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Regulatory Phosphorylation of C_4 Phosphoenolpyruvate Carboxylase[1]

A Cardinal Event Influencing the Photosynthesis Rate in *Sorghum* and Maize

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C_4 leaf phosphoenolpyruvate carboxylase (PEPC; EC 4.1.1.31) is subject to a day/night regulatory phosphorylation cycle. By using the cytoplasmic protein synthesis inhibitor cycloheximide (CHX), we previously reported that the reversible in vivo light activation of the C_4 PEPC protein-serine kinase requires protein synthesis. In the present leaf gas-exchange study, we have examined how and to what extent the CHX-induced inhibition of PEPC protein kinase activity/PEPC phosphorylation in the light influences C_4 photosynthesis. Detached *Sorghum vulgare* and maize (*Zea mays*) leaves fed 10 μM CHX showed a gradual but marked decrease in photosynthetic CO_2 assimilation capacity. A series of control experiments designed to assess deleterious secondary effects of the inhibitor established that this reduction in C_4 leaf CO_2 assimilation was not due to (a) an increased stomatal resistance to CO_2 diffusion, (b) a decrease in the activation state of other photoactivated C_4 cycle enzymes, and (c) a perturbation of the Benson-Calvin C_3 cycle, as evidenced by the absence of an inhibitory effect of CHX on leaf photosynthesis by a C_3 grass (*Triticum aestivum*). It is notable that the CHX-induced decrease in CO_2 assimilation by illuminated *Sorghum* leaves was highly correlated with a decrease in the apparent phosphorylation status of PEPC and a concomitant change in carbon isotope discrimination consistent with a shift from a C_4 to a C_3 mode of leaf CO_2 fixation. These collective findings indicate that the light-dependent activation of the PEPC protein-serine kinase and the resulting phosphorylation of serine-8 or serine-15 in *Sorghum* or maize PEPC, respectively, are fundamental regulatory events that influence leaf C_4 photosynthesis in vivo.

Regulatory phosphorylation of the C_4 leaf photosynthetic PEPC by a protein-Ser kinase is now a well-established process, both in vitro and in vivo (reviewed by Jiao and Chollet, 1991). The PEPC protein kinase specifically phosphorylates Ser^8 (*Sorghum* PEPC), or its structural homolog in maize (*Zea mays*; Ser^{15}), located in a consensus sequence of the N-terminal region of the target enzyme (Jiao and Chollet, 1990; Jiao et al., 1991b; Wang et al., 1992). This posttranslational modification dominates in the light by virtue of the reversibly light-activated PEPC protein kinase (Echevarria et al., 1990; Jiao et al., 1991a; McNaughton et al., 1991; Bakrim et al., 1992; Jiao and Chollet, 1992) and confers PEPC with altered functional and regulatory properties. Specifically, the phosphorylated form of the enzyme is less sensitive to the feedback inhibitor, L-malate, and exhibits increased activity at suboptimal levels of pH and PEP. In addition, PEPC is believed to be under metabolic control by different photosynthesis-related compounds in the cytosolic compartment of C_4 mesophyll cells (Doncaster and Leegood, 1987; Jiao and Chollet, 1992). Therefore, the fine tuning of carboxylating activity and, thus, atmospheric CO_2 fixation would result from a delicate balance between positive (Glc-6-P, triose-P) and negative (L-malate) effectors whose influence would be further modulated by the regulatory phosphorylation process (Jiao and Chollet, 1991; Bakrim et al., 1992; Jiao and Chollet, 1992). Finally, PEPC activity also depends on the availability of its substrates, PEP and CO_2 (i.e. HCO_3^-), the concentration of which varies greatly during a day/night cycle (Leegood and Furbank, 1984; Stitt and Heldt, 1985; Usuda, 1985; Doncaster and Leegood, 1987).

It has been postulated that C_4 PEPC activity must be curtailed in the night such that there is no interference with energy-producing pathways (e.g. glycolysis) and subsequently activated during the light period following the opening of stomata and the buildup of photosynthetic PEP by the light-activated PPDK reaction (Doncaster and Leegood, 1987). Then, coordination of the C_4 cycle and the Benson-Calvin C_3 cycle could be achieved by an adequate supply of photosynthetic compounds acting as metabolic messages at the level of PEPC and resulting in photosynthetic homeostasis. In this respect, the role of PEPC phosphorylation is still unclear and a matter of some debate (Jiao and Chollet, 1991; McNaughton et al., 1991). During C_4 photosynthesis in *Sorghum* and maize, the malate concentration is quite high (>15–20 mM [Stitt and Heldt, 1985; Doncaster and Leegood,

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Abbreviations: CHX, cycloheximide; MDH, malate dehydrogenase; PEP, phosphoenolpyruvate; PEPC, phosphoenolpyruvate carboxylase; PPDK, pyruvate Pi dikinase.

1987]) to maintain the requisite diffusion gradient between the two photosynthetic leaf cell types. Because phosphorylation not only improves the catalytic activity of PEPC but, more notably, decreases its sensitivity to L-malate, this regulatory process might prove essential for the functioning of C_4 photosynthesis.

Recently, it was shown that PEPC phosphorylation and PEPC protein kinase activity are suppressed in vivo by cytosolic protein synthesis inhibitors, e.g. CHX (Carter et al., 1991; Jiao et al., 1991a; Bakrim et al., 1992). These experiments led to the suggestion that a rapid, light-dependent protein biosynthesis event is somehow involved in the light activation of the C_4 PEPC protein kinase and, thus, the control of phosphorylation. In the present work, we have taken advantage of these recent findings with CHX (Carter et al., 1991; Jiao et al., 1991a; Bakrim et al., 1992) to assess how the regulatory phosphorylation of PEPC influences C_4 photosynthesis by the intact leaf.

MATERIALS AND METHODS

Plant Material

Sorghum vulgare (cv Tamaran), maize (*Zea mays* L., cv F7F2), and wheat (*Triticum aestivum*, cv H 16 IN 80164) were grown in a greenhouse for approximately 30 d with supplemental lighting that provided 300 μmol of photons m^{-2} s^{-1} (400–700 nm), in the absence of sunlight, at leaf level, and in soil watered with Hoagland solution. The photoperiod was 15 h (25°C day/18°C night).

Treatment of Leaves and Gas-Exchange Analyses

Leaves were excised under water, inserted into a beaker containing 50 mL of distilled water, and placed in an assimilation chamber connected to an open IRGA system described by Prioul et al. (1980). To ensure uniform leaf sampling, the 5th leaf (about 100 cm^2) was taken from plants of similar ages. Net photosynthesis was measured at an incident light intensity of 750 μmol of photons m^{-2} s^{-1} (400–700 nm) provided by a high-pressure sodium lamp (Osram; 400 W). The CO_2 concentration in air was usually 330 μL L^{-1}. The leaf temperature in the chamber was maintained at approximately 25°C. Both leaf and air temperatures were continuously recorded. The air flow was passed through the chamber at a constant rate of 3 L min^{-1}. After CO_2 assimilation had reached steady state, freshly prepared CHX (10 μM, final concentration) was added to distilled water and allowed to be taken up by the leaves in the transpiration stream. Resistance to CO_2 diffusion and the intercellular CO_2 concentration were calculated from the rates of CO_2 and H_2O vapor exchange using the equation of Prioul et al. (1980).

On-Line Carbon Isotope Measurements

These analyses were performed essentially as described previously (Von Caemmerer and Farquhar, 1981; Bowman et al., 1989). The open IRGA system (Prioul et al., 1980) was used with a CO_2-trapping system to measure leaf CO_2 exchange and the carbon isotope composition of the air stream that had passed over the leaf. Before the experiment, the entire gas-exchange system was flushed with compressed air for 36 h at 2 L min^{-1}. Attached leaves from 1-month-old plants (three leaves/100 cm^2) were placed in the assimilation chamber and then equilibrated with the same compressed air for 24 h (8 h of light/16 h of dark); they were then excised under water and illuminated at 750 μmol of photons m^{-2} s^{-1}. Gas samples (3 L) were taken at the exit of the assimilation chamber before and after CHX feeding of the leaves. CO_2 was cryogenically purified, and the carbon isotope composition was measured by MS (Funnigan S). Discrimination was determined according to the method of Evans et al. (1986).

Enzyme Extraction and Activity Assays

Leaf tissue sampling was performed at the indicated times. Two square centimeters of leaf blade were immediately extracted in 300 μL of extraction medium (100 mM Tris-HCl [pH 8], 10 mM $MgCl_2$, 1 mM EDTA, 10% [v/v] glycerol, 10 mM DTT, 2% [w/v] insoluble PVP, and some washed sand) in a precooled mortar (0°C). The homogenate was rapidly centrifuged for 1 min at maximal speed in an Eppendorf centrifuge. The following enzyme activities were determined using the crude supernatant fluid: (a) PEPC was measured in a 1-mL assay containing 100 mM Hepes-NaOH (pH 7.3), 2.5 mM PEP, 5 mM $NaHCO_3$, 5 mM $MgCl_2$, 0.2 mM NADH, and 10 units of commercial NAD-MDH. Malate inhibition was determined by adding a final concentration of 1 mM L-malate (pH adjusted) to the assay medium; (b) NADP-MDH (EC 1.1.1.82) and (c) PPDK (EC 2.7.9.1) were assayed as described by Bakrim et al. (1992). In all cases, the decrease in absorbance was recorded at 340 nm and 30°C. One enzyme unit is defined as that amount of enzyme that catalyzes the transformation of 1 μmol of substrate/min under the described experimental conditions.

RESULTS AND DISCUSSION

CHX Inhibits Both PEPC Phosphorylation and the Rate of Net CO_2 Assimilation by Detached *Sorghum* and Maize Leaves

The in vivo light activation of the protein-Ser kinase that, in turn, phosphorylates Ser^8 or Ser^{15} of *Sorghum* or maize PEPC, respectively, is prevented by the cytosolic protein synthesis inhibitor CHX, whereas chloramphenicol is without effect (Jiao et al., 1991a). Therefore, a rapid protein turnover event appeared to be a component of the C_4 PEPC regulatory phosphorylation cascade (Jiao et al., 1991a; Bakrim et al., 1992). We have taken advantage of this fact to assess the role of PEPC phosphorylation with regard to the functioning of C_4 photosynthesis in the intact leaf. To this end, the effect of this inhibitor on photosynthesis by detached C_4 and C_3 leaves has been measured using an open IRGA system.

In preliminary experiments, net CO_2 uptake was shown to reach steady state after 30 to 60 min of illumination at 750 μmol of photons m^{-2} s^{-1}. For both *Sorghum* (Fig. 3A) and maize (not shown), an external CO_2 concentration of 300 μL L^{-1} was saturating under the experimental conditions used. For the three species examined (*Sorghum*, maize, and wheat), CO_2 assimilation, after stabilization, remained nearly constant for >7 h in continuous light.

When leaf photosynthesis was at steady state, CHX (10 μM) was fed to the excised leaves via the transpiration stream, and the recording of CO_2 assimilation was continued. Within 1 h after the *Sorghum* (Fig. 1) or maize (not shown) leaves were allowed to take up the inhibitor, their CO_2 assimilation rate gradually declined, eventually stabilizing at about 10 to 20% of the initial, uninhibited value after 4 to 5 h of CHX treatment. It was demonstrated previously with maize (Jiao et al., 1991a) and *Sorghum* (Bakrim et al., 1992) leaves that the main effect of CHX was to inhibit the synthesis of either the PEPC protein-Ser kinase itself or a putative protein factor required for its maximal activity, thereby blocking PEPC phosphorylation. In the absence of the protein synthesis inhibitor, a 2-h illumination of a *Sorghum* leaf caused PEPC activity and malate inhibition values to change about 2-fold, indicating that the regulatory phosphorylation of the target enzyme had occurred (Table I). These light-induced changes were maintained throughout the duration of the experiment. In contrast, after 6 h of CHX treatment under continuous light, when the rate of net CO_2 assimilation had reached its lowest level (Fig. 1), sensitivity to L-malate and the activity of PEPC reached values characteristic of the dark-form enzyme; such behavior was indicative of a dephosphorylated status (Table I).

A time-course experiment was next performed with illuminated *Sorghum* leaves to determine the variation in the malate sensitivity/apparent phosphorylation status of PEPC and the corresponding leaf CO_2 assimilation rate as a function of CHX treatment. In Figure 2, malate inhibition values of PEPC activity during the course of CHX treatment are plotted as a function of CO_2 assimilation rate. The high degree of correlation between these two parameters ($R^2 = 0.977$) indicates that they are closely related physiological events.

As a working hypothesis we propose that a possible cause-and-effect relationship exists between the CHX-mediated decrease in PEPC phosphorylation status and the decreasing ability of the C_4 leaf to fix atmospheric CO_2. A variety of control experiments was designed to address any possible detrimental side effects of CHX on *Sorghum* leaf photosynthesis.

Table I. *Light/dark and CHX effects on the activity of PEPC, PPDK, and NADP-MDH in detached Sorghum leaves*

Samples (100 mg) of dark (D) and light-adapted (2-h exposure, L) control leaves were rapidly homogenized in 300 μL of extraction medium, and the supernatant fluids were used immediately to determine enzyme activities at 30°C. PEPC activity was assayed at suboptimal conditions in the presence or absence of 1 mM L-malate. The same experiment was also conducted with illuminated leaves with or without a 6-h CHX treatment (10 μM). Values are means; SE = 5%; $n = 3$. The 100% activity values (in units/g fresh weight) for PEPC, PPDK, and NADP-MDH were 30, 0.3, and 15, respectively.

Parameter	PEPC		PPDK		NADP-MDH	
	D	L	D	L	D	L
Activity (%)	50	100	10	100	12	100
Malate inhibition (%)	70	30				
	Light ± CHX					
	−	+	−	+	−	+
Activity (%)	100	50	100	95	100	100
Malate inhibition (%)	30	70				

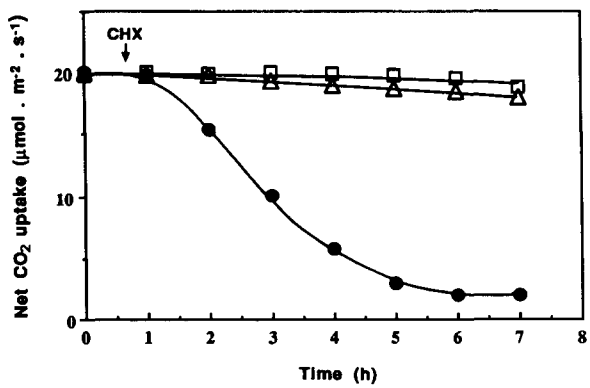

Figure 1. Effect of CHX on the rate of net CO_2 assimilation by *Sorghum* (C_4) and wheat (C_3) leaves. Mature detached leaves (4th to 5th leaf) were inserted into the assimilation chamber of the open IRGA system (Prioul et al., 1980) with the basal part of the leaf immersed in water. After the rate of net CO_2 uptake reached steady state (0.5–1 h), the leaves were fed 10 μM CHX, and the recording of CO_2 exchange was continued. Control leaves were maintained in water. Net assimilation was calculated from the difference between the CO_2 concentrations in the incoming and outgoing gas by means of an IRGA in the absolute mode. Water vapor pressure modifications by the leaf were also monitored to allow calculation of transpiration, resistance to CO_2 and H_2O diffusion, and internal leaf CO_2 concentration. □, Control *Sorghum*; ●, CHX-treated *Sorghum*; △, wheat ± CHX.

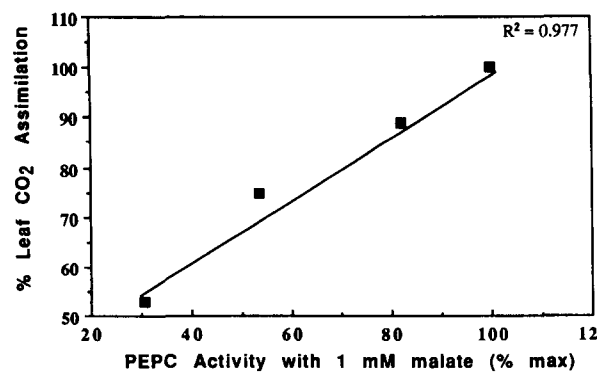

Figure 2. Variations in *Sorghum* leaf CO_2 assimilation as a function of malate inhibition of PEPC. Detached leaves were inserted into the assimilation chamber of the IRGA system, and net CO_2 uptake was recorded. After steady state was reached, the illuminated leaves were fed CHX (10 μM). Leaf samples were extracted at various times after CHX treatment, and PEPC activity and malate inhibition (which reflects the apparent phosphorylation status of PEPC) were determined at pH 7.3, 2.5 mM PEP, ± 1 mM L-malate. PEPC activity values in the presence of 1 mM L-malate were plotted against the relative leaf CO_2 assimilation rate at the corresponding time after CHX addition. The maximum value was arbitrarily set at 100% (x axis). Results are the means of triplicate experiments (SE = 5%).

CHX Has No Effect on the Functioning of the Benson-Calvin C_3 Cycle in Wheat Leaves

In a C_4 plant such as *Sorghum*, photosynthesis is achieved by the concerted functioning of two metabolic cycles, the intercellular C_4 cycle and the Benson-Calvin pathway of the bundle sheath chloroplast. With respect to the inhibitory effect of CHX on net CO_2 uptake by *Sorghum* (Fig. 1) and maize leaves (not shown), the most obvious metabolic pathway to be examined was the Benson-Calvin cycle. Unfortunately, because the C_4 and C_3 cycles are functionally linked, any perturbation at a given point will have an effect on this integrated metabolic system. Thus, direct assessment of a possible CHX effect on the C_3 pathway in a C_4 leaf is difficult. Indirect evidence was obtained by using a graminaceous C_3 plant that lacks the C_4 cycle. Feeding CHX to an illuminated, detached wheat leaf did not cause a significant inhibition of its CO_2 assimilation capacity during the 6-h treatment period (Fig. 1). By analogy, we assume that the marked reduction of C_4 photosynthesis by CHX in *Sorghum* (Fig. 1) and maize leaves was not due to any deleterious effect of the inhibitor at the level of the C_3 cycle.

CHX Has No Effect on the Activation State of Other C_4-Cycle Enzymes

The C_4 mesophyll-chloroplast enzymes NADP-MDH and PPDK are reversibly light activated (Edwards et al., 1985). These "on or off" photoregulation cycles are rapid and take place in <10 to 15 min, and they are influenced, at least in part, by the rates of photosynthetic electron transport (thioredoxin-mediated reductive activation of NADP-MDH) and ATP synthesis (dephosphorylation/activation of PPDK) (Edwards et al., 1985; Nakamoto and Edwards, 1986). Whereas a 6-h CHX treatment of an illuminated *Sorghum* leaf caused PEPC dephosphorylation (monitored by L-malate inhibition) and a marked inhibition of CO_2 assimilation (Fig. 1, Table I), this inhibitor had essentially no effect on the photoactivation state of PPDK and NADP-MDH (Table I). Similarly, the light-induced increase in activity of these two stromal target enzymes and of cytosolic Suc-P synthase in a darkened maize leaf is also unaffected by CHX treatment (Jiao et al., 1991a). Consequently, these collective results indicate that an illuminated, CHX-treated C_4 leaf maintains its capacity for the continued light activation of mesophyll cell photosynthetic enzymes except for cytosolic PEPC.

CHX Has No Apparent Effect on Stomatal Movements

Another potential site through which CHX could exert its negative effect on atmospheric CO_2 assimilation by a C_4 leaf could be the stomata. It is conceivable that feeding the inhibitor to the leaf could result in stomatal closure and a decrease in the CO_2 assimilation rate. To obtain insight into the stomatal response to CHX, the following gas-exchange experiment was devised. In this study, the external CO_2 concentration was decreased stepwise from 480 to 0 μL L^{-1}, and the corresponding steady-state assimilation rate in the control and CHX-treated (approximately 80% inhibition) *Sorghum* leaves was recorded. Figure 3A shows that net photosynthesis was saturated at about 300 μL L^{-1} of external

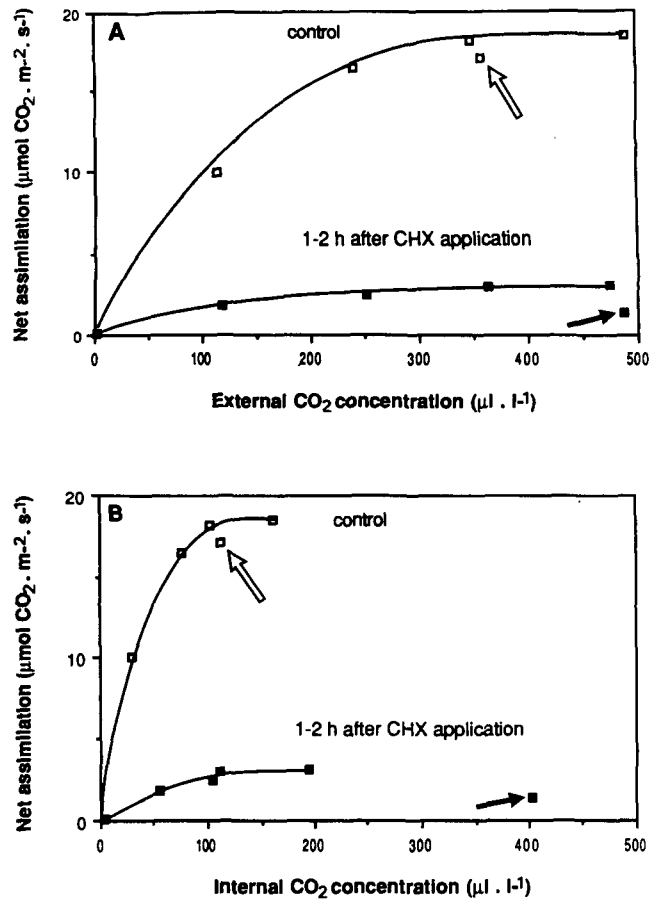

Figure 3. CO_2 response curves of net assimilation by control and CHX-treated *Sorghum* leaves. The curves were obtained by decreasing the CO_2 concentration in the assimilation chamber before (control, □) and after 1 to 2 h of 10 μM CHX treatment (■), when the rate of net photosynthesis had nearly plateaued at about 20% of the initial value. Leaves were allowed to equilibrate initially at 480 μL L^{-1} of CO_2 and 750 μmol of photons m^{-2} s^{-1}. The external CO_2 concentration was decreased stepwise (350, 250, and 125 μL L^{-1}) to zero and then rapidly readjusted to 380 (control, arrow) or 480 μL L^{-1} (CHX-treated leaves, arrow). At each step net CO_2 assimilation was rapidly measured before going to the next. The final value indicated by an arrow should be compared to the initial value at the corresponding external CO_2 concentration (A). Internal CO_2 concentration (*Ci*) was calculated from the H_2O vapor pressure data according to the equation $Ci = Cext - A/g$, where *Cext* is the external CO_2 concentration, *A* is a CO_2 assimilation, and *g* is the stomatal conductance. The response curve as a function of *Ci* (B) represents assimilation by the leaf at the cellular level (i.e. stomata excluded).

CO_2 in the control leaves, as expected for a C_4 plant, whereas the treated leaves exhibited a markedly reduced capacity for fixation at any level of external CO_2 examined. Figure 3B clearly demonstrates that the low rates of CO_2 assimilation by CHX-treated *Sorghum* leaves are not due to a decrease in leaf internal CO_2 (*Ci*) but, rather, are the consequence of a much reduced carboxylation efficiency. This was confirmed by the effect on net CO_2 assimilation of rapidly readjusting the external CO_2 from zero, where stomata were fully open,

to 400 to 500 $\mu L\ L^{-1}$. Because this increase was rapid, the stomata did not have sufficient time to close; therefore, the internal CO_2 became closer to the external CO_2 value (Fig. 3). Despite the large increase in internal CO_2 concentration, the net CO_2 assimilation rate by the CHX-treated leaf remained at a very low level. This further rules out a possible stomatal effect on the observed inhibition of net photosynthesis.

CHX Causes a Shift in C_4 Leaf Carbon Isotope Discrimination

C_3 plants discriminate carbon isotopes much more than C_4 plants (Deleens et al., 1983; Farquhar et al., 1989). This discrimination against ^{13}C is largely due to the biochemical mechanism of Rubisco. In contrast, C_4 plants are characterized by a lower carbon isotope discrimination value (Δ) owing to their primary carboxylase being PEPC, which shows very little discrimination, and the fact that the majority of CO_2 released in the bundle sheath cells is refixed by PEPC in the surrounding mesophyll. Thus, it would be predicted that, if PEPC is progressively inhibited in a *Sorghum* leaf by CHX, the relative C_3 to C_4 mode of CO_2 fixation would gradually increase, accompanied by a corresponding shift in Δ values.

The results presented in Figure 4 clearly show that the gradual CHX-induced inhibition of CO_2 assimilation by an illuminated *Sorghum* leaf is accompanied by the predicted shift in Δ value from 5‰ to about 30‰ when leaf CO_2 assimilation is inhibited by about 80%. Control plants displayed a constant, C_4-specific Δ value throughout the experiment (5‰). This demonstrates that the marked decrease in C_4 leaf CO_2 fixation is correlated with the CHX-mediated inactivation of PEPC, which is primarily a consequence of the inactivation of its protein-Ser kinase (Jiao et al., 1991a; Bakrim et al., 1992) and the resulting changes in the phosphorylation status of the target enzyme (Table I).

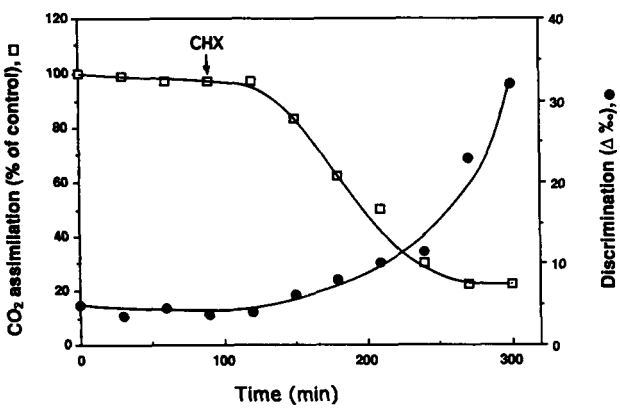

Figure 4. Net CO_2 assimilation and carbon isotope discrimination (Δ) of a CHX-treated *Sorghum* leaf. Leaves were inserted into the IRGA system and CO_2 assimilation (□) measured as described for Figure 1. At the indicated times, samples of the air stream that had passed over the leaf were collected, and the carbon isotope composition of the purified CO_2 was analyzed by MS (●). The arrow indicates the time of addition of 10 μM CHX.

CONCLUDING REMARKS

It has long been known for tropical grasses (e.g. *Sorghum*, maize) that exhibit the C_4 pathway of photosynthesis that PEPC activity (measured at optimal pH and PEP concentrations) in leaf extracts far exceeds the observed rate of net photosynthetic CO_2 fixation. However, when measured at suboptimal, but physiological, levels of PEP and pH (approximately pH 7), the carboxylating capacity of PEPC is severalfold lower and possibly less than is needed for maximum leaf photosynthesis. With recent advances in both C_4 and CAM PEPC research (Andreo et al., 1987; Doncaster and Leegood, 1987; Jiao and Chollet, 1991), it has become increasingly apparent that this enzyme is subject to metabolic control. The mechanism involves a balanced regulation between positive (Glc-6-P, triose-P) and negative (L-malate) effectors, such that it is now considered one of the key regulatory enzymes determining carbon flow in the C_4 pathway. In addition, owing to the photosynthetic origin of these metabolite effectors, the interplay between their positive and negative feedback effects would participate in the coordination of the C_4 and Benson-Calvin C_3 cycles and, thus, the homeostasis of the overall C_4 pathway of photosynthesis (Doncaster and Leegood, 1987).

In maize, extensive accumulation of L-malate was measured in the mesophyll cells (Leegood, 1985), the exclusive site of C_4 PEPC, approximately 20 min after the leaf was illuminated. This buildup is necessary to establish an intercellular diffusion gradient between the mesophyll and bundle-sheath cells, which are thought to be the driving force determining malate transfer to the decarboxylation and Rubisco carboxylation sites in the bundle sheath (Stitt and Heldt, 1985). Much of this malate pool is presumably photosynthetically inactive. However, to account for the observed rates of C_4 leaf photosynthesis, a minimal intercellular difference of about 10 mM has been calculated (Stitt and Heldt, 1985), thus suggesting that the concentration of L-malate in the vicinity of PEPC is likely greater than this value. Because the apparent K_i (L-malate) of PEPC in an illuminated C_4 leaf is approximately 0.5 to 1 mM (Nimmo et al., 1987; Jiao et al., 1991a; Jiao and Chollet, 1992), extensive inhibition of the enzyme would be predicted. However, the inhibitory effect of L-malate is partially offset by the opposing effects of Glc-6-P, triose-P, and PEP (Doncaster and Leegood, 1987), which accumulate in the C_4 leaf after the onset of illumination.

Recently, it was proposed that a protein phosphorylation cycle imposes an additional, but related, level of regulation on C_4 and CAM PEPC (Jiao and Chollet, 1991). In vivo or in vitro phosphorylation of an N-terminal Ser residue on dark-form C_4 PEPC results in an increase in catalytic activity and, more notably, a 2- to 3-fold decrease in malate sensitivity (Jiao and Chollet, 1988; Jiao and Chollet, 1991; Arrio-Dupont et al., 1992). In the work described herein, we investigated how and to what extent the in vivo regulatory phosphorylation of PEPC influences C_4 photosynthesis in intact leaves. To this end, we have made use of the cytoplasmic protein synthesis inhibitor, CHX, previously found to prevent C_4 (or CAM) PEPC phosphorylation in an illuminated (or darkened) leaf and the resulting change in malate sensitivity (Carter et al., 1991; Jiao et al., 1991a; Bakrim et al., 1992).

The present data show that feeding a C_4 leaf this inhibitor markedly perturbs both the apparent phosphorylation status (i.e. malate sensitivity) of PEPC and C_4 photosynthesis in a highly correlated manner (Fig. 2). To ascertain the validity of this inhibitor-based observation, we have verified that the protein synthesis inhibitor did not exert its negative effects through secondary detrimental action on photosynthesis. It was observed that CHX neither decreased significantly stomatal aperture nor photoactivation of other C_4 mesophyll-cell enzymes (Table I, Fig. 3B; Jiao et al., 1991a; Bakrim et al., 1992). To ascertain any detrimental effect on the Benson-Calvin cycle, the C_3 grass, wheat, was used as a control species; in contrast to *Sorghum* and maize, no inhibition of wheat leaf photosynthesis was observed (Fig. 1). A similar trend with excised wheat leaves was reported recently by Williams et al. (1992). Also supporting our findings with C_4 leaves is the response of the CAM plant *Bryophyllum fedtschenkoi* to the cytoplasmic protein synthesis inhibitors puromycin and CHX (Carter et al., 1991).

Finally, carbon isotope analysis performed on the air stream, which had passed over a CHX-treated *Sorghum* leaf, revealed a shift from the C_4 to the C_3 mode of photosynthesis. Recent short-term measurements in several C_4 species showed that carbon isotope discrimination was very stable with respect to CO_2 partial pressure and temperature (Henderson et al., 1992). This was interpreted as resulting from a close coupling between the C_4 and C_3 photosynthetic cycles. The observed CHX effect on *Sorghum* could support the idea that phosphorylation of PEPC represents a part of the coupling mechanism.

It has been established that (a) upon return to darkness, maize leaf PEPC protein kinase activity/PEPC phosphorylation is decreased to the original dark level within about 2 h and (b) feeding an illuminated *Sorghum* or maize leaf CHX resulted in the rapid blocking of PEPC protein kinase activity, thus resulting in dephosphorylation of PEPC in <4 h (Jiao et al., 1991a; Bakrim et al., 1992). These and the present results suggest that the activity of PEPC protein kinase in a C_4 mesophyll cell is dependent on a short-term protein synthesis event. Therefore, the inhibition kinetics of leaf CO_2 assimilation, as observed in the present work, are consistent with the time frame needed to inactivate this complex PEPC regulatory system. The apparent specificity of the inhibitory effect of CHX is further supported by the observations that both the PEPC type 2A protein phosphatase and other light-activated leaf protein kinases remain unaffected, as judged from in vivo and in vitro experiments, respectively (Jiao et al., 1991a; Bakrim et al., 1992). Phosphorylation increases both the catalytic activity and apparent K_i (L-malate) value of PEPC, but the latter is probably not sufficient to ensure full protection of the enzyme against the high malate concentration that prevails during maximum C_4 photosynthesis. It is assumed that antagonistic, positive effectors of PEPC, notably sugar-P, act in concert with the regulatory PEPC phosphorylation cycle to desensitize the enzyme to L-malate (Jiao and Chollet, 1992). Both effects would be required for PEPC to continue to carboxylate PEP at high rates in the presence of millimolar levels of the inhibitor.

The present results, however, indicate that PEPC phosphorylation would not simply be an additive protection effect because when this modification is specifically prevented in vivo by CHX treatment, the CO_2 assimilation rate is severely impaired. It was recently demonstrated that DL-glyceraldehyde, which blocks the Benson-Calvin cycle, also inhibits PEPC protein kinase activity/PEPC phosphorylation (Bakrim et al., 1992; Jiao and Chollet, 1992). It was thus inferred that the signal transduction pathway involves an intercellular signal emanating from the Benson-Calvin cycle, in the bundle sheath, presumably a photosynthesis-related metabolite. Recently, we obtained evidence to suggest that the light activation of PEPC kinase activity/PEPC phosphorylation depends on the prior alkalization of the C_4 mesophyll-cell cytosol (Pierre et al., 1992). The overall effect of alkalization is to improve enzyme activity by increasing both its phosphorylation status and V_{max} and decreasing its malate sensitivity. Along these lines, it can be speculated that inhibition of C_4 photosynthesis of *Sorghum* and maize by CHX is through a marked reduction of PEPC activity following dephosphorylation in the light and a decrease in cytosolic pH and enzyme V_{max}.

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