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Effect of Light and NO_3^- on Wheat Leaf Phospho*enol*pyruvate Carboxylase Activity

Evidence for Covalent Modulation of the C_3 Enzyme

Le Van Quy, Christine Foyer, and Marie-Louise Champigny*

Photosynthèse et Métabolisme (U.R.A. 1128), Bâtiment 430, Université Paris-Sud, F-91405 Orsay cedex, France (L.V.Q., M.-L.C.); and Métabolisme et Nutrition des Plantes, Institut National de la Recherche Agronomique, F-78000 Versailles, France (C.F.)

ABSTRACT

Phospho*enol*pyruvate carboxylase (PEPcase) activity was studied in excised leaves of wheat (*Triticum aestivum* L.) in the dark and in the light, in presence of either N-free (low-NO_3^- leaves) or 40 millimolar KNO_3 (high-NO_3^- leaves) nutrient solutions. PEPcase activity increased to 2.7-fold higher than that measured in dark-adapted tissue (control) during the first 60 minutes and continued to increase more slowly to 3.8-fold that of the control. This level was reached after 200 minutes exposure of the leaves to light and high NO_3^-. In contrast, the lower rate of increase recorded for low-NO_3^- leaves ceased after 60 minutes of exposure to light at 2.3-fold the control level. The short-term NO_3^- effect increased linearly with the level of NO_3^- uptake. In immunoprecipitation experiments, the antibody concentration for PEPcase precipitation increased with the protein extracts from the different treatments in the order: control, illuminated low-NO_3^- leaves, illuminated high-NO_3^- leaves. This order also applied with regard to a decreasing sensitivity to malate and an increasing stimulation by okadaic acid (an inhibitor of P-protein phosphatases). Following these studies, ^{32}P labeling experiments were carried out *in vivo*. These showed that the light-induced change in the properties of the PEPcase was due to an alteration in the phosphorylation state of the protein and that this effect was enhanced in high-NO_3^- conditions. Based on the responses of PEPcase and sucrose phosphate synthase in wheat leaves to light and NO_3^-, an interpretation of the role of NO_3^- as either an inhibitor of P-protein phosphatase(s) or activator of protein kinase(s) is inferred. In the presence of NO_3^-, the phosphorylation state of both PEPcase and sucrose phosphate synthase is increased. This causes activation of the former enzyme and inhibition of the latter. We suggest that NO_3^- modulates the relative protein kinase/protein phosphatase ratio to favor increased phosphorylation of both enzymes in order to redirect carbon flow away from sucrose synthesis and toward amino acid synthesis.

A decrease in the rate of sucrose synthesis in illuminated wheat leaves upon transition from the low-NO_3^- to the high-NO_3^- state has recently been reported (30). This effect is observed within minutes of feeding NO_3^- to detached nitrogen-deficient leaves. The decrease in sucrose synthesis is linearly correlated with the rate of NO_3^- uptake and assimilation. The diversion of ^{14}C-labeled photosynthetic carbon away from carbohydrate synthesis toward that of organic acids and amino acids provided evidence that, in the presence of NO_3^-, the regulation of sucrose synthesis is modified to allow partitioning of C between the NO_3^- assimilation and carbohydrate biosynthesis pathways in wheat leaves (8).

Measurements of the level of intermediary metabolites and the changes of the fructose-2,6-bisphosphate concentration have revealed that the increased demand for carbon skeletons created by high rates of NO_3^- assimilation is associated with (a) a concomitant decrease in phospho*enol*pyruvate contents showing that PEPcase[1] (EC 4.1.1.31) and/or pyruvate kinase (EC 2.7.1.40) are activated, and (b) a decrease of SPS (EC 2.4.1.14) activity, which restricts the rate of sucrose synthesis (our unpublished data). Thus, it appears that in wheat leaves, light and NO_3^- might regulate the activity of two enzymes that are involved in primary (PEPcase) and secondary (SPS) carbon metabolism in an inverse fashion.

The results reported in the present paper are consistent with this hypothesis. We provide evidence that PEPcase is activated and SPS is deactivated when low-NO_3^- wheat leaves are challenged by high-NO_3^- nutrition in the light.

PEPcase has been described as the protein whose changes are most affected by N availability in leaves (26); however, the regulation of PEPcase in C_3 plants is not well understood. This is in marked contrast to the situation for PEPcase activity in C_4 species and CAM plants, in which the light effect on PEPcase activity has been described repeatedly and thoroughly studied (2). The mechanism of the light modulation of the C_4 and CAM plant PEPcase is considered to involve an interaction among the two effectors (glucose-6-phosphate and malate), phosphorylation, and aggregation/disaggregation of the enzyme-protein (13, 15, 20, 21, 27). Although it is generally sensitive to the same effectors, the PEPcase of C_3 plants is thought to need less "tight" regulation than the C_4 and CAM enzyme (18).

Because the evidence for *in vivo* leaf PEPcase activation was first obtained from a C_3 plant, the work reported here has focused on wheat leaf PEPcase with the objective of establish-

[1] Abbreviations: PEPcase, phospho*enol*pyruvate carboxylase; SPS, sucrose phosphate synthase, I_{50}, concentration of malate for 50% inhibition of the PEP case activity.

ing and characterizing the dependency of PEPcase activity on light and nitrate. Assuming that the mechanism of the activation of C_3 plant PEPcase might be similar to that of the C_4 and CAM plant enzymes, the approach undertaken to elucidate the underlying mechanisms in this study was as follows: (a) comparison of the immunological properties; (b) sensitivity of PEPcase to the effectors malate and glucose-6-P (32); (c) sensitivity to okadaic acid (an inhibitor of phosphoprotein phosphatase activity) (9); and (d) sensitivity to cycloheximide (an inhibitor of protein synthesis) in crude extracts from low-NO_3^- and high-NO_3^- wheat leaves in the light as well as from low-NO_3^- leaves sampled in the dark (control). In addition, treatment of the leaves with ^{32}Pi was performed in order to determine whether or not the wheat leaf PEPcase was modulated by reversible phosphorylation, as is the PEPcase from C_4 and CAM plants, and SPS.

The results presented here provide evidence for modulation of PEPcase by light and NO_3^- in mature wheat leaves and establish that the covalent modification of the enzyme is due to reversible phosphorylation. An interpretation of the interaction of NO_3^- with the light effect, based on the comparison of the responses of wheat leaf PEPcase and SPS, is proposed.

MATERIALS AND METHODS

Plant Material

Wheat seeds (*Triticum aestivum* L. var Fidel) were germinated on moist vermiculite in the greenhouse. After 7 d, plantlets were transferred to pots containing aerated nutrient solution. Nitrogen was given as 0.5 mM KNO_3 added to the N-free solution, which contained 0.375 mM KH_2PO_4, 0.125 mM K_2HPO_4, 0.375 mM $MgSO_4$, 0.1 mM NaCl, 1.25 mM $CaSO_4$, 10 mg/L Fe-EDTA, and the micronutrients (1), pH 6.

Treatment to Provide Low-NO_3^- and High-NO_3^- Mature Leaves

At the age of 18 d, plants had four leaves of which the youngest mature leaf was No. 3 counted from the base. Plants were transferred to the N-free solution 48 h before sampling to deplete the NO_3^- content of the tissues. Leaf No. 3 was then detached at the end of a 16 h night and placed in either 1 mL N-free nutrient solution (low-NO_3^- leaves) or nutrient solution containing KNO_3 at concentrations as indicated in the text for transition from the low-NO_3^- to the high-NO_3^- state (high-NO_3^- leaves). The leaf was then immediately illuminated or frozen in liquid nitrogen (control).

Enzyme Extraction

Leaves were sampled at intervals after start of illumination as indicated in "Results." They were immediately frozen in liquid N_2 and stored at −80°C prior to extraction. Extraction was achieved by grinding frozen leaves one by one (120–150 mg fresh weight) with liquid N_2 in a chilled mortar and resuspending the powder in 1 mL 50 mM Hepes-KOH, pH 7.4, 12 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA, 1 mM DTT, 10% glycerol, 2 mM benzamidine, 2 mM ϵ-amino-*n*-caproic acid, according to Siegl and Stitt (24). After 10 s mixing with vortex, the suspension was stored at −80°C until assay of the enzymes (less than 1 d later).

Enzyme Assays

The samples were allowed to thaw to 4°C and the extract was immediately centrifuged (16,000*g* for 2 min). The supernatant obtained in this way was desalted by centrifugal filtration at 5000*g* at 5°C with Sephadex G-25 equilibrated with the extraction buffer supplemented with 0.1% BSA, according to the method of Marques *et al.* (17).

PEPcase was immediately measured spectrophotometrically by coupling the reaction to NADH-oxidation mediated by malate dehydrogenase. The standard assay medium contained in 50 mM Tris-HCl, pH 7.6: 20 μmol $NaHCO_3$, 130 nmol NADH, 10 μmol $MgCl_2$, 5 μmol DTT, 1 unit malate dehydrogenase, 50 μL desalted enzyme solution (1 mL final volume). Reactions, at 30°C, were initiated by addition of 3.25 μmol phospho*enol*pyruvate. As indicated in the text, some reaction mixtures were supplemented with malate or glucose-6-phosphate at concentrations of 1 to 5 mM. The activity is expressed in nmol NADH oxidized/mg Chl·min, or unit, with 1 unit = 1 μmol NADH oxidized/min. Pyruvate kinase was assayed after PEPcase, by starting the reaction with addition of 0.5 mM ADP (22). SPS was assayed as in Stitt *et al.* (25), including 2 mM Fru-6-P, 10 mM Glu-6-P, 3 mM UDPG, and 5 mM Pi in the assay to differentiate between the "V_{max} assay" and the "limiting assay" (24).

Treatment of Leaves with Okadaic Acid or Cycloheximide

Low-NO_3^- leaves were detached 4 h before the dark period and placed in 1 mL N-free solution containing either 0.4 μM okadaic acid or 5 μM cycloheximide. They were allowed to absorb the inhibitors through the transpiration stream for 4 h in the light, followed by 16 h in the dark. At the end of the dark period, NO_3^- was added at final concentration 40 mM to half of the okadaic acid-treated leaves and half of the cycloheximide-treated leaves.

Immunoprecipitation

Immunoprecipitation was carried out by incubating enzyme preparations (from Sephadex G-25) with various amounts (0–100 μL) of rabbit antiserum against the *Sorghum* green-leaf PEPcase holoenzyme (4). Samples were incubated for 1 h at 4°C and then centrifuged at 16,000*g* for 2 min. The amount of enzyme activity remaining in the supernatant, after removal of the immune complex, was then determined.

Labeling with ^{32}P and Analysis of PEPcase

Detached low-NO_3^- leaves were allowed to take up carrier-free ^{32}P (33 μCi in 80 μL H_2O/leaf) for 6 h in the light and 16 h in the dark. Water was replaced as it was depleted via transpiration and the total volume adjusted to 1 mL for the night period. The leaves were then transferred to the unlabeled N-free or 40 mM KNO_3 nutrient solution and illuminated. The extraction procedure of PEPcase was the same as above. Immunoprecipitation was achieved with the amount of anti-

serum necessary to sediment the totality of the PEPcase activity in the extracts. After centrifugation (16,000*g*, 2 min), the pellet was washed five times with 1 mL of 50 mM Tris, pH 7.4, 1 M NaCl, and 3% (v/v) Triton X-100. The pellet was then resuspended in 70 μL SDS sample buffer, boiled for 5 min, and analyzed by SDS-PAGE (10%). The molecular mass marker proteins were α-macroglobuline (180 kD), β-galactosidase (116 kD), fructose-6-P kinase (84 kD), pyruvate kinase (58 kD), fumarase (48.5 kD), lactate dehydrogenase (36.5 kD), and triose-P isomerase (26.6 kD). Proteins on the gel were stained with Coomassie brilliant blue. Autoradiography was carried out using Fuji x-ray film. The PEPcase band was cut out and the radioactivity of the protein was determined with a Beckman scintillation spectrophotometer (LS 6000IC).

Protein Assay

The protein concentration in the crude enzyme extracts was determined by the dye-binding method as described by Sedmak and Grossberg, using BSA as standard (23).

Chl Determination

The Chl content of the pellets obtained after centrifugation of the enzyme crude extracts was solubilized in methanol and the absorbance was measured at 652 nm according to the method of Bruinsma (3).

Nitrate Uptake Determination

The nitrate content of the nutrient solution was determined by the colorimetric method of Cataldo *et al.* (7). Nitrate uptake was measured via the depletion of the nutrient solution.

Experiments were repeated at least twice and generally four times. Each leaf was extracted and analyzed separately. Values are means ot a minimum of three and a maximum of five leaves. Results are expressed per mg Chl. No change in tissue Chl content was observed during the short-term nitrate treatment applied to the leaves.

RESULTS

Time Course of NO_3^- Uptake, PEPcase, SPS, and Pyruvate Kinase Activity in the Light

The PEPcase and SPS activities of low-NO_3^- leaves increased 2.3-fold within 10 to 30 min after detached leaves were transferred to the light (Fig. 1A–C). In leaves provided with 40 mM NO_3^-, the transition to the high-NO_3^- state was concomitant with illumination. In this situation, the light-dependent activation of PEPcase appeared sooner and the activity increased more rapidly than in the low-NO_3^- leaves (2.7 times within 60 min and 3.8 times within 200 min as compared with the control). The time-course of PEPcase activity was very similar to that of NO_3^- uptake (Fig. 1B). In contrast, supply of NO_3^- to the leaves led to an immediate decrease of SPS activity in V_{max} assay (Fig. 1C) as well as in limiting assay (Fig. 1D). Later, inhibition of the activity was resumed, but the level of SPS remained lower in the high-NO_3^- than in the low-NO_3^- leaves. Thus, feeding the leaves

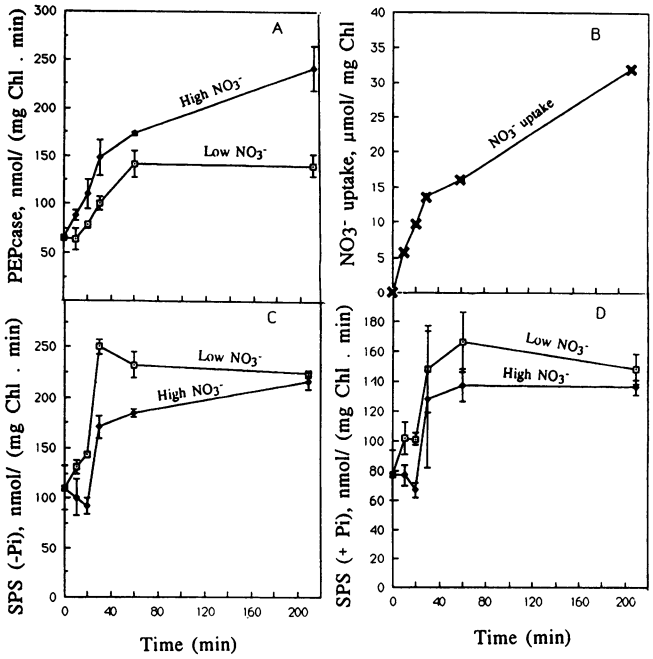

Figure 1. Effect of light and NO_3^- on the time-course of A, PEPcase activity; B, NO_3^- uptake; C, SPS (−Pi) V_{max} assay; D, and SPS (+Pi) limiting assay of detached wheat leaves. Seedlings were in darkness for 16 h on the N-free solution before the youngest mature leaves (No. 3 counted from the base) were detached and transferred onto (□) N-free solution (low-NO_3^- leaves) or (♦) 40 mM KNO_3 (high-NO_3^- leaves) and illuminated (520 μmol m^{-2} s^{-1}). The results are given as the mean ± SE (n = 3).

with NO_3^- resulted in a significant short-term enhancement of the light stimulation of PEPcase activity and reduced the light-dependent activation of SPS. Pyruvate kinase was not affected by light or by NO_3^- (data not shown).

PEPcase Activity in Response to Light and Nitrate

Light intensity affected the degree of activation of PEPcase (Fig. 2). Both NO_3^- uptake and the magnitude of the stimulation of PEPcase activity depended on the NO_3^- concentration in the uptake solution (Fig. 3A). The correlation coefficient between NO_3^- uptake and PEPcase activation was 0.97 (Fig. 3B).

Immunoreactivity of PEPcase

The rabbit antiserum against *Sorghum* leaves PEPcase has affinity for the wheat leaf enzyme (19). The amounts of antiserum required to immunoprecipitate 50% of the same initial activity (1.4 10^{-3} units) from the extracts of control, illuminated low-NO_3^-, and illuminated high-NO_3^- leaves were 12.3, 7.3, and 5.0 μL, respectively (Fig. 4). Different amounts of antiserum were required in each case. One interpretation of this result is that the PEPcase proteins from the three kinds of leaves were not immunochemically identical.

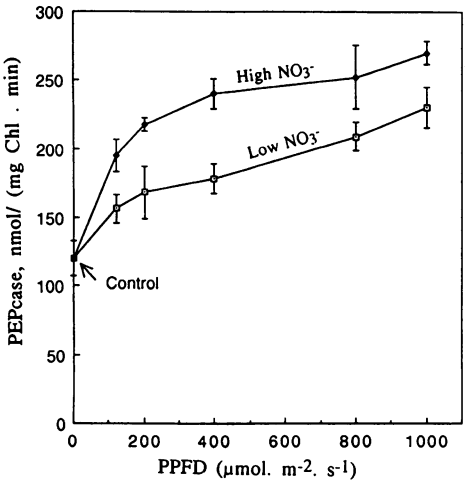

Figure 2. Effect of light intensity on PEPcase activity of detached wheat leaves. Seedlings were in darkness for 16 h on the N-free solution before the youngest mature leaves (No. 3 counted from the base) were detached and transferred onto (⊡) N-free solution (low-NO_3^- leaves) or (♦) 40 mM KNO_3 (high-NO_3^- leaves) and illuminated for 60 min (120–1000 μmol m^{-2} s^{-1}). The results are given as the mean ± SE (n = 3).

Sensitivity of PEPcase to Inhibition by Malate and Activation by Glucose-6-P

Malate inhibited the PEPcase from low-NO_3^-, high-NO_3^-, and control leaves, but the percentage inhibition differed significantly (Fig. 5). The activity from control leaves was completely inhibited at 4 mM malate, and I_{50}(malate) was about 1.45 mM. The PEPcase from illuminated high-NO_3^- leaves was about 2.5 times less sensitive, with an I_{50}(malate) of 3.4. The I_{50}(malate) of the low-NO_3^- leaves was 1.7.

Differences in glucose-6-P activation were also observed. The PEPcase from leaves of all treatments was activated to about the same extent (maximal activation 3.5-fold). However, the A_{50}(glucose-6-P) was slightly higher for the control enzyme compared with the low-NO_3^- and high-NO_3^- enzymes (5.3, 4.1, and 3.5, respectively).

Effects of Okadaic Acid and Cycloheximide on PEPcase Activity

The short-term increase in PEPcase activity in response to illumination and also to the transition from the low-NO_3^- to high-NO_3^- state was enhanced by the inclusion of 0.4 μM okadaic acid into the uptake solution (Table I). Okadaic acid is known to be a potent inhibitor of the protein phosphatase that dephosphorylates PEPcase in *Bryophyllum fedtschenkoi* leaves (6). This demonstrates that at least some of the increase in PEPcase activity was due to phosphorylation and that the leaf tissue contained a PEPcase phosphatase that prevented the enzyme from attaining its maximal potential activity.

Inclusion of 5 μM cycloheximide did not affect the short-term activation of PEPcase, but suppressed the low long-term increase of the activity (Table I). Although cycloheximide is known to have effects on systems other than protein synthesis (11), the most probable explanation of this result is that

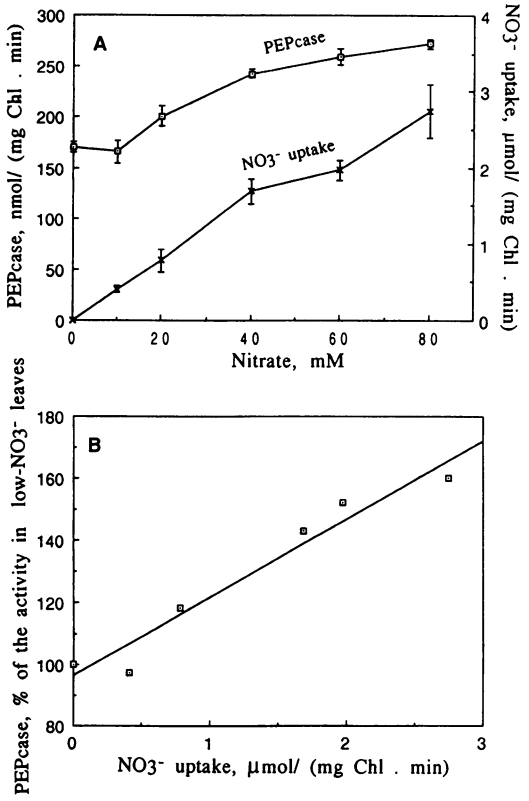

Figure 3. Effect of NO_3^- concentration in the solution on NO_3^- uptake and PEPcase activity. Mature wheat leaves were detached from seedlings after 16 h in darkness and transferred onto KNO_3 at 0 to 80 mM, for 60 min in the light (520 μmol m^{-2} s^{-1}). A, NO_3^- uptake (×) and PEPcase activity (⊡); the results are given as the mean (n = 3). B, Relationship between the effect of NO_3^- on the light-dependent activation of PEPcase and the NO_3^- uptake rate. Data are enhancements as percentages of the activity in leaves on N-free solution in the light. 100% = 243 nmol/mg Chl·min. Regression equation y = 108.8449 + 4.5458 x, (r^2 = 0.97).

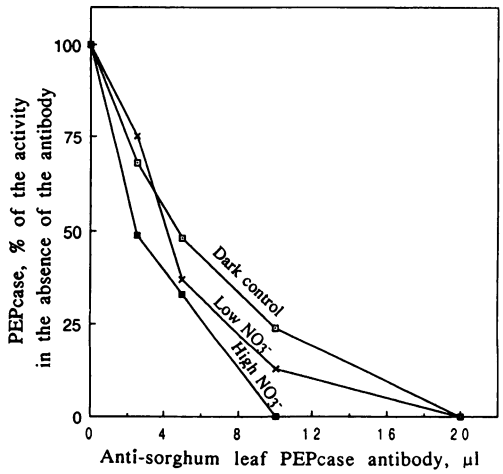

Figure 4. Immunotitration with anti-sorghum leaf PEPcase antibodies of PEPcase extracted from wheat leaves detached from seedlings in darkness (⊡), then transferred onto N-free solution (×) or 40 mM KNO_3 (■), and illuminated (520 μmol m^{-2} s^{-1}) for 60 min.

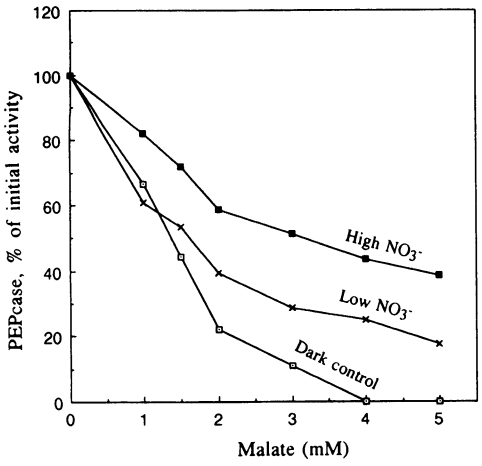

Figure 5. Effect of light and NO_3^- on the sensitivity of PEPcase to malate. PEPcase was extracted from leaves of wheat seedlings after 16 h in darkness (⊡); and transferred onto (×) N-free solution or (■) 40 mM KNO_3 for 60 min in the light (520 μmol m^{-2} s^{-1}). Data are inhibition as percentages of the activity with no addition of effector (control). 100% = 0.005 unit.

stimulation of protein synthesis in high-NO_3^- leaves was an additional aspect of the PEPcase increase.

Labeling of PEPcase with ^{32}P

The PEPcase activity layered onto the SDS-PAGE gels was equivalent to 0.5 mg Chl and to 0.06 unit for the control, 0.08 unit for the low-NO_3^-, and 0.1 unit for the high-NO_3^- protein, as estimated before immunoprecipitation. As judged by Coomassie brilliant blue staining (Fig. 6), these amounts corresponded to similar amounts of a protein of subunit mol wt 117,000, which was shown to be PEPcase (10). The enzyme extracted from leaves after 16 h darkness following prelabeling contained the least ^{32}P. When darkness was followed by 2 h light, labeling of the PEPcase from low-NO_3^- and high-NO_3^- leaves was 416 and 665% of the control, respectively. It decreased to 133 and 216% when illumination was followed by 10 h darkness (Fig. 6).

DISCUSSION AND CONCLUSION

The results obtained in the present study clearly demonstrate that the demand for carbon skeletons created by high rates of NO_3^- assimilation is met by a concomitant increase of PEPcase and decrease of SPS activity. It is clearly demonstrated that PEPcase in wheat leaves is subjected to some form of light-dependent modulation. This new observation on a C_3 species PEPcase shows that there is a similarity in the regulation of this enzyme type to that already described in C_4 plants, even though PEPcase from C_3 plants is a distinct enzyme and fulfills different functions (28). In addition, this study shows that NO_3^- enhances the light effect in wheat leaves. In the present context, it is not possible to discern whether the effect of NO_3^- is through uptake, storage, or assimilation, as rates of NO_3^- utilization in leaf tissues depend on the rate of uptake (8). These effects can result from NO_3^- itself or some derivative of its assimilation, such as the level of NH_4^+ or the glutamine/glutamate ratio, as in *Selenastrum minutum* (29).

The okadaic acid and cycloheximide studies show that NO_3^- has a dual effect, one in the long term and the other in the short term. The long-term effect, which is inhibited by cycloheximide, can be explained by an increased rate of protein synthesis due to the transition from the low-NO_3^- to the high-NO_3^- nutrition. It implies regulation at the level of transcription and translation and the development of the processes of NO_3^- assimilation and protein synthesis. A similar NO_3^--dependent increase in PEPcase activity has already been reported in leaves of *Phaseolus vulgaris* L. after several days of growing the plants on NO_3^- (22).

The short-term effect of NO_3^- on leaf PEPcase activity, which appears within 10 min of the transition from the low-NO_3^- to the high-NO_3^- state, cannot be explained by *de novo* synthesis of the protein. The simplest hypothesis is that light modulates PEPcase activity and that NO_3^- enhances the light effect. An alternative hypothesis is that the effect of NO_3^- is at another level of control and additional to that of light.

Although the exact mechanism for the light modulation is not known, it appears to involve changes in protein conformation. This is suggested by the observations that the I_{50} of immunotitration and the sensitivity to malate and glucose-6-phosphate were altered. This type of regulation also appears

Table I. *Effect of Pretreatment with Cycloheximide (5 μM) or Okadaic Acid (0.4 μM) on PEPcase Activity in Detached Wheat Leaves*

Pretreatments were carried out for 4 h in the light followed by 16 h in the dark. Leaves were then frozen in liquid nitrogen (control) or transferred onto N-free solution (low-NO_3^-) or 40 mM KNO_3 (high-NO_3^-) and illuminated (520 μmol m^{-2} s^{-1}) for 30, 60, and 120 min. The results are given as the mean ± SE (n = 3).

Pretreatment	Control	PEPcase Activity					
		Low NO_3^-			High NO_3^-		
		30 min	60 min	120 min	30 min	60 min	120 min
		nmol/mg Chl·min					
None	118 ± 9		159 ± 7	194 ± 11		291 ± 13	380 ± 14
Cycloheximide	118 ± 9		159 ± 8	132 ± 15		286 ± 10	281 ± 14
None	89 ± 7	101 ± 5	136 ± 7		125 ± 3	185 ± 8	
Okadaic acid	89 ± 7	149 ± 5	173 ± 7		152 ± 9	198 ± 5	

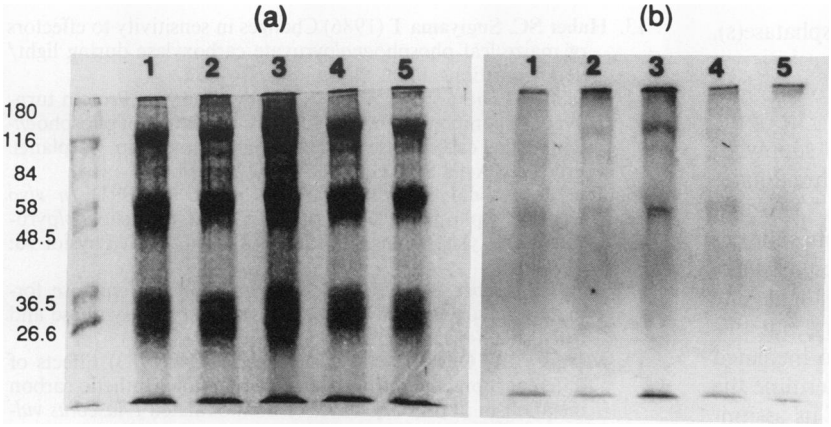

Figure 6. SDS-PAGE of extracts from ^{32}P-labeled leaves. Detached wheat leaves were labeled with ^{32}P for 6 h and then maintained in darkness for 16 h. They were then extracted immediately (track 1); after 90 min illumination on N-free solution (track 2); after 90 min illumination on 40 mM NO_3^- (track 3); after 2 h illumination on N-free solution followed by 10 h darkness (track 4); after 2 h illumination on 40 mM NO_3^- followed by 10 h darkness (track 5). a, Coomassie brilliant blue-stained gel; b, autoradiograph. The numbers on the left indicate mol wt values $\times 10^{-3}$ of the marker proteins. The experiment was repeated four times.

to depend on NO_3^-, because similar but higher-intensity alterations were observed with the enzyme from illuminated high-NO_3^- leaves. Thus, differences in relative sensitivity to effectors strongly suggest that some posttranslational modification during the dark to light and low-NO_3^- to high-NO_3^- transitions modulates activity in C_3 leaves. It also corroborates the hypothesis that the NO_3^- dependent and light-controlled mechanism of PEPcase modulation are the same and that the role of NO_3^- is to enhance the light-dependent modification of the protein from a low-activity to a high-activity form.

Phosphorylation-mediated posttranslational modification is known to modulate the activity of PEPcase in C_4 and CAM plants (14). Evidence that the modulation of PEPcase in wheat leaves is also due to the phosphorylation state of the protein is suggested by the assay with okadaic acid, and demonstrated by the ^{32}P-labeling experiments presented here. The level of phosphorylation of PEPcase appeared to correlate with the magnitude of the stimulation. The reversibility of the control was also demonstrated.

The proposed activation of wheat leaf PEPcase by phosphorylation rendering the enzyme less sensitive to feedback inhibition by malate is very similar to that proposed for PEPcase from *Zea mays* and several other C_4 or CAM plants whose PEPcase is normally active during the day in the former case or at night in the latter, when the malate level is high in the leaves (5, 13). A reduction in the sensitivity of PEPcase to feedback inhibition by malate in NO_3^--fed leaves would be expected to make the enzyme more active *in vivo,* because the malate concentration is high in nitrate-assimilating wheat leaves (data not shown).

When illuminated wheat leaves were supplemented with NO_3^-, PEPcase increased, whereas SPS activity was transiently greatly decreased and thereafter remained at a lower level than in the leaves on N-free solution, although the difference was not as marked as during the first minutes of NO_3^- feeding (Fig. 1). It is now well known that the regulation of SPS activity is one of the components that controls the flux of carbon into sucrose (16, 25). It has been established that changes in SPS activity with light/dark transitions involved a covalent modification (31). Reversible phosphorylation was implicated, with the phosphorylated form being the low-activity form (12, 24). Thus, PEPcase and SPS are both comodulated by light via protein phosphorylation, but the relationship is inverse. Whereas PEPcase is activated by phosphorylation, SPS is activated by dephosphorylation. In both cases, phosphorylation of the protein is reversible. The phosphorylation (kinase) and dephosphorylation (phosphatase) reactions are active at the same time. The relative activities of the protein kinase and phosphatase enzymes determine the overall level of phosphorylation, which, hence, results in a steady-state enzyme activity. The high activity of PEPcase depends on a relatively high protein-kinase to protein-phosphatase ratio, whereas the reverse is true for SPS, the high-activity form of which depends on a low kinase to phosphatase ratio. By comparison of the responses to NO_3^- of PEPcase and SPS in illuminated wheat leaves, it is possible to suggest that the evidence supports the hypothesis that NO_3^- favors the phosphorylated form of both enzymes (Fig. 7). It is tempting to suggest that NO_3^- or a derivative stimulates the protein-kinase(s), as does the light with the maize leaf PEP-

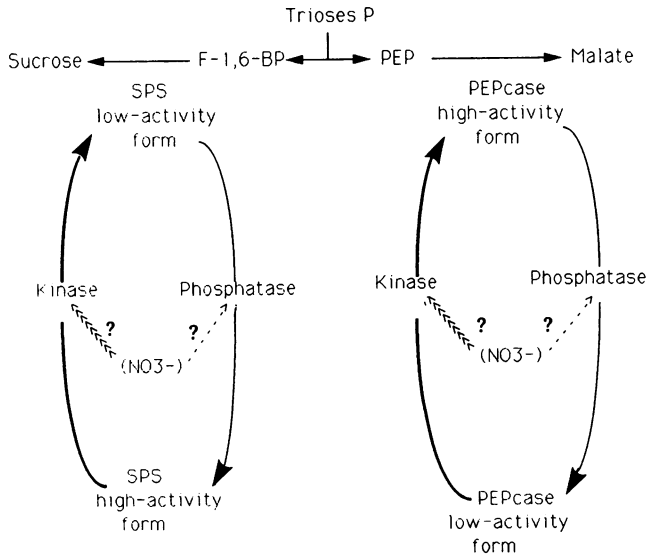

Figure 7. A diagrammatic representation of the proposed interaction of NO_3^- with the light-dependent activation of PEPcase and deactivation of SPS in detached mature wheat leaves. >>>>>, kinase stimulation; - - - - >, phosphatase inhibition.

case-kinase (15), and/or inhibits the protein-phosphatase(s), thereby activating PEPcase and deactivating SPS.

In conclusion, modulation by phosphorylation of the mature wheat leaf PEPcase is now well established as part of the regulatory mechanism that directly controls the supply of carbon to the anapleurotic pathway and indirectly modulates the provision of carbon skeletons needed for NO_3^- assimilation. We show here that this regulation involves phosphorylation of the PEPcase protein. However, the sites of phosphorylation on the protein have to be identified. Light may regulate the process in a more or less direct way, but this remains to be elucidated. This modulation is also mediated by NO_3^-, and further studies are necessary to determine the relationship between NO_3^- or some derivative of its assimilation and the phosphorylation state of PEPcase.

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