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In Vivo Regulation of Wheat-Leaf Phospho*enol*pyruvate Carboxylase by Reversible Phosphorylation[1]

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Regulation of C_3 phospho*enol*pyruvate carboxylase (PEPC) and its protein-serine/threonine kinase (PEPC-PK) was studied in wheat (*Triticum aestivum*) leaves that were excised from low-N-grown seedlings and subsequently illuminated and/or supplied with 40 mM KNO_3. The apparent phosphorylation status of PEPC was assessed by its sensitivity to L-malate inhibition at suboptimal assay conditions, and the activity state of PEPC-PK was determined by the in vitro ^{32}P labeling of purified maize dephospho-PEPC by [γ-^{32}P]ATP/Mg. Illumination ($\pm NO_3^-$) for 1 h led to about a 4.5-fold increase in the 50% inhibition constant for L-malate, which was reversed by placing the illuminated detached leaves in darkness (minus NO_3^-). A 1-h exposure of excised leaves to light, KNO_3, or both resulted in relative PEPC-PK activities of 205, 119, and 659%, respectively, of the dark/0 mM KNO_3 control tissue. In contrast, almost no activity was observed when a recombinant sorghum phosphorylation-site mutant (S8D) form of PEPC was used as protein substrate in PEPC-PK assays of the light plus KNO_3 leaf extracts. In vivo labeling of wheat-leaf PEPC by feeding ^{32}P-labeled orthophosphate showed that PEPC from light plus KNO_3 tissue was substantially more phosphorylated than the enzyme in the dark minus-nitrate immunoprecipitates. Immunoblot analysis indicated that no changes in relative PEPC-protein amount occurred within 1 h for any of the treatments. Thus, C_3 PEPC activity in these detached wheat leaves appears to be regulated by phosphorylation of a serine residue near the protein's N terminus by a Ca^{2+}-independent protein kinase in response to a complex interaction in vivo between light and N.

An important area of research in the field of leaf metabolism involves the interactions between carbon and nitrogen assimilation in the chloroplast stroma and the cytosol (Huppe and Turpin, 1994). It is probable that these pathways are highly coordinated and involve several steps of reciprocal control (Robinson, 1988; Huppe and Turpin, 1994). It has been shown in C_3 leaves that three likely targets of such control are the reactions catalyzed by cytosolic SPS, NR, and PEPC. The modulation of the activities of these three enzymes is believed to contribute to the integration of nitrogen assimilation, carbon fixation, and carbon partitioning (Champigny and Foyer, 1992; Huber et al., 1992a, 1992b, 1992c, 1994). The activities of all three target enzymes respond to light/dark transitions and appear to be modulated by regulatory protein phosphorylation in response to light, N, or both (Sicher and Kremer, 1984; Van Quy et al., 1991a; Huber et al., 1992a, 1992b, 1992c, 1994). Therefore, it has been suggested that the requisite protein kinases and protein phosphatases that regulate PEPC, SPS, and NR function to coordinate these major metabolic pathways in the C_3-leaf cytosol (Huber et al., 1994).

The posttranslational regulation of PEPC in C_4 and CAM leaves has been extensively studied and recently reviewed (Leegood and Osmond, 1990; Jiao and Chollet, 1991; Nimmo, 1993; Huber et al., 1994; Lepiniec et al., 1994). It consists of an interaction between this allosteric enzyme and its negative (L-malate) and positive (G6P, triose-P) effectors and a complex regulatory phosphorylation cycle that modulates several important kinetic parameters of PEPC, most notably K_i (L-malate) and K_a (G6P) (Duff et al., 1995). In contrast, relatively little is known about the possible posttranslational regulation of the nonphotosynthetic C_3 enzyme, although it too is subject to allosteric control (Outlaw, 1990; Schuller et al., 1990a, 1990b; Wang et al., 1994). However, only a single report has appeared dealing directly with the in vivo phosphorylation of the C_3-leaf enzyme (Van Quy et al., 1991a). It was reported recently that a Ca^{2+}-independent protein-Ser/Thr kinase exists in tobacco leaves that is capable of phosphorylating purified maize and tobacco PEPC in vitro (Wang and Chollet, 1993), but no data were presented on whether phosphorylation changed the kinetic properties of the target enzyme. However, in vitro phosphorylation of soybean-nodule PEPC and the recombinant sorghum C_3 isoform decreased the enzyme's sensitivity to L-malate (Pacquit et al., 1993; Schuller and Werner, 1993). Finally, it was also reported that guard-cell PEPC activity in closed stomata from *Vicia faba* leaves is significantly more inhibited by L-malate than the enzyme in opening stomata, thus suggesting in vivo changes in its apparent phosphorylation status (Zhang et al., 1994).

The possibility that C_3-leaf PEPC is regulated by reversible phosphorylation has important ramifications with regard to the interactions between C and N metabolism by

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Abbreviations: CHX, cycloheximide; G6P, Glc-6-P; $I_{0.5}$, 50% inhibition constant; MV, methylviologen; NR, nitrate reductase; PEPC, PEP carboxylase; PEPC-PK, PEPC protein-Ser/Thr kinase; SPS, Suc-P synthase.

virtue of the enzyme's provision of C skeletons for N assimilation into amino acids. Thus, in the present study we have initiated an evaluation of such a possibility using excised leaves from low-N-grown wheat (*Triticum aestivum*) seedlings. We have assessed the apparent phosphorylation status (sensitivity to L-malate), specific activity, protein amount, and phosphorylation state (in vivo labeling of PEPC with [^{32}P]Pi) of PEPC and the activity of PEPC-kinase in these detached leaves during light-dark transitions and/or low-high nitrate feedings.

MATERIALS AND METHODS

Reagents

All biochemicals were purchased from Sigma except as noted. [^{32}P]Pi and [γ-^{32}P]ATP were from Amersham and microcystin-LR was from Calbiochem. Immunochemicals were obtained from Bio-Rad. Rabbit polyclonal antibody against the immunopurified sorghum-leaf PEPC holoenzyme was a generous gift from Dr. Jean Vidal (Université de Paris-Sud, Orsay, France).

Plant Material and Experimental Treatments

Low-N-grown wheat (*Triticum aestivum* L. cv Brule) plants were germinated and grown in vermiculite following the N regimen described by Van Quy et al. (1991a) at a PPFD of 150 μmol photons m^{-2} s^{-1}, a light/dark temperature cycle of 23/21°C, and a photoperiod of 13.5 h. The N regimen consisted of a 7-d germination period with deionized water, a subsequent 11-d period with 0.5 mM KNO_3 added to N-free nutrient solution (Van Quy et al., 1991a), followed by a 2-d treatment with only the N-free nutrient solution. These low-N-grown plants were then placed in darkness for 16 h, after which the various treatments were initiated. High-N-grown wheat plants were propagated following the same protocol (Van Quy et al., 1991a) but with daily 10 mM KNO_3 for 20 d and without the initial 7-d and final 2-d treatments with 0 mM KNO_3. At the end of the 16-h dark treatment all four leaves of the 20-d-old low-N- and high-N-grown seedlings were detached under water and transferred to a 500-mL beaker containing either N-free nutrient solution (−N) or nutrient solution containing 40 mM KNO_3 (+N). The excised leaves were then immediately either illuminated at a PPFD of 800 to 1000 μmol photons m^{-2} s^{-1} (L) or placed in darkness (D) for the indicated period of time at 25°C. For CHX or MV pretreatment of the detached, darkened wheat leaves the method of Li and Chollet (1993) was used. At the end of the various treatment periods the excised leaves were quickly frozen in liquid N_2, pulverized, and stored at −80°C until used (never more than 4 d after treatment).

Maize (*Zea mays* L. cv Golden Bantam) plants were obtained from a local greenhouse.

Enzyme Extractions and Assays

All steps in the extraction or processing of leaf-protein samples were carried out at 4°C. All buffers were pH adjusted and degassed at room temperature. Samples (1–2 g fresh weight) from excised wheat leaves extracted for PEPC-activity measurements, soluble-protein determinations, PEPC immunoblotting, immunoprecipitation of in vivo ^{32}P-labeled PEPC, or nitrate measurements were ground with sand in a chilled mortar and pestle at a 1:4 (w/v) tissue:buffer ratio using PEPC-extraction buffer (100 mM Tris-HCl, pH 8.0, 5 mM L-malate, 5 mM NaF, 1 mM fresh PMSF, 10 μg/mL chymostatin, 10 μg/mL leupeptin, 14 mM 2-mercaptoethanol, 1 μM microcystin-LR, 5% [v/v] glycerol, 1.5% [w/v] insoluble PVP). The homogenates were clarified by centrifugation at 24,000*g* for 5 min. For PEPC-activity measurements and some protein determinations, protein in aliquots of the clarified crude homogenates was precipitated by the addition of ultrapure ammonium sulfate to 60% saturation and allowed to remain for 10 min at 4°C. After centrifugation for 10 min at 24,000*g* the pelleted protein sample was resuspended in 100 to 500 μL of PEPC-PK extraction buffer (50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM EDTA, 5% [v/v] glycerol, 14 mM 2-mercaptoethanol, 1 mM fresh PMSF, 10 μg/mL chymostatin). Following clarification by centrifugation, 50-μL aliquots were desalted against this same buffer medium using a 0.5-mL Sephadex G-25 spin column (International Biotechnologies, Inc., New Haven, CT). PEPC-activity assays or protein measurements were performed immediately. Leaf samples extracted for PEPC-PK assays were ground with sand in a chilled mortar and pestle at a 1:4 (w/v) ratio with PEPC-PK extraction buffer plus 1.5% (w/v) insoluble PVP. The samples were then processed as described above for PEPC-activity measurements.

PEPC-Activity Assays, Kinetic Analysis, and Soluble-Protein Determination

All PEPC reactions were initiated with an aliquot of the concentrated and desalted protein sample and initial rates (30–90 s) were determined at 30°C at the indicated pH. PEPC activity was determined by a coupled assay using exogenous malate dehydrogenase and following the oxidation of NADH at 340 nm. All 1-mL reaction mixtures contained 50 mM Hepes-KOH (pH as indicated), 0.2 mM NADH, 10 mM $MgCl_2$, 1 mM $NaHCO_3$, 4 units of porcine-heart mitochondrial malate dehydrogenase (Sigma, No. M-2634), and the indicated concentrations of pH-adjusted PEP and L-malate. $I_{0.5}$ (L-malate) values at the indicated pH and PEP concentrations were determined from a Job plot (Job et al., 1978) using 0 to 1.5 mM L-malate. Apparent K_m (total PEP) values were evaluated from Lineweaver-Burk plots over the range of 0.04 to 1.0 mM PEP.

Soluble-protein concentration was measured by a standard dye-binding method (Bradford, 1976) using the Bio-Rad dye-reagent and BSA as the standard.

Nitrate Determination

Nitrate concentration in the crude centrifuged wheat-leaf extracts was determined by an established colorimetric method (Cataldo et al., 1975).

Electrophoresis, Electroblotting, and Western Blots

SDS-PAGE was performed using a Bio-Rad mini-gel apparatus according to an established protocol (Laemmli, 1970). Electroblotting and PEPC immunoblots were performed as previously described (Duff et al., 1991) using polyclonal rabbit antibody raised against the SDS-PAGE-purified maize-PEPC monomer (Budde and Chollet, 1986). Laser densitometry of the immunoblots and determination of wheat-leaf PEPC relative amounts were performed as described previously (Duff et al., 1991) except that calibration standards were not used and only PEPC amounts relative to total leaf soluble-protein were assessed.

PEPC Purification and Wheat-Leaf PEPC-PK Assays

Maize dark-form (dephospho) PEPC and the recombinant sorghum phosphorylation-site mutant (S8D) enzyme were purified to electrophoretic homogeneity as described by Jiao et al. (1991b) and Wang et al. (1992), respectively. In vitro phosphorylation of purified PEPC by the concentrated and desalted wheat-leaf protein samples was performed for 45 min at 30°C in a final volume of 60 μL in the presence of [γ-^{32}P]ATP/Mg (2–3 μCi; 3 Ci/mmol), 14 μg of exogenous maize or sorghum-mutant PEPC, 5 nM microcystin-LR, and 0.2 mM EGTA as previously described for maize leaf extracts (Jiao and Chollet, 1992; Li and Chollet, 1993). Following SDS-PAGE, visualization and quantitation of ^{32}P incorporation into the approximately 110-kD PEPC monomer were performed by phosphorimaging as described by Li and Chollet (1994).

In Vivo ^{32}P Labeling and Immunoprecipitation of PEPC

In vivo labeling was performed by feeding excised wheat leaves [^{32}P]Pi (500 μCi; 10 mCi/mL) exactly as described by Jiao et al. (1991b) for detached maize leaves. Indirect immunoprecipitation of the ^{32}P-labeled PEPC from the clarified leaf extracts was performed using antibodies to native sorghum PEPC and protein A-agarose (Lin et al., 1989). The immunoprecipitated pellets were washed six times with PBS (40 mM KH_2PO_4/K_2HPO_4, 150 mM NaCl, pH 7.1), resuspended in SDS-sample buffer, heated to 100°C for 2 to 5 min, and analyzed by SDS-PAGE. Visualization by phosphorimaging was performed as described by Li and Chollet (1994).

RESULTS

Kinetic Analysis of PEPC from Wheat Leaves

Since the magnitude of the increase in $I_{0.5}$ (L-malate) due to N-terminal seryl-phosphorylation of PEPC varies depending on the conditions at which the target enzyme is assayed relative to its kinetics properties (Wang and Chollet, 1993; Duff et al., 1995), we first determined K_m (total PEP) for wheat-leaf PEPC from high-N-grown plants. The apparent K_m (total PEP) values at pH 7.3 and pH 8.0 were 0.8 and 0.1 mM, respectively. $I_{0.5}$ (L-malate) values at pH 7.3 and 0.25 mM PEP (which is 2.5 times the K_m value at pH 8.0 [similar relative conditions as in Wang et al., 1992, and Wang and Chollet, 1993, for the C_4 and C_3 enzymes, respectively]) for PEPC extracted from dark- and light-adapted (3 h) leaves were 0.07 and 0.14 mM, respectively, indicating that a modest difference in apparent phosphorylation status of PEPC exists between the light- and dark-adapted N-sufficient leaves.

Apparent Phosphorylation of PEPC during Illumination and N-Status Transitions

Low-N-grown wheat leaves contained low amounts of total PEPC activity on a g fresh weight basis (approximately 0.01 μmol min^{-1} g^{-1}), making it difficult to measure specific activity and especially malate sensitivity in the 0 to 60% ammonium sulfate-concentrated and desalted extracts at the extremely suboptimal assay conditions of 0.25 mM PEP and pH 7.3. Determination of $I_{0.5}$ (L-malate) at various suboptimal pH values and PEP concentrations indicated that a suitable compromise between having adequate activity to reliably measure this parameter while maintaining substantial differences in $I_{0.5}$ (L-malate) between light-form and dark-form PEPC was to perform measurements at pH 7.5, 0.3 mM PEP. Table I summarizes the specific activity and $I_{0.5}$ (L-malate) values of PEPC at these assay conditions that was extracted from excised leaves treated with 0 (−N) or 40 mM nitrate (+N) and 0 (D) or 800 to 1000 μmol photons m^{-2} s^{-1} PPFD (L) for 1 h from both high- and low-N-grown wheat seedlings. It is clear that illumination causes significant increases in $I_{0.5}$ (L-malate) of approximately 2-fold for the high-N-grown plants and 4.5-fold for the low-N-grown plants. Because the latter plants showed a more substantial effect of illumination on the apparent phosphorylation status [i.e. $I_{0.5}$ (L-malate)] of wheat-leaf PEPC, all further experiments were performed with low-N-grown plants. Illumination for 1 h also caused about a 50% increase in PEPC specific activity in both sets of plants. However, nitrate feeding appeared to have little or no effect on $I_{0.5}$ (L-malate) or specific activity of PEPC, regardless of illumination status or growth conditions (Table I).

Table I. *Composite L-malate inhibition and specific activity values of PEPC from excised wheat leaves*

Excised Leaf Treatment[a]	Low-N-Grown[b]		High-N-Grown[c]	
	$I_{0.5}$ (L-malate)[d]	S.A.[e]	$I_{0.5}$ (L-malate)[d]	S.A.[e]
	mM	*units/mg*	*mM*	*units/mg*
Dark (D − N)	0.27	0.06	0.35	0.06
Dark + nitrate (D + N)	0.26	0.07	0.38	0.06
Light (L − N)	1.10	0.09	0.62	0.09
Light + nitrate (L + N)	1.23	0.10	0.65	0.09

[a] Treatment for 1 h at 25°C and ±800 to 1000 μmol photons m^{-2} s^{-1} and ±40 mM KNO_3. [b] Plants grown with 0.5 mM KNO_3. [c] Plants grown with 10 mM KNO_3. [d] Measured at pH 7.5, 0.3 mM PEP, 0 to 1.5 mM L-malate. [e] S.A., Specific activity, in units (μmol/min) per mg soluble protein, at pH 7.5, 0.3 mM PEP. Specific activity and $I_{0.5}$ (L-malate) values are the average of three or four separate experiments and are reproducible to within ±15% SE.

Figure 1 depicts the changes in $I_{0.5}$ (L-malate) and specific activity of PEPC from excised leaves of low-N-grown wheat seedlings as a function of treatment time. The specific activity of PEPC increased rapidly for 2 h in the light plus nitrate (L + N) and light minus nitrate (L − N) detached leaves and showed no signs of leveling off (Fig. 1A). Much smaller changes in specific activity, if any, were observed in the dark, plus or minus nitrate (D + N, D − N). The extent and rapidity of change was greatest in the L + N leaves and least in the D − N leaves. Placing the detached leaves in the control (D − N) conditions after 2 h led to a slow but significant decrease in specific activity of PEPC over the next 3 h (Fig. 1A). $I_{0.5}$ (L-malate) increased rapidly in the L + N and L − N leaves and leveled off after

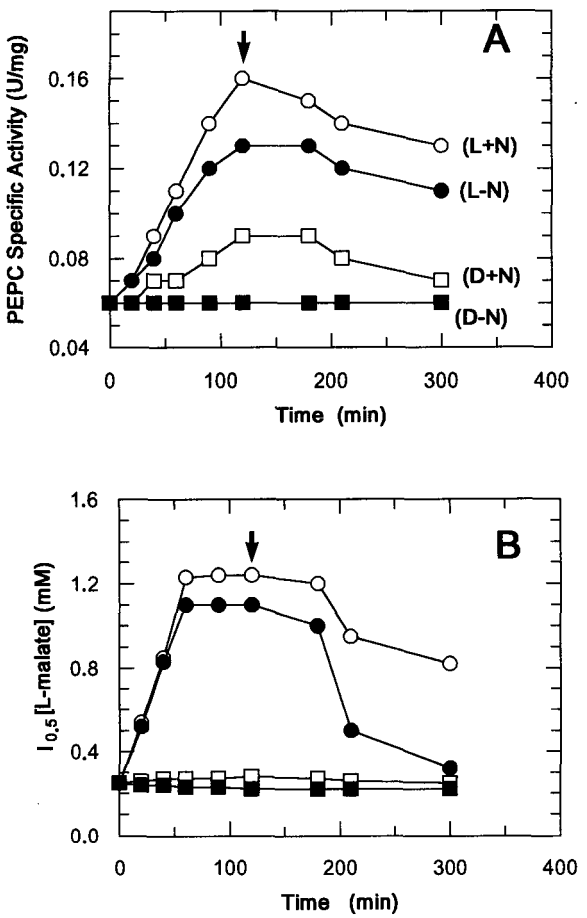

Figure 1. Time course of in vivo changes in PEPC activity and $I_{0.5}$ (L-malate) of 0 to 60% $(NH_4)_2SO_4$ concentrated and desalted extracts of excised leaves from low-N-grown wheat seedlings. PEPC specific activity (μmol min^{-1} mg^{-1} protein) (A), and $I_{0.5}$ (L-malate) (B) were measured at pH 7.5, 0.3 mM PEP, 0 to 1.5 mM L-malate (see "Materials and Methods"). The darkened, detached leaves were treated as follows: ○, 800 to 1000 μmol photons m^{-2} s^{-1} plus 40 mM KNO_3 (L + N); ●, 800 to 1000 μmol photons m^{-2} s^{-1} alone (L − N); □, darkness plus 40 mM KNO_3 (D + N); ■, darkness (D − N). The vertical arrow at 2 h indicates when the detached leaves were placed in the dark minus nitrate (D − N) control conditions. Specific activity and $I_{0.5}$ (L-malate) values were the average of three separate experiments and were reproducible to within ±15% SE and ±12% SE, respectively.

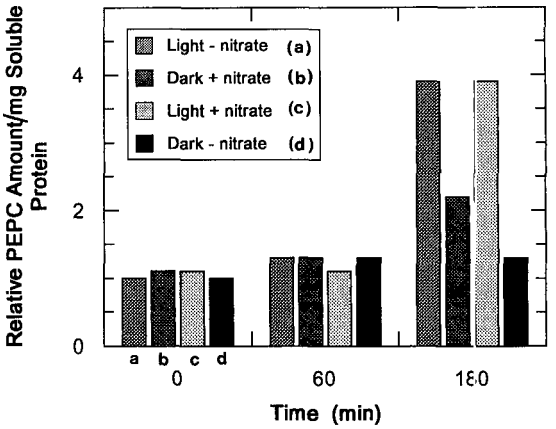

Figure 2. Time course of changes in relative PEPC-protein amounts as determined by immunoquantification on western blots by laser densitometry. The excised leaves from low-N-grown wheat plants were treated for the indicated periods of time (see legend to Fig. 1). Relative PEPC amounts were the average of two separate experiments, which did not vary by more than 12%.

1 h, whereas no significant change was observed in this regulatory property of PEPC from D − N and D + N leaves (Fig. 1B). Placing the excised leaves in the D − N control conditions after 2 h caused an almost complete reversal of the increase in the $I_{0.5}$ (L-malate) value in the L − N leaves but only a partial reversal in the L + N tissue. Since nitrate feeding appeared to have no major effect on the apparent phosphorylation status of PEPC during the first 2 h of treatment (Fig. 1B; Table I), we performed nitrate assays with crude centrifuged extracts of the D + N and D − N leaves to verify that the excised tissue was, in fact, accumulating exogenous nitrate. D + N leaves quickly accumulated nitrate to 3 times the concentration of the D − N tissue within 1 h, after which the accumulation slowed markedly (data not shown).

Synthesis of PEPC Protein during Nitrate Feeding and Illumination

Since both illumination and nitrate supply led to increases in specific activity of PEPC in leaves detached from low-N-grown plants (Fig. 1A), it was important to determine if these changes were, in fact, due to increases in the amount of PEPC protein rather than covalent modification. It is well documented that phosphorylation of C_4 PEPC causes an increase in its specific activity when the enzyme is assayed under suboptimal (but not optimal) conditions (Duff et al., 1995). Figure 2 shows amounts of PEPC protein relative to the total soluble protein extracted from excised leaves that had undergone the various light and nitrate treatments for 1 or 3 h. None of the treatments led to a significant change in relative PEPC-protein amount during the 1st h, but the L + N, L − N, and D + N detached leaves had substantial increases in PEPC-protein amount relative to the D − N control after 3 h. Thus, the increases in PEPC specific activity observed within 1 h of treatment that are depicted in Figure 1A appear not to be due to changes in

PEPC-protein amount relative to other soluble leaf proteins but rather protein phosphorylation.

In Vivo Phosphorylation of PEPC

Since decreases in the malate sensitivity of plant PEPC can be due not only to regulatory phosphorylation but also to N-terminal proteolysis of the protein (Wang et al., 1992; Lepiniec et al., 1994; Duff et al., 1995), it was imperative to document the actual in vivo phosphorylation of PEPC in the excised wheat leaves. The detached leaves were fed [^{32}P]Pi and then treated under the L + N and D − N conditions for 1 h, the time required for the maximum increase in $I_{0.5}$ (L-malate) (Fig. 1B). Figure 3A depicts the phosphorimager analysis of the in vivo-labeled and immunoprecipitated PEPC resolved by SDS-PAGE. A clearly observable [^{32}P]PEPC band is detected in the L + N sample (lane 1), which is substantially decreased in the D − N lane (lane 2). This difference clearly indicates that illumination and/or nitrate feeding causes an increase in the in vivo phosphorylation status of wheat-leaf PEPC and substantiates our decision to use the $I_{0.5}$ (L-malate) value as an indication of apparent phosphorylation state. Figure 3B is the PEPC immunoblot corresponding to the phosphorimage and documents two important points: (a) the difference in [^{32}P]PEPC labeling between the two 1-h treatments is not due to PEPC amount (see also Fig. 2); and (b) the radiolabeled band on the phosphorimage corresponding to the interface between the stacking and separating gels (Fig. 3A, see asterisk) from the D − N sample is not PEPC.

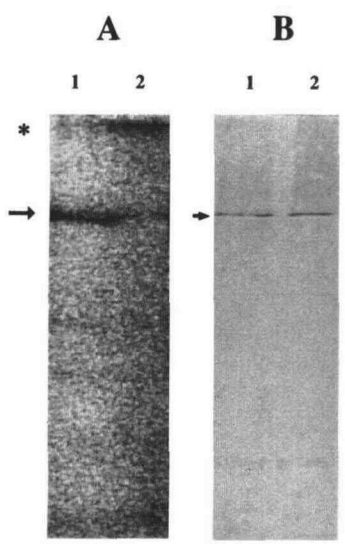

Figure 3. In vivo phosphorylation of PEPC in leaves detached from low-N-grown wheat seedlings. A, Phosphorimager analysis of immunoprecipitated PEPC following SDS-PAGE. The asterisk marks the stacking/separating gel interface. B, Corresponding western blot of immunoprecipitated PEPC. Lane 1, 1-h L + N sample (see legend to Fig. 1); lane 2, 1-h D − N sample. The ^{32}P-labeling results were representative of two separate experiments. The arrows indicate the approximately 110-kD PEPC monomer.

Table II. *Relative PEPC-kinase activity in the concentrated and desalted extracts of leaves excised from low-N-grown wheat seedlings*

Excised Leaf Treatment[a]	Relative PEPC-Kinase Activity[b]
	%
Dark (D − N)	100
Dark + nitrate (D + N)	119
Light (L − N)	205
Light + nitrate (L + N)	659
Light + nitrate (L + N)	14 (S8D)[c]
Light + nitrate (L + N) (+CHX)[d]	253
Light + nitrate (L + N) (+MV)[d]	12

[a] Except where noted below, all treatments were for 1 h (see the legend to Table I for further details). [b] Relative PEPC-PK activity was measured by in vitro phosphorylation of exogenous maize-leaf or recombinant sorghum phosphorylation-site mutant (S8D) PEPC by [γ-^{32}P]ATP/Mg, and subsequent resolution by SDS-PAGE followed by phosphorimager analysis (see "Materials and Methods"). All 60-μL phosphorylation assays contained 0.2 mM EGTA, 5 nM microcystin-LR, and, except where noted otherwise, maize dephospho-PEPC (0.23 mg/mL) as the exogenous protein substrate. All values correspond to the ^{32}P labeling of the approximately 110-kD PEPC monomer relative to the D − N control sample (100%) and are the average of at least two separate experiments, which did not vary by more than 15%. [c] Mutant PEPC (0.23 mg/mL) used as exogenous protein substrate. [d] Detached leaves were first pretreated with 5 μM CHX or 0.2 mM MV for 4 h in the dark (D − N) and subsequently transferred to L + N conditions for 1 h.

Regulation of PEPC-PK Activity by Light and Nitrate Feeding

Table II summarizes the results from the in vitro phosphorylation of maize dephospho-PEPC (or the recombinant sorghum S8D mutant enzyme) by the concentrated and desalted extracts from excised wheat leaves that had undergone the various experimental treatments for 1 h. Although nitrate feeding alone (D + N) had little effect on the relative PEPC-kinase activity (119% D − N control), 1 h of illumination (L − N) substantially increased PEPC-PK activity (205% D − N control). Somewhat surprisingly (cf. Fig. 1B), a further substantial increase in kinase activity was observed with the L + N sample (659% D − N control). Pretreatment of the excised leaves in the dark with 5 μM CHX for 4 h caused a substantial reduction in the subsequent L + N-induced PEPC-kinase activity, and pretreatment with 0.2 mM MV caused an almost complete elimination of detectable PEPC-PK activity (12% D − N control). There was also a striking effect of using the recombinant phosphorylation-site mutant (S8D) PEPC as exogenous substrate for wheat PEPC-kinase. Not only was the in vitro phosphorylation reaction specific for the regulatory Ser residue near the N terminus of plant PEPC (less than 3% of the corresponding L + N control sample when the mutant substrate was employed), but it was also Ca^{2+} independent given the presence of 0.2 mM EGTA in the routine PEPC-PK assay medium.

DISCUSSION

The results obtained in this study clearly establish the existence of the regulatory phosphorylation of C_3 PEPC by a protein-Ser/Thr kinase in N-deficient wheat leaves. This covalent modification likely occurs near the protein's N terminus at a target Ser residue homologous to Ser^8 in sorghum PEPC or Ser^{15} in the maize enzyme (Jiao et al., 1991b; Lepiniec et al., 1994). Three previous studies have presented results suggesting that C_3-leaf PEPC is regulated by reversible phosphorylation in vivo (Van Quy et al., 1991a; Van Quy and Champigny, 1992; Manh et al., 1993). These prior studies, however, relied primarily on measurements of PEPC activity at near-optimal (approximately V_{max}) conditions (3.25 mM PEP, pH 7.6) to provide indirect evidence of in vivo or in vitro changes in apparent phosphorylation status of PEPC. In contrast, little attempt was made to differentiate between the several factors that are known to cause changes in malate sensitivity (N-terminal proteolysis versus phosphorylation) and specific activity (phosphorylation versus enzyme amount) of C_4 PEPC (Wang et al., 1992; Lepiniec et al., 1994; Duff et al., 1995). It is also established that assay of C_4 PEPC specific activity or $I_{0.5}$ (L-malate) under near-optimal conditions is a poor indicator of its phosphorylation state, since under these conditions there are little or no differences in V_{max}, K_m (total PEP), K_a (G6P), and K_i (L-malate) between the phospho and dephospho enzyme forms (Wang et al., 1992; Duff et al., 1995). One study of wheat-leaf PEPC did report changes in apparent phosphorylation status [$I_{0.5}$ (L-malate)] and actual in vivo phosphorylation in response to light and N, although the $I_{0.5}$ values were measured under near-optimal conditions (Van Quy et al., 1991a). In a related report, ATP/Mg-dependent changes in PEPC specific activity measured under near-optimal conditions were used to indicate in vitro PEPC-PK activity, but changes in neither $I_{0.5}$ (L-malate) nor PEPC amount were assessed (Manh et al., 1993).

Throughout this investigation we have chosen $I_{0.5}$ (L-malate) at 0.3 mM PEP (3 times K_m, at pH 8.0) and pH 7.5 as a marker for apparent changes in the in vivo phosphorylation state of C_3-leaf PEPC from low-N-grown wheat plants. We have shown that when assayed under these suboptimal conditions light increases the $I_{0.5}$ (apparent phosphorylation status) and activity of PEPC in vivo, but that nitrate feeding has little or no major effect (Table I; Fig. 1); however, at least some of the increased activity is caused by an increase in the relative amount of PEPC protein (Fig. 2). We have also observed that treatment with light plus nitrate causes an increase in the actual phosphorylation state of the target enzyme in vivo (Fig. 3A) and that light and light plus nitrate supply lead to increases in activity of a Ca^{2+}-independent PEPC-kinase that modifies the target Ser residue near the N terminus exclusively (Table II).

There appear to be several similarities between the regulatory phosphorylation of C_3- and C_4-leaf PEPC. For example, in both cases (a) PEPC-kinase and thereby its target enzyme are activated by light in vivo in a MV-sensitive manner (Fig. 1; Table II) (Jiao and Chollet, 1991, 1992; Li and Chollet, 1993); (b) PEPC-PK is a Ca^{2+}-independent protein-Ser/Thr kinase that phosphorylates PEPC near its N terminus and thereby decreases the malate sensitivity of this allosteric target enzyme (Fig. 1B; Table II) (Jiao and Chollet, 1991; Li and Chollet, 1993; Wang and Chollet, 1993); and (c) the activity of PEPC-kinase is sensitive to CHX pretreatment in vivo (Table II) (Jiao et al., 1991a; Li and Chollet, 1993). It is also notable that although not completely understood at this time, there is a definite effect of leaf nitrate status on the regulatory phosphorylation of C_3 PEPC on at least three levels, including (a) changes in $I_{0.5}$ (L-malate) are attenuated considerably in high-N-grown plants (Table I), (b) nitrate feeding appears to affect the reversibility of the light-induced increase in $I_{0.5}$ (Fig. 1B), and (c) in illuminated leaves nitrate feeding appears to have a major effect on the activity of PEPC-PK (Table II). Another notable observation is that illumination, with or without nitrate feeding, also causes an increase in relative PEPC-protein amount in excised wheat leaves over the long term (i.e. 3 h; Fig. 2).

The major difference between the present and related previous results (Van Quy et al., 1991a; Manh et al., 1993) is that we were unable to observe much of a short-term (1–2 h) effect of nitrate feeding on the activity and malate sensitivity (apparent phosphorylation status) of C_3 PEPC in either illuminated or darkened wheat leaves detached from low-N-grown plants (Table I; Fig. 1). In contrast, a rather dramatic effect on PEPC-PK activity was observed with illuminated excised leaves fed exogenous nitrate (Table II). Why this should be the case is unclear. The overall phosphorylation state of C_3 PEPC must be the result of the competing activities of PEPC-kinase and a heretofore uninvestigated PEPC-protein phosphatase. Therefore, the simplest explanation is that a concomitant increase in PEPC-phosphatase activity accounts for the observed anomaly between PEPC-kinase activity (measured in the presence of microcystin-LR) and the apparent phosphorylation state of PEPC in the L + N versus L − N leaves. It is also possible that despite the striking similarities to the C_4 system described above, PEPC is phosphorylated in vitro by more than one protein kinase in these wheat-leaf extracts, although only one of them is the in vivo PEPC-kinase. Finally, the smaller increase in PEPC-PK activity observed in the excised L − N leaves (2-fold) may be sufficient to completely phosphorylate the relatively low levels of PEPC-protein in the nitrate-deficient tissue, whereas the higher level of kinase activity in the L + N leaves (6.6-fold) would still be only saturating.

Since SPS, NR, and C_3 PEPC are all regulated by reversible phosphorylation and are all key enzymes in leaf N and/or C metabolism, it has been suggested that the protein kinases and protein phosphatases that modify these target enzymes function in the coordination of these major metabolic pathways in the cytosol (Champigny and Foyer, 1992; Huber et al., 1994). Van Quy et al. (1991a, 1991b) and Champigny and Foyer (1992) hypothesized that in illuminated C_3 seedlings, nitrate or a downstream product of its assimilation would activate PEPC and inactivate SPS by modulating the respective protein kinase/phosphatase ac-

tivity ratios to favor increased phosphorylation of both enzymes in order to redirect carbon flow away from Suc synthesis and toward amino-acid biosynthesis. It has been shown that when nitrate is supplied to excised, N-deficient wheat leaves, C-assimilates are diverted from Suc to organic acids and amino acids (Van Quy et al., 1991b; Manh et al., 1993), thus indicating that since N and C assimilation are competitive, regulation of the two pathways must exist. Although the initial signals that activate and/or deactivate these protein kinases and protein phosphatases are not known, obvious candidates are the end products of C and N metabolism (Manh et al., 1993; Huber et al., 1994). Along these lines, it has been suggested recently that Gln and Glu are positive and negative regulatory effectors of C_3 PEPC-kinase, respectively (Manh et al., 1993), and that an increase in endogenous Gln could lead to the reciprocal modulation of SPS and PEPC activity by changes in phosphorylation state (Foyer et al., 1994). Vanlerberghe et al. (1990) reported that Gln and Glu play a key role in regulating PEPC activity in a green alga, but this may simply be due to an allosteric effect on the enzyme (Schuller et al., 1990a). It has also been shown that Gln induces the accumulation of PEPC mRNA in detached C_4 and C_3 leaves (Sugiharto et al., 1992; Manh et al., 1993), although the direct effect of Gln or Glu on PEPC-PK activity in an in vitro ^{32}P-based assay has not been determined. Therefore, we included nitrate, Gln, Glu, or Asp (each at 5 mM) in our routine wheat-leaf PEPC-PK assay medium and found that PEPC-kinase activity in a concentrated and desalted L − N extract was 107, 100, 62, and 80% of the corresponding no-addition control (100%; relative activities are the average of two separate experiments, which varied by ≤15%). Similar results were obtained using D − N and L + N wheat-leaf extracts (data not shown). Therefore, neither nitrate, Gln, nor Glu has a direct and specific effect on C_3 PEPC-PK activity in vitro, although we cannot rule out an indirect effect that might occur in vivo. Although there is yet no evidence of any effect of NO_3^- supply on the protein phosphatases or kinases that act on SPS or NR, there does seem to be a major effect of NO_3^- feeding on PEPC-PK activity in illuminated, N-deficient wheat leaves (Table II). Regulatory phosphorylation of PEPC appears to be more complicated in C_3 plants, and therefore future studies must address both the signal transduction pathway and the initial (and direct?) signal(s) involved.

In conclusion, PEPC in C_3 leaves excised from low-N-grown wheat plants is regulated in the short term by reversible protein phosphorylation and in the long term by de novo synthesis in response to changes in illumination and nitrate supply. This regulatory phosphorylation of C_3 PEPC is catalyzed by a Ca^{2+}-independent protein-Ser/Thr kinase and most probably occurs at the invariant Ser residue near the N terminus of the plant protein. Like PEPC-kinase in C_4 leaves, the activity of C_3 PEPC-PK is up-regulated in vivo by illumination (although most especially in the presence of nitrate) and down-regulated by inhibition of either cytosolic protein synthesis (+CHX) or photosynthetic electron transport from H_2O to NADP (+MV).

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LITERATURE CITED

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:** 248–254

Budde RJA, Chollet R (1986) *In vitro* phosphorylation of maize leaf phospho*enol*pyruvate carboxylase. Plant Physiol **82:** 1107–1114

Cataldo DA, Haroon M, Schrader LE, Youngs VL (1975) Rapid colorimetric determination of nitrate in plant tissue by nitration of salicylic acid. Commun Soil Sci Plant Anal **6:** 71–80

Champigny ML, Foyer C (1992) Nitrate activation of cytosolic protein kinases diverts photosynthetic carbon from sucrose to amino acid biosynthesis. Basis for a new concept. Plant Physiol **100:** 7–12

Duff SMG, Andreo CS, Pacquit V, Lepiniec L, Sarath G, Condon SA, Vidal J, Gadal P, Chollet R (1995) Kinetic analysis of the non-phosphorylated, *in vitro* phosphorylated, and phosphorylation-site mutant (Asp8) forms of intact recombinant C_4 phospho*enol*pyruvate carboxylase from sorghum. Eur J Biochem **228:** 92–95

Duff SMG, Plaxton WC, Lefebvre DD (1991) Phosphate-starvation response in plant cells: *de novo* synthesis and degradation of acid phosphatases. Proc Natl Acad Sci USA **88:** 9538–9542

Foyer CH, Noctor G, Lelandais M, Lescure JC, Valadier MH, Boutin JP, Horton P (1994) Short-term effects of nitrate, nitrite and ammonium assimilation of photosynthesis, carbon partitioning and protein phosphorylation in maize. Planta **192:** 211–220

Huber JL, Huber SC, Campbell WH, Redinbaugh MG (1992a) Reversible light/dark modulation of spinach leaf nitrate reductase activity involves protein phosphorylation. Arch Biochem Biophys **296:** 58–65

Huber SC, Huber JL, Campbell WH, Redinbaugh MG (1992b) Comparative studies of the light modulation of nitrate reductase and sucrose-phosphate synthase activities in spinach leaves. Plant Physiol **100:** 706–712

Huber SC, Huber JL, McMichael RW Jr (1994) Control of plant enzyme activity by reversible protein phosphorylation. Int Rev Cytol **149:** 47–98

Huber SC, Huber JL, McMichael RW Jr, Campbell WH, Redinbaugh MG (1992c) Regulation of cytoplasmic C- and N-metabolism by protein phosphorylation. *In* N Murata, ed, Research in Photosynthesis, Vol 3. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 675–682

Huppe HC, Turpin DH (1994) Integration of carbon and nitrogen metabolism in plant and algal cells. Annu Rev Plant Physiol Plant Mol Biol **45:** 577–607

Jiao J-A, Chollet R (1991) Posttranslational regulation of phospho*enol*pyruvate carboxylase in C_4 and Crassulacean acid metabolism plants. Plant Physiol **95:** 981–985

Jiao J-A, Chollet R (1992) Light activation of maize phospho*enol*-pyruvate carboxylase protein-serine kinase activity is inhibited by mesophyll and bundle sheath-directed photosynthesis inhibitors. Plant Physiol **98:** 152–156

Jiao J-A, Echevarría C, Vidal J, Chollet R (1991a) Protein turnover as a component in the light/dark regulation of phospho*enol*-pyruvate carboxylase protein-serine kinase activity in C_4 plants. Proc Natl Acad Sci USA **88:** 2712–2715

Jiao J-A, Vidal J, Echevarría C, Chollet R (1991b) *In vivo* regulatory phosphorylation site in C_4-leaf phospho*enol*pyruvate carboxylase from maize and sorghum. Plant Physiol **96:** 297–301

Job D, Cochet C, Dhien A, Chambaz EM (1978) A rapid method for screening inhibitor effects: determination of I_{50} and its standard deviation. Anal Biochem **84:** 68–77

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680–685

Leegood RC, Osmond CB (1990) The flux of metabolites in C_4 and CAM plants. *In* DT Dennis, DH Turpin, eds, Plant Physiology, Biochemistry and Molecular Biology. Longman Scientific and Technical, Essex, UK, pp 274–298

Lepiniec L, Vidal J, Chollet R, Gadal P, Cretin C (1994) Phospho*enol*pyruvate carboxylase: structure, regulation and evolution. Plant Sci **99:** 111–124

Li B, Chollet R (1993) Resolution and identification of C_4 phospho*enol*pyruvate-carboxylase protein-kinase polypeptides and their reversible light activation in maize leaves. Arch Biochem Biophys **307:** 416–419

Li B, Chollet R (1994) Salt induction and the partial purification/characterization of phospho*enol*pyruvate carboxylase protein-serine kinase from an inducible Crassulacean-acid-metabolism (CAM) plant, *Mesembryanthemum crystallinum* L. Arch Biochem Biophys **314:** 247–254

Lin M, Turpin DH, Plaxton WC (1989) Pyruvate kinase isozymes from the green alga, *Selenastrum minutum.* I. Purification and physical and immunological characterization. Arch Biochem Biophys **269:** 219–227

Manh CT, Bismuth E, Boutin J-P, Provot M, Champigny M-L (1993) Metabolite effectors for short-term nitrogen-dependent enhancement of phospho*enol*pyruvate carboxylase activity and decrease of net sucrose synthesis in wheat leaves. Physiol Plant **89:** 460–466

Nimmo HG (1993) The regulation of phospho*enol*pyruvate carboxylase by reversible phosphorylation. *In* NH Battey, HG Dickinson, AM Hetherington, eds, Society for Experimental Biology Seminar Series 53: Post-Translational Modifications in Plants. Cambridge University Press, London, pp 161–170

Outlaw WH Jr (1990) Kinetic properties of guard-cell phospho*enol*pyruvate carboxylase. Biochem Physiol Pflanzen **186:** 317–325

Pacquit V, Santi S, Cretin C, Bui V-L, Vidal J, Gadal P (1993) Production and properties of recombinant C_3-type phospho*enol*-pyruvate carboxylase from *Sorghum vulgare: in vitro* phosphorylation by leaf and root PyrPC protein serine kinases. Biochem Biophys Res Commun **197:** 1415–1423

Robinson JM (1988) Spinach leaf chloroplast CO_2 and NO_2^- photoassimilations do not compete for photogenerated reductant. Manipulation of reductant levels by quantum flux density titrations. Plant Physiol **88:** 1373–1380

Schuller KA, Plaxton WC, Turpin DH (1990a) Regulation of phospho*enol*pyruvate carboxylase from the green alga *Selenastrum minutum.* Plant Physiol **93:** 1303–1311

Schuller KA, Turpin DH, Plaxton WC (1990b) Metabolite regulation of partially purified soybean nodule phospho*enol*pyruvate carboxylase. Plant Physiol **94:** 1429–1435

Schuller KA, Werner D (1993) Phosphorylation of soybean (*Glycine max* L.) nodule phospho*enol*pyruvate carboxylase in vitro decreases sensitivity to inhibition by L-malate. Plant Physiol **101:** 1267–1273

Sicher RC, Kremer DF (1984) Changes of sucrose-phosphate synthase activity in barley primary leaves during light/dark transitions. Plant Physiol **76:** 910–912

Sugiharto B, Suzuki I, Burnell JN, Sugiyama T (1992) Glutamine induces the N-dependent accumulation of mRNAs encoding phospho*enol*pyruvate carboxylase and carbonic anhydrase in detached maize leaf tissue. Plant Physiol **100:** 2066–2070

Vanlerberghe GC, Schuller KA, Smith RG, Feil R, Plaxton WC, Turpin DH (1990) Relationship between NH_4^+ assimilation rate and in vivo phospho*enol*pyruvate carboxylase activity. Regulation of anaplerotic carbon flow in the green alga *Selenastrum minutum.* Plant Physiol **94:** 284–290

Van Quy L, Champigny ML (1992) NO_3^- enhances the kinase activity for phosphorylation of phospho*enol*pyruvate carboxylase and sucrose phosphate synthase proteins in wheat leaves. Evidence from the effects of mannose and okadaic acid. Plant Physiol **99:** 344–347

Van Quy L, Foyer C, Champigny ML (1991a) Effect of light and NO_3^- on wheat leaf phospho*enol*pyruvate carboxylase activity. Evidence for covalent modulation of the C_3 enzyme. Plant Physiol **97:** 1476–1482

Van Quy L, Lamaze T, Champigny ML (1991b) Short-term effects of nitrate on sucrose synthesis in wheat leaves. Planta **185:** 53–57

Wang X-C, Outlaw WH Jr, De Bedout JA, Du Z (1994) Kinetic characterization of phospho*enol*pyruvate carboxylase extracted from whole-leaf and from guard-cell protoplasts of *Vicia faba* L. (C_3 plant) with respect to tissue pre-illumination. Histochem J **26:** 152–160

Wang Y-H, Chollet R (1993) In vitro phosphorylation of purified tobacco-leaf phospho*enol*pyruvate carboxylase. FEBS Lett **328:** 215–218

Wang Y-H, Duff SMG, Lepiniec L, Crétin C, Sarath G, Condon SA, Vidal J, Gadal P, Chollet R (1992) Site-directed mutagenesis of the phosphorylatable serine (Ser^8) in C_4 phospho*enol*pyruvate carboxylase from sorghum. The effect of negative charge at position 8. J Biol Chem **267:** 16759–16762

Zhang SQ, Outlaw WH Jr, Chollet R (1994) Lessened malate inhibition of guard-cell phospho*enol*pyruvate carboxylase velocity during stomatal opening. FEBS Lett **352:** 45–48