^{32}P-labeled ATP had *no* effect on PPDK activity, yet there was acid-stable labeling of the 94 to 100 kD band and numerous other lower mol wt polypeptides (Fig. 1, lane D). To determine whether the ATP-dependent phosphorylation of the 94 to 100 kD band was labeling of PPDK or PEPCase, two-dimensional (urea-IEF/SDS-PAGE) gel electrophoresis was employed. Coomassie blue-stained gels revealed two intense, well separated bands in the 94 to 100 kD region (Fig. 2A, arrows). One had an isoelectric point of 5.5 to 6.0, identical to that previously reported for PPDK (1, 6), and the other was situated at the cathodic (basic) end of the urea-IEF tube gel. The identity of these two bands as PPDK and PEPCase, respectively, was verified by immunoblotting. Probing with anti-PEPCase antibodies resulted in a band with some streaking away from the cathodic end of the tube gel (Fig. 2B), whereas when probed with anti-PPDK antibodies a single band with an isoelectric point of 5 to 6 was visualized (Fig. 2C). When two-dimensional gels were used to analyze soluble leaf protein phosphorylation under identical reaction conditions as in Figure 1, the acid-stable ATP-dependent phosphorylation of the 94 to 100 kD band was localized specifically in PEPCase and not PPDK (Fig. 2D). As expected (1, 2, 6), the ADP-dependent phosphorylation resulted in the exclusive labeling of PPDK (Fig. 2E).

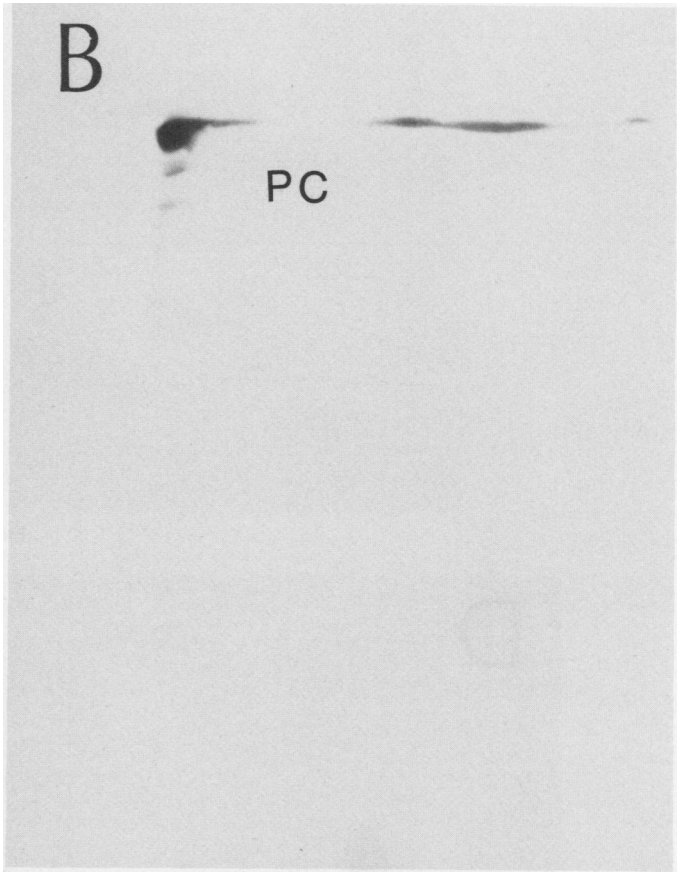

FIG. 2B

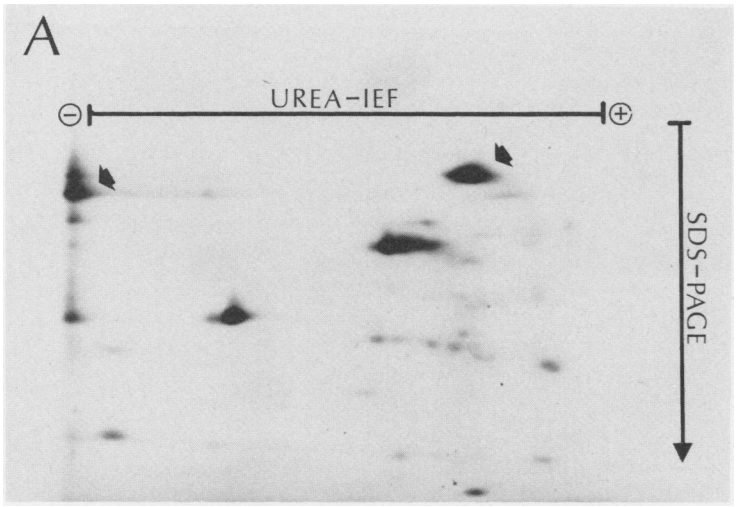

FIG. 2A

FIG. 2. Separation and identification of phosphorylated PPDK and PEPCase in the soluble leaf protein sample by two-dimensional gel electrophoresis and immunoblotting. A, A Coomassie-blue stained gel (60 μg protein load) of maize leaf soluble proteins. The arrows mark the two 94 to 100 kD polypeptides; B, localization of PEPCase (PC) by immunoblotting; C, localization of PPDK by immunoblotting (B and C: 40 μg protein loads); D, autoradiograph of soluble leaf polypeptides phosphorylated by ATP; E, autoradiograph of soluble leaf polypeptides phosphorylated by ADP (D and E: 60 μg protein loads, 48-h film exposure).

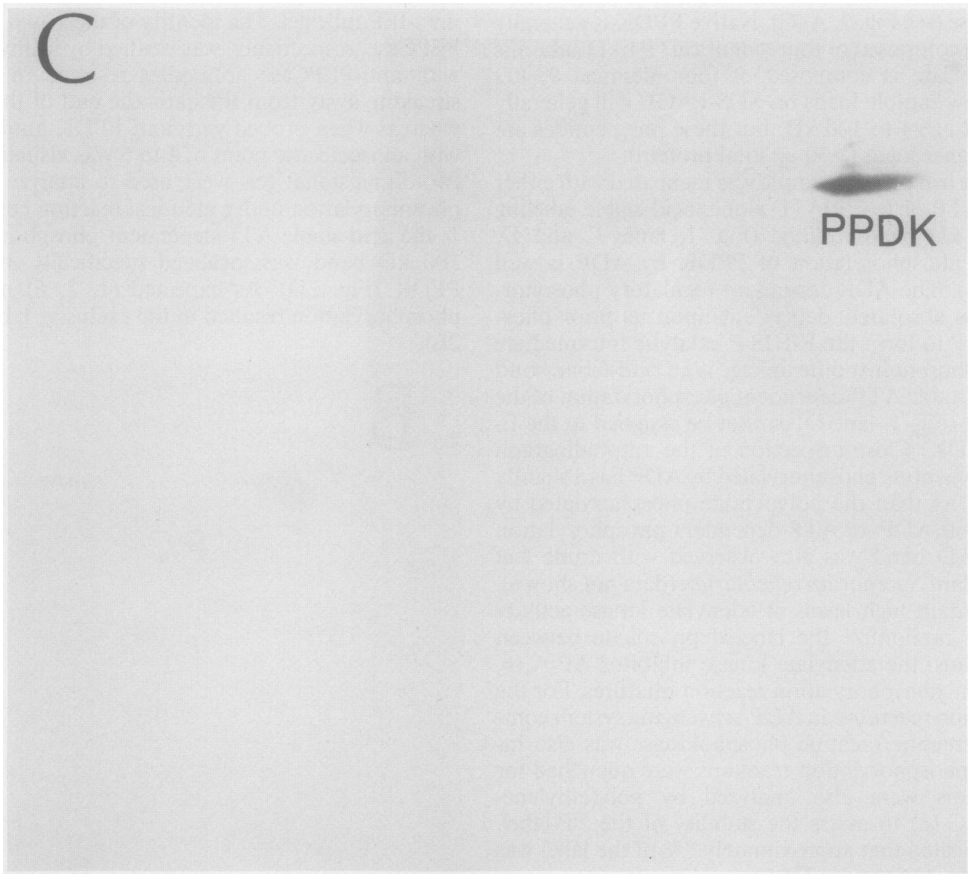

FIG. 2C

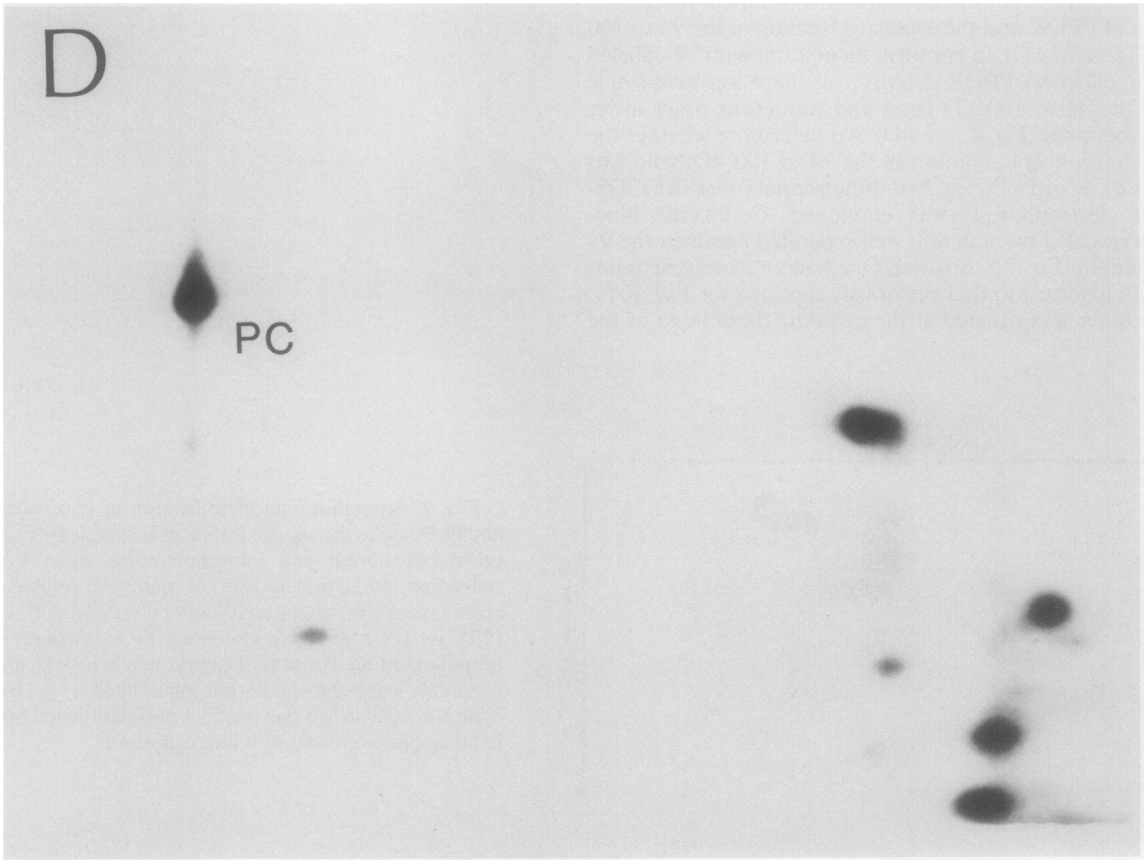

FIG. 2D

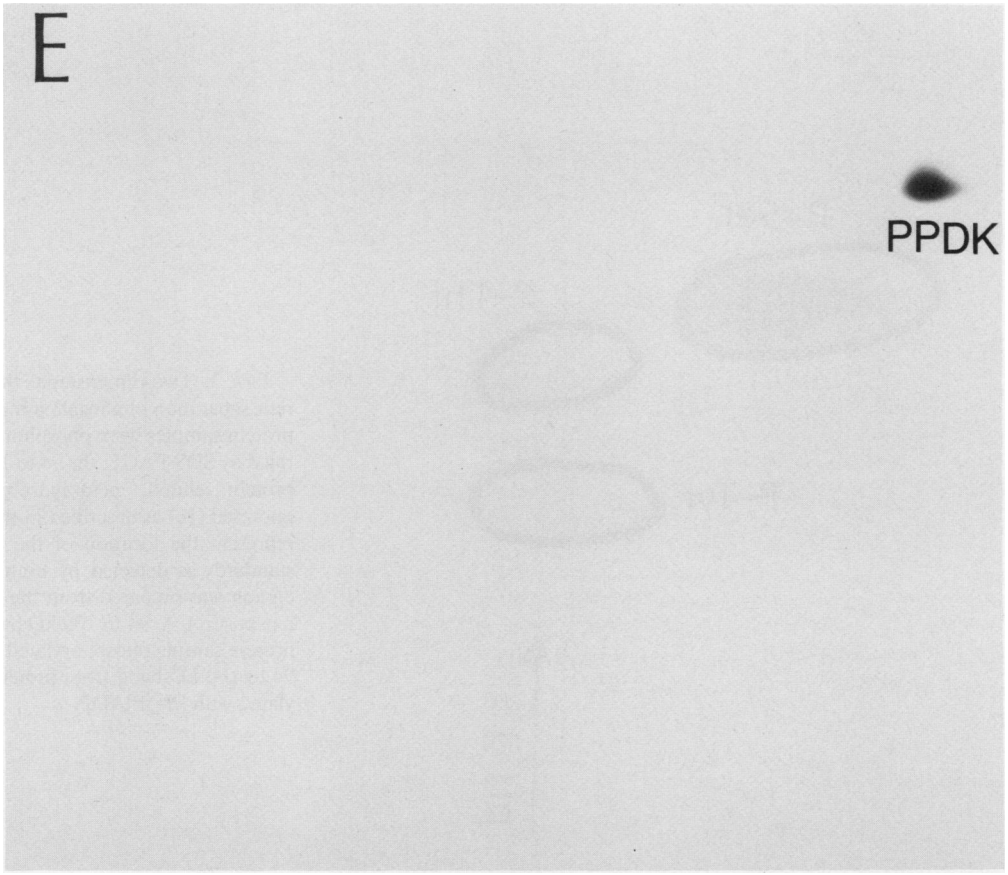

FIG. 2E

Phosphoamino Acid Analysis. Soluble leaf polypeptides from [β-^{32}P]ADP- and [γ-^{32}P]ATP-dependent protein phosphorylation reactions were separated on preparative (3 mm-thick) SDS-PAGE slab gels. The 94 to 100 kD band was excised, the protein extracted, hydrolyzed in 6 N HCl (105°C, 4 h), and phosphoamino acid analysis performed. Two-dimensional thin-layer electrophoresis (16) indicated that the ^{32}P-label from ATP-phosphorylated PEPCase was located exclusively in P-serine (Fig. 3A). As expected (1, 6), the ^{32}P-label in the ADP-phosphorylated PPDK was located specifically in P-threonine (Fig. 3B).

Effect of Phosphorylation on PEPCase Activity. The soluble leaf protein samples were incubated with [γ-^{32}P]ATP as described above except that 2 mg/ml BSA was added to help stabilize PEPCase. After incubation with the phosphorylation reaction mixture, the protein sample was diluted with 50% glycerol in desalting buffer (minus Pi) to ~1 unit of PEPCase activity (in μmol/min) per ml (determined spectrophotometrically) and aliquots assayed radiometrically. The results summarized in Table I are from one experiment and were reproducible from day to day. Incubation with ATP consistently resulted in a partial inactivation of PEPCase from illuminated tissue when measured at either saturating or nonsaturating PEP concentrations. The inhibitory effect (11–13% inactivation) of phosphorylation was about doubled when assayed at pH 7 and subsaturating levels of PEP. A similar effect of pH and PEP concentration has recently been reported by Karabourniotis *et al.* (17) in relation to the photoactivation of PEPCase in C_4 plants. Inhibition by L-malate or activation by glucose-6-P, when assayed at subsaturating levels of PEP, was not significantly affected by prior phosphorylation of PEPCase with ATP.

Aggregation State of Native PEPCase. A model of PEPCase regulation in CAM plants has recently been proposed in which the enzyme exists as a malate-sensitive homodimer during illumination of the plant and a malate-insensitive homotetramer in the dark (30). Nimmo *et al.* (21) and Brulfert *et al.* (5) have suggested that, perhaps, the diurnal changes in malate sensitivity and quaternary structure of CAM PEPCase may be due to the phosphorylation (night)/dephosphorylation (day) of both seryl and threonyl groups in the protein. To determine if there are similar changes in the aggregation state of C_4 leaf PEPCase, soluble leaf proteins from intact maize plants previously exposed to the light or dark were electrophoresed on nondenaturing gels, transferred to nitrocellulose, and probed immunologically for PEPCase. Samples from dark-adapted plants gave a single high mol wt band irregardless of protein load (Fig. 4, lanes A and B). In marked contrast, samples from illuminated plants yielded a single band of high mol wt only when at least 4 μg of protein was loaded onto the gel (Fig. 4, lane C). Lesser protein loads from illuminated tissue consistently dissociated into two bands (Fig. 4, lane D), presumably the dimeric and monomeric forms of PEPCase, under these electrophoretic conditions. These differences in dissociation properties of PEPCase from darkened *versus* illuminated leaf tissue cannot be attributed to large differences in the relative amounts of PEPCase protein in the crude leaf extracts since Huber and Sugiyama (15) have reported that illumination of dark-adapted (15 h) maize leaves caused only about a 10% increase in the protein. While the dilution of protein necessary to effect dissociation under these conditions questions if such a phenomenon would occur *in vivo*, these findings demonstrate a real physical difference between PEPCase from illuminated *versus* darkened maize plants.

CONCLUDING REMARKS

Besides C_4 leaf PPDK and NADP-malate dehydrogenase (9), the phosphorylation of maize leaf PEPCase documented in this

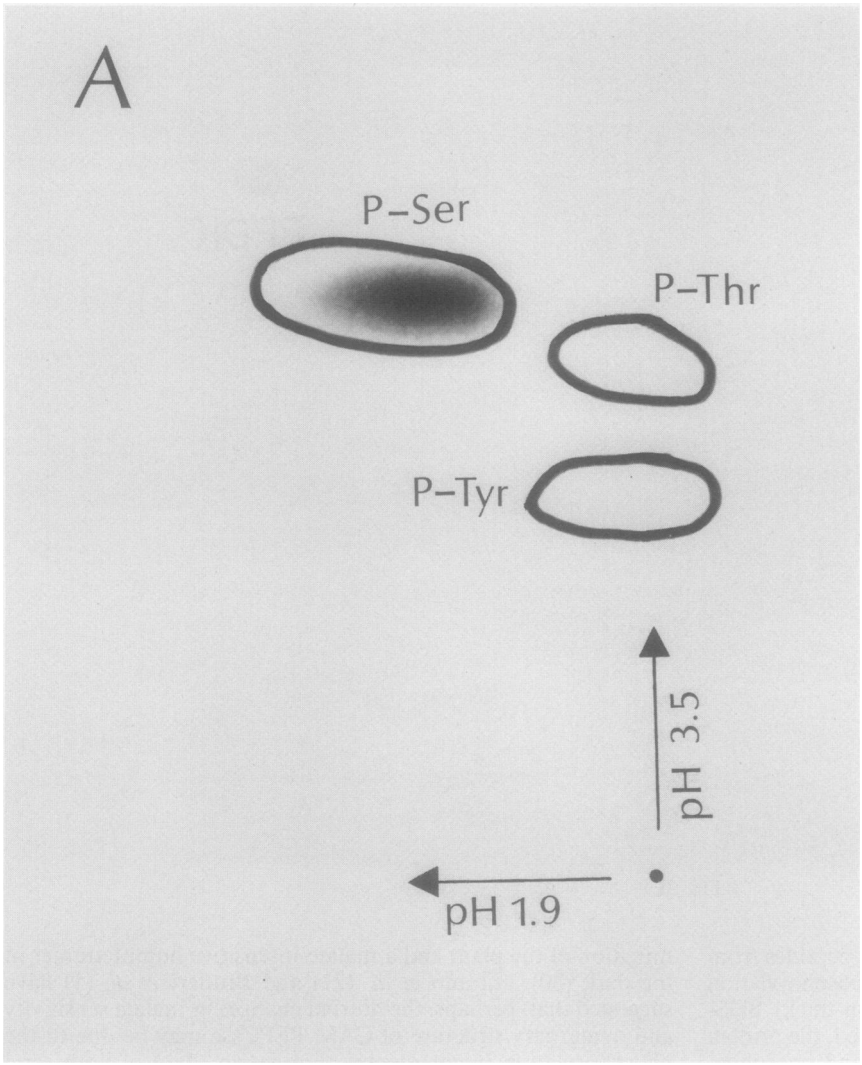

FIG. 3A

FIG. 3. Two-dimensional thin-layer electrophoretic separation of phosphoamino acids. Soluble leaf protein samples were phosphorylated *in vitro*, separated by SDS-PAGE, the 94 to 100 kD band excised, protein eluted, acid-hydrolyzed, and electrophoresed (16) as described in the text. Circled areas represent the location of the phosphoamino acid standards as detected by ninhydrin. An autoradiograph was prepared from the thin-layer plate (72-h exposure). A, 94 to 100 kD band from soluble leaf protein sample phosphorylated with [γ-^{32}P]ATP; B, 94 to 100 kD band from protein sample phosphorylated with [β-^{32}P]ADP.

report provides information on the posttranslational modification of yet another key C_4-cycle enzyme. It is now realized that protein phosphorylation is a major means of metabolic regulation. With enzymes, phosphorylation generally results in activation or inactivation. Our attempts, to date, have indicated that the *in vitro* phosphorylation of C_4 leaf PEPCase from illuminated tissue by an endogenous protein kinase does not completely inactivate the enzyme, in contrast to the situation with PPDK (1, 2, 6). This lack of complete inactivation may be due to the protein being only partially phosphorylated. Our studies have indicated that the *in vitro* phosphorylation reaction mixture allows for maximum phosphorylation within 15 to 20 min, after which the level of phosphorylation declines to a lower steady state level (data not shown). With the PPDK system it is easier to achieve complete phosphorylation/inactivation since one can experimentally manipulate the dephosphorylation step *in vitro* by omitting the nonprotein substrate, Pi (1, 2, 6).

The need for PEPCase regulation in C_4 plants is, perhaps, not as apparent as that in CAM species (30). During illumination, PEPCase is the primary carboxylating enzyme in C_4 photosynthesis and its activity may control the intercellular distribution of metabolites between the two photosynthetic cell-types (27). An intriguing question is how does PEPCase function in the illuminated C_4 mesophyll cell in the presence of 35 mM malate *in vivo* (27), given that the enzyme is inhibited by more than 75% *in vitro* by 6 mM malate? Similarly, if during periods of darkness the cytosolic PEP is utilized primarily in glycolysis by pyruvate kinase, are the differences in K_m(PEP) between PEPCase and pyruvate kinase sufficiently large to alleviate the diversion of glycolytic PEP to four-carbon dicarboxylic acids by the large amounts of PEPCase (~10–15% of the total soluble leaf protein [13]) present in the C_4 leaf cytosol? These factors suggest that PEPCase is likely a key regulatory enzyme in C_4 metabolism. Future research will hopefully provide insight into the following questions: Does phosphorylation of C_4 leaf PEPCase occur *in vivo* and if so, what is its physiological role?; Is phosphorylation of the protein related to the light-induced changes in activity and effector sensitivity of C_4 PEPCase observed by Karabourniotis *et al.* (17), and Huber and Sugiyama (15), and/or the light/dark-mediated change in aggregation state documented in this report for the C_4 enzyme and elsewhere (30) for CAM plants?

As an important sidelight, it is our opinion that the almost identical relative mol wt of the PPDK and PEPCase monomers on SDS-PAGE has resulted in the incomplete identification of this 94 to 100 kD band in several cases. A paper on maize PEPCase (13) illustrates this point, in which the protein was purified in a manner in which PPDK would not be separated entirely from PEPCase (28). The final product on one-dimensional SDS-PAGE, a doublet at low protein loads, was used to make antibodies for further studies of PEPCase during the greening of maize leaves (26). Similarly, others (4) have used the excised 94 to 100 kD band from mesophyll protoplast extracts

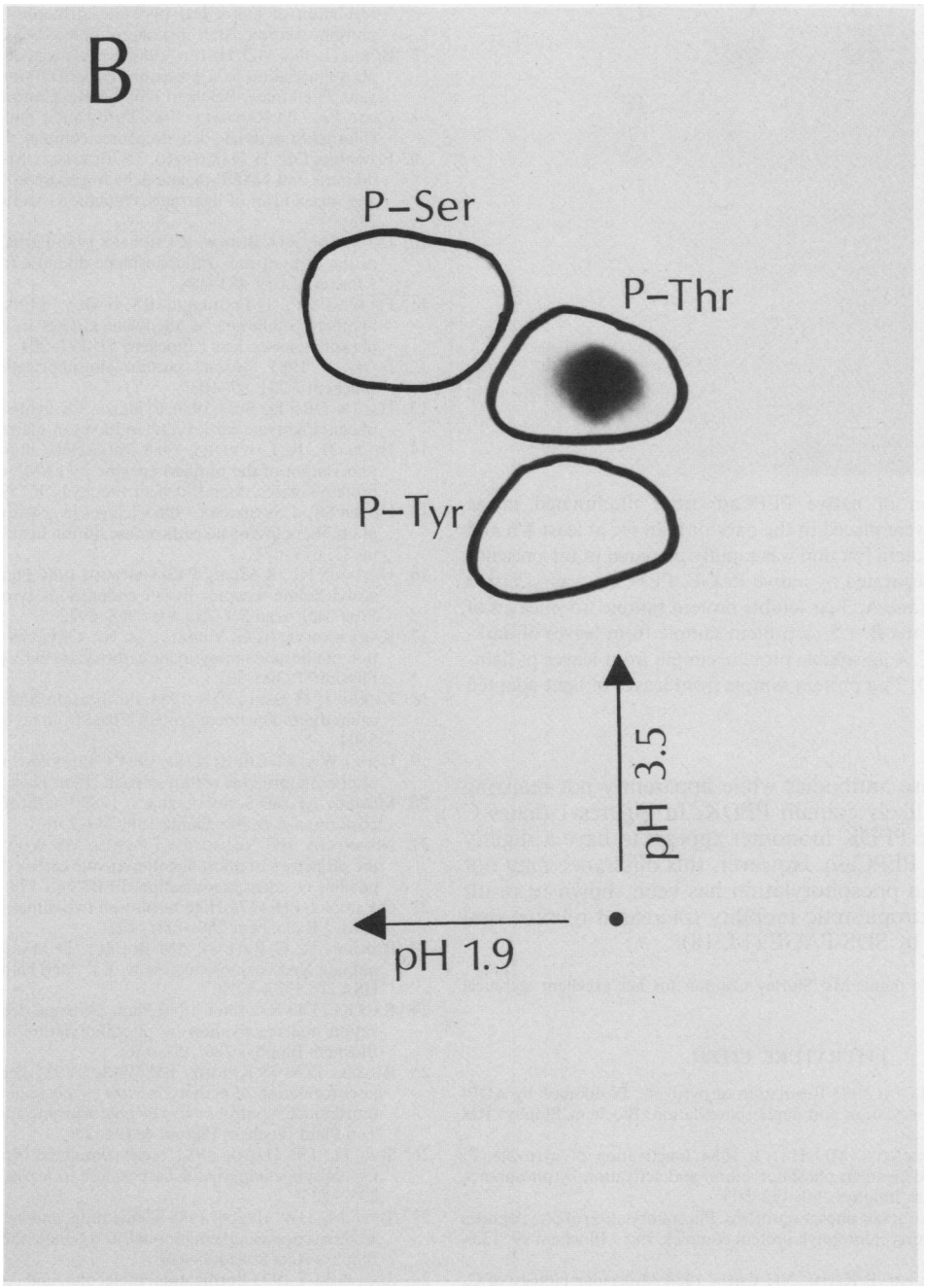

FIG. 3B

Table I. *Effect of Phosphorylation by an Endogenous Protein Kinase on the Activity and Regulatory Properties of PEPCase Extracted from Illuminated Tissue and Assayed at pH 7 and pH 8*

Addition	+0.75 mM ATP		−ATP	
	pH 7	pH 8	pH 7	pH 8
	μmol/min·mg protein			
6 mM PEP	1.47 ± 0.07[a]	1.23 ± 0.01	1.68 ± 0.07	1.39 ± 0.18
1 mM PEP	0.19 ± 0.007	0.85 ± 0.058	0.26 ± 0.024	0.95 ± 0.044
+ 2 mM glucose-6-P	2.15 ± 0.08	2.91 ± 0.06	2.38 ± 0.04	2.57 ± 0.22
+ 6 mM L-malate	0.002 ± 0.0015	ND[b]	0.006 ± 0.0002	ND

[a] The results represent the mean ($n = 4$), ± SD, PEPCase specific activity. [b] Not determined.

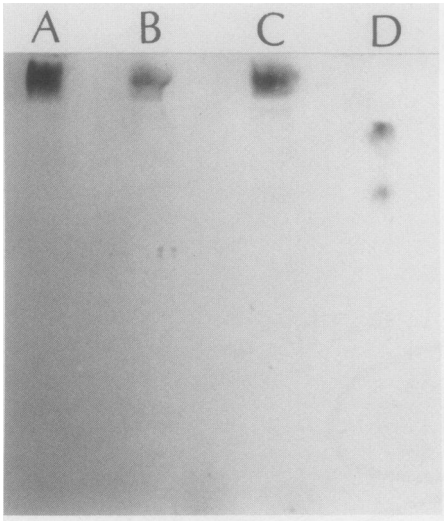

FIG. 4. Dissociation of native PEPCase from illuminated maize leaves. Whole plants were placed in the dark or light for at least 4 h and a crude soluble leaf protein fraction was rapidly prepared in the presence of 25% glycerol and separated by native PAGE. PEPCase was localized by immunoblotting. Lane A, 3 μg soluble protein sample from leaves of dark-adapted plants; lane B, 1.5 μg protein sample from leaves of dark-adapted plants; lane C, 4 μg soluble protein sample from leaves of light-adapted plants; lane D, 2 μg protein sample from leaves of light-adapted plants.

for making PEPCase antibodies while apparently not realizing that it would also likely contain PPDK. In Figures 1 (lanes C and D) and 2A, the PPDK monomer appears to have a slightly larger mol wt than PEPCase. However, this difference may not be real since protein phosphorylation has been shown to result in a decreased electrophoretic mobility (increased relative mol wt) when analyzed by SDS-PAGE (14, 18).

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