Role of Metabolites in the Reversible Light Activation of Pyruvate, Orthophosphate Dikinase in Zea mays Mesophyll Cells in Vivo

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ABSTRACT

Whole leaf and mesophyll cell concentrations of pyruvate, phosphoenolpyruvate (PEP), ATP, and ADP were determined in Zea mays during the reversible light activation of pyruvate, orthophosphate dikinase in vivo. Mesophyll cell levels of the four metabolites were estimated by extrapolation from values in freeze-quenched leaf samples that were fractionated by differential filtration through nylon mesh nets (adapted from M Stitt, HW Heldt [1985] Planta 164: 179–188). During the 5 minutes required for complete light activation of dikinase, pyruvate levels in the mesophyll cell decreased (from 166 ± 15 to 64 ± 10 nanomoles per milligram of chlorophyll [nmol/mg Chl]) while PEP levels increased (from 31 ± 4 to 68 ± 4 nmol/mg Chl, with a transient burst of 133 ± 16 nmol/mg Chl at 1 minute). Mesophyll cell levels of ATP increased (from 22 ± 4 to 48 ± 3 nmol/mg Chl) and ADP levels decreased (from 18 ± 4 to 7 ± 6 nmol/mg Chl) during the first minute of illumination. Upon darkening of the leaf and inactivation of dikinase, pyruvate levels initially increased in the mesophyll (from 160 ± 30 to a maximum of 625 ± 40 nmol/mg Chl), and then slowly decreased to about the initial value in the light over an hour. PEP levels dropped (from 176 ± 5 to 47 ± 3 nmol/mg Chl) in the first 3 minutes and remained low for the remainder of the dark period. Mesophyll levels of ATP and ADP rapidly decreased and increased, respectively, about twofold upon darkening. The trends observed for these metabolite levels in the mesophyll cell during the light/dark regulation of pyruvate, orthophosphate dikinase activity suggest that pyruvate and PEP do not play a major role in vivo in regulating the extent of light activation (dephosphorylation) or dark inactivation (ADP-dependent threonyl phosphorylation) of dikinase by its bifunctional regulatory protein. While the changes in ADP levels appear qualitatively consistent with a regulatory role for this metabolite in the light activation and dark inactivation of dikinase, they are not of a sufficient magnitude to account completely for the tenfold change in enzyme activity observed in vivo.

Pyruvate, orthophosphate dikinase (EC 2.7.9.1) catalyzes the conversion of pyruvate, ATP and Pi to PEP, AMP, and PPI during photosynthesis in the mesophyll chloroplasts of C₄ plants (14). The two-step catalytic reaction sequence is shown in the top line of Figure 1. The enzyme is regulated by a light-dependent, DCMU- and photophosphorylation inhibitor-sensitive activation (dephosphorylation) and dark-dependent inactivation (phosphorylation) cycle (1, 3, 21). The reactions of this cyclic cascade are catalyzed by a bifunctional stromal regulatory protein (4, 7, 23) which uses ADP as the substrate for phosphorylation and Pi as acceptor for dephosphorylation of a threonine residue in the active-site domain of dikinase (1, 3, 24) (Fig. 1). The form of dikinase that is inactivated (phosphorylated) by the regulatory protein is the enzyme catalytic intermediate, DK-HisP, which is phosphorylated on an essential histidine residue during partial catalysis with PEP or ATP and Pi (6). There are two forms of inactive dikinase that can be activated by the regulatory protein. The initial product of inactivation, DK-HisP-ThrP, is activated five times more slowly than the free histidyl form, DK-His-ThrP (5, 7). The dephosphorylation of the essential histidine residue can occur in vitro by either the dikinase half-reaction AMP + PPI → ATP + Pi, or simply by hydrolysis (Fig. 1). This half-reaction probably does not occur in vivo due to the high level of pyrophosphatase activity present in the C₄ mesophyll chloroplast (14).

The current model for the mechanism of dikinase activation (dephosphorylation) and inactivation (threonyl phosphorylation) (Fig. 1) shows no direct link with light. Several possible modes of regulation can be disallowed: (a) The regulatory protein does not appear to be regulated by a covalent modification. When partially purified from light-adapted leaves, it catalyzes both the activation and inactivation of dikinase (3). Similarly, desalted crude extracts from dark- or light-adapted leaves can be used to catalyze either reaction (4). (b) Changes in pH or [Mg²⁺] in the mesophyll chloroplast stroma are unlikely to alter the activation state of dikinase because the pH profiles of the regulatory protein-catalyzed activation and inactivation reactions are essentially identical and both reactions require Mg²⁺ (4, 7).

Another possibility is that the dikinase regulatory cascade is controlled by the availability of the substrates of the regulatory protein (Fig. 1). For example, one would predict that if the ratio of stromal [ADP] to [Pi] decreases in the light, then more of the dikinase will become activated. This prediction has been verified in part by the observation that the activation state of the dikinase in vitro increases with increasing adenylate energy charge (20). The other substrate of the regulatory
protein to consider is dikinase. The level of dikinase per se does not change rapidly in response to light. However, the amount of DK-HisP, the requisite enzyme form phosphorylated by the regulatory protein, available for inactivation could be decreased upon illumination by increasing the ratio of stromal [pyruvate] to [PEP]. Indeed, pyruvate has been shown to prevent the regulatory phosphorylation (inactivation) reaction in vitro (4, 8), consistent with its interaction with the requisite enzymic intermediate DK-HisP (Fig. 1). Alternatively, the amount of DK-HisP would be decreased by increasing the ratio of [AMP] plus [PPI] to [ATP] plus [Pi], but PPI levels in the C₄ mesophyll chloroplast are probably extremely low due to the high pyrophosphatase activity present (14).

In this paper we report the changes in ATP, ADP, pyruvate, and PEP levels in Zea mays whole leaves and MC cells in vivo during dark=light transitions. Leaf samples were freeze-quenched during the first 15 min of illumination and the first 70 min of darkness, during which time DK was activated and inactivated, respectively. The changes in the metabolite levels are compared with the changes in the activation state of dikinase.

**MATERIALS AND METHODS**

**Plant Material**

Zea mays L., cv B73Ht, was grown in a greenhouse with supplemental lighting (12-h photoperiod, 27 to 29°C day/21°C night) during March and April. Five- to 6-week-old plants were used. Plants were transported to two environmentally controlled growth chambers with the same temperatures and photoperiod 20 h prior to sampling. One growth chamber remained dark the day of the experiment, while the other continued to operate with the same photoperiod.

**Leaf Samples**

For the dark inactivation of dikinase, three leaves of similar age were excised from two plants which had been illuminated (600–800 µE/m²·s, PAR) for 5 to 8 h. The leaves were placed in a beaker of water and illuminated for 15 min at 1000 to 1200 µE/m²·s prior to being transferred to darkness for up to 70 min. After a given period in the dark at 24°C the leaves were rapidly deribbed and plunged into liquid N₂ in total darkness. For the light activation of dikinase, the plants were dark-adapted for 19 to 22 h. Individual excised leaves were placed in water and illuminated at 1000 to 1200 µE/m²·s and 26 to 28°C for up to 15 min in a trough made from a split polyvinylchloride pipe, which allowed the leaf to be freeze-quenched in liquid N₂ during illumination. The frozen leaf was deveined and poured into a beaker containing liquid N₂. The sample for a given illumination period consisted of two leaves from the same plant. The frozen samples were pulverized with a mortar and pestle and transferred to vials in liquid N₂ after which they were stored for up to 5 months at −80°C. Leaf material was fractionated into samples with different ratios of BSC and MC by differential filtration through nylon mesh nets of varying apertures (200-, 80-, and 40-µm [27, 28]). For the determination of dikinase activity, frozen leaf material (50–200 mg) was homogenized in 0.8 ml 50 mM Tris, 5 mM MgSO₄, 10 mM DTT, 5 mM pyruvate, 1% (w/v) BSA, 0.1% (v/v) Triton X-100 (pH 8.0) (homogenization buffer) with a ground-glass homogenizer. Dikinase activity was determined spectrophotometrically at 340 nm and 25°C (15) after removing a 150-µL aliquot for Chl determination (31) and centrifuging the remaining suspension for 5 s at room temperature.

**Marker Enzymes**

A small amount of frozen fractionated leaf material (about 20 mg) was homogenized in 500 µl of homogenization buffer minus pyruvate. An aliquot (150 µL) was removed for Chl determination (31), the remaining suspension centrifuged briefly, and the clarified solution either used immediately or frozen in a clean vial in liquid N₂ for subsequent determination of marker enzyme activity. One unit of activity (IU) corresponds to 1 µmol per min at 25°C.

NADP-malic enzyme activity (bundle-sheath marker) was measured spectrophotometrically at 340 nm with 25 mM malate, 25 mM MgSO₄, 50 mM Bicine, 0.5 mM NADP, and 1 mM EDTA (pH 8.3). PEP carboxylase activity (mesophyll marker) was measured spectrophotometrically at 340 nm with 2.5 mM PEP, 10 mM NaHCO₃, 5 mM MgSO₄, 1 mM glucose 6-phosphate, 50 mM Bicine, 1 mM DTT, 1 mM EDTA, 0.2 mM NADH, and 5 IU/mL malate dehydrogenase (pH 8.3). Ribulosebisphosphate carboxylase activity (bundle-sheath marker) was measured radiochemically. The enzyme was activated for 20 min in 20 mM NaHCO₃, 20 mM MgSO₄, 50 mM Tris (pH 8.3). The assay solution had final concentrations of 10 mM NaHCO₃, 10 mM MgSO₄, 0.4 mM ribulosebisphosphate (pH 8.3). After 30 s the reaction was quenched with 3 N formic acid in methanol. The solution was dried in an oven at 30°C and acid-stable [¹⁴C]-dpm determined. NADP-malate dehydrogenase activity (mesophyll marker) was measured spectrophotometrically at 340 nm in 2.5 mM oxaloacetate, 0.2 mM NADPH, 50 mM Chs, 1 mM EDTA (pH 9.0). Chl was determined in 96% (v/v) ethanol (31).

**Metabolites**

Frozen fractionated leaf material (200–300 mg) was homogenized in 700 µL of 10% (v/v) HCIO₄ at 4°C. The ground-
glass homogenizer was rinsed with three or four 200-μL aliquots of 10% HClO₄ and the extract centrifuged. The pellet was rinsed with 200 μL of 2% HClO₄ and stored in a refrigerator for subsequent measurement of Chl as pheophytin (31). The combined supernatant fluids were adjusted to pH 7.0 to 7.1 with 3 M K₂CO₃, 0.2 M Mops (pH 12). After removing the precipitated KClO₄ by centrifugation, a 200-μL sample was stored at −80°C for subsequent measurement of ATP and ADP concentrations. Activated charcoal (20 mg/mL) was added to the remaining supernatant fluid, which was then centrifuged and stored at −80°C. Aliquots of standard solutions of each metabolite to be measured were added to two of the 10% HClO₄ solutions prior to homogenization of the frozen fractionated leaf material. Pyruvate showed 103% recovery, PEP 95%, ATP 105%, and ADP 102%.

Pyruvate and PEP concentrations were measured in a Cary model 219 double-beam spectrophotometer. Samples (600 μL) of the neutralized, decolorized supernatant fluids were mixed with assay solution (400 μL), bringing the final concentrations to 50 mM Mops, 5 mM MgSO₄, 30 mM KCl, 5 mM ADP, and 0.2 mM NADH (pH 7.4). The pyruvate assay was initiated by the addition of 5 IU (10 μL) lactate dehydrogenase. The endpoint of this reaction was verified to have reached at least 98% completion by the addition of a standard solution of pyruvate to the plant extract. The PEP assay was initiated in the same cuvette after the endpoint of the pyruvate assay had been reached by the addition of 6 IU (10 μL) pyruvate kinase. All changes in absorbance at 340 nm were corrected for the minor dilution upon the addition of the coupling enzyme(s).

ATP and ADP were measured using a luciferin/luciferase-based assay. Measurements were done in a Perkin-Elmer MPF-44 spectrofluorometer with the emission monochromator set at 0, the slit width at 20 nm, and the dynode voltage at 1000 V. Either an adenine nucleotide standard solution or dilute, neutralized plant extract (200 μL) was injected into the assay solution (1 mL) through a hole in the lid of the instrument. Emission versus time was recorded as a peak, whose height was proportional to [ATP]. The stock assay solution consisted of 250 mg firefly lantern extract (No. FLE-50, Sigma Chemical Co.) in 125 mL 40 mM Mops, 15 mM MgSO₄ (pH 7.4). The solution was centrifuged after stirring for 3 h, after which luciferin (3.7 mg) was added to the supernatant fluid. The peak heights of standards (0.5–5.5 μM ATP) were constant for up to 12 h. The concentration of the ATP standard solution was determined with 4 IU hexokinase and 1 IU glucose 6-phosphate dehydrogenase in 3 mL 50 mM Tris, 5 mM glucose, 1.5 mM MgSO₄, 0.3 mM NADP (pH 8.0). The concentration of the ADP standard was determined with pyruvate kinase and lactate dehydrogenase (see PEP assay described above, employing 1.5 mM PEP instead of 5 mM ADP). The standard lines for the luciferase assay had a correlation coefficient of at least 0.993. The neutralized extracts were diluted with 50 mM Mops, 5 mM K₂SO₄, 5 mM MgSO₄ (pH 7.4) (usually 50 μL: 600 μL, respectively) to prevent quenching of the signal as verified by the addition of 0.5 μM ATP. ADP was converted to ATP by diluting the extract as above, but including 0.1 mM PEP and 4 IU/mL pyruvate kinase. The solution was incubated for 30 min at 30°C prior to measurement of ADP as ATP.

RESULTS

Determination of MC Metabolite Levels by Differential Filtration

Freeze-quenched Z. mays leaf tissue was pulverized with a mortar and pestle and fractionated using various sizes of nylon mesh nets (27, 28). The extent of separation of the MC and BSC was determined by measuring the activities of several marker enzymes in each fraction (Table I). The enzyme activities in each fraction are expressed as a percent of the total units recovered. It should be noted that both BSC marker enzymes cofractionated, as did the two MC markers, thus verifying the reproducibility of the fractionation technique (Table I).

The equation derived for calculating the level of a given metabolite in the MC is based on the assumption that the metabolites of interest are located entirely in the MC and BSC. The amount measured (e.g. PEPobs) can be expressed as a weighted average of the concentration in the two cell types:

\[
P_{\text{PEP}}^{\text{obs}} = \frac{\text{mg Chl}_{\text{MC}}}{\text{mg Chl}_{\text{BSC}} + \text{mg Chl}_{\text{BSC}}} + \frac{\text{mg Chl}_{\text{BSC}}}{\text{mg Chl}_{\text{MC}} + \text{mg Chl}_{\text{BSC}}} \]

(1)

where PEPobs is expressed in nmol/mg Chl. The measured marker enzyme activities (expressed in IU/mg Chl) can be described similarly by Eqs. 2 and 3:

\[
P_{\text{PEP}}^{\text{MC}} = \frac{\text{mg Chl}_{\text{MC}}}{\text{mg Chl}_{\text{MC}} + \text{mg Chl}_{\text{BSC}}} \]

(2)

\[
\text{NADP-ME}_{\text{obs}} = \frac{\text{mg Chl}_{\text{BSC}}}{\text{mg Chl}_{\text{MC}} + \text{mg Chl}_{\text{BSC}}} \]

(3)

where PEPobs and NADP-MEobs are the measured activities of PEP carboxylase and NADP-malic enzyme, respectively, and PEPMC and NADP-MEMBSC are the theoretical values of these two marker enzyme activities in pure MC and BSC, respectively. These enzyme activities can be related to each other as expressed in Eq. 4:

\[
P_{\text{PEP}}^{\text{obs}} = \frac{\text{NADP-ME}_{\text{obs}}}{\text{NADP-ME}_{\text{BSC}}} \]

(4)

From the equations given above, one can derive a linear equation relating metabolite levels to marker enzyme activities, as shown, for example, in Eq. 5:

\[
P_{\text{PEP}}^{\text{obs}} = \left[\frac{\text{NADP-ME}_{\text{obs}} - \text{NADP-ME}_{\text{BSC}}}{\text{NADP-ME}_{\text{BSC}}}\right] \text{NADP-ME}_{\text{obs}} + \text{NADP-ME}_{\text{BSC}} \]

(5)

One can estimate the level of a given metabolite in the MC by plotting, for example, the observed activity of the bundle-sheath marker NADP-malic enzyme (NADP-MEobs in IU/mg Chl) versus the experimentally determined level of PEP in the same fraction (PEPobs in nmol/mg Chl) and extrapolating to zero malic enzyme activity, i.e. no BSC contamination (see Figure 2 for estimation of PEPMC). The results presented here
were calculated using a linear regression least-squares best fit computer program. The metabolite values thus obtained are presented as nmol per mg Chl. In contrast to equations previously presented (12, 28), the derivation shown here takes into account the difference in distribution of total leaf Chl between maize MC and BSC. (This value does not have to be determined directly, however, because it drops out of the final equation [Eq. 5].) This is important when the concentration of the metabolites and marker enzyme activities are expressed per mg Chl.

One can derive a similar equation to solve for metabolite levels in the BSC. We found, however, that the range of PEP carboxylase activity in the various leaf fractions (14–21 IU/mg Chl) was too small to give values without substantial error. It is also possible to use these equations to estimate a given marker enzyme’s activity in the MC and BSC. We found NADP-EME_{BSC} to be 29 ± 2 IU/mg Chl and PEP_{MC} to be 27 ± 2 IU/mg Chl.

**Light/Dark Effects on Dikinase Activity**

The kinetics of light activation and dark inactivation of DK are shown in Figure 3. Dikinase activity in each leaf sample was measured under optimal conditions and therefore does not represent enzyme activity in vivo. It reflects, instead, the activation state of dikinase, i.e. the extent of threonyl phosphorylation, at each given time point. Light activation was complete after 3 min at the light intensity employed (approximately half of full sunlight), whereas dark inactivation occurred over at least 70 min (Fig. 3, A and B, respectively).

**Table I.** Distribution of MC and BSC Marker Enzyme Activities

<table>
<thead>
<tr>
<th>Marker Enzyme*</th>
<th>Aperture of Net (μm)</th>
<th>% recovered activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADP-malic enzyme (BSC)</td>
<td>200 80 40 0</td>
<td>31 35 21 14</td>
</tr>
<tr>
<td>Ribulosebisphosphate carboxylase (BSC)</td>
<td>32 36 18 14</td>
<td></td>
</tr>
<tr>
<td>PEP carboxylase (MC)</td>
<td>22 19 29 31</td>
<td></td>
</tr>
<tr>
<td>NADP-malate dehydrogenase (MC)</td>
<td>21 19 31 29</td>
<td></td>
</tr>
</tbody>
</table>

* BSC, BSC marker; MC, MC marker.

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Figure 2. PEP levels versus NADP-malic enzyme activity in freeze-quenched Z. mays leaf material fractioned by nylon mesh nets. The level of PEP in the MC (PEP_{MC}) is estimated by extrapolation to zero malic enzyme activity, i.e. no bundle-sheath contamination (see text for discussion).

Figure 3. Light activation (A) and dark inactivation (B) of DK in excised Z. mays leaves. Light activation was performed at 1000 to 1200 μE/m^2·s, PAR. For each data point, n = 3. The standard deviations are less than 0.1 IU DK/mg Chl.

**Pyruvate and PEP Levels**

Pyruvate and PEP levels in MC and the whole Z. mays leaf during the initial 15 min of illumination are shown in Figure 4, whereas those during the 70-min dark inactivation period are given in Figure 5. In general, parallel trends are seen in the MC and whole leaf for these two metabolites. A comparison of the pyruvate levels after 15 min of illumination (Fig. 4A) with those obtained at the beginning of the dark period after at least 5 h of light (0-min values in Fig. 5A) suggests that with extended illumination, the concentration of pyruvate continues to increase, with the largest increase occurring in the BSC. PEP concentrations in the whole leaf and MC are comparable and undergo similar changes upon illumination and darkening. PEP levels show a marked burst during the first 1 to 2 min of illumination of a dark-adapted leaf (Fig. 4B). PEP concentrations decreased to about 80 nmol/mg Chl during 3 to 15 min of illumination (Fig. 4B) but, like pyruvate, would appear to increase during prolonged illumination (0-min values in Fig. 5B). PEP levels in both the whole leaf and MC dropped rapidly upon darkening the leaf (Fig. 5B).

The maximum and minimum possible pyruvate levels in the mesophyll chloroplast during the 15-min illumination...
extrapolation as of extrapolation as described in the text and in Figure 2. For each data point, n = 3 to 5. The error bars represent 1 sd. The theoretical minimum pyruvate concentration possible in the mesophyll chloroplast (A, - - -) was calculated based on the rates at which pyruvate is transported into the chloroplast stroma (22) and converted to PEP by dikinase (see text for discussion).

Figure 4. Pyruvate (A) and PEP (B) levels in Z. mays whole leaves and MC during light activation of dikinase. Levels in the whole leaf were measured directly, whereas those in the MC were estimated by extrapolation as described in the text and in Figure 2. For each data point, n = 3 to 5. The error bars represent 1 sd. The theoretical minimum pyruvate concentration possible in the mesophyll chloroplast (A, - - -) was calculated based on the rates at which pyruvate is transported into the chloroplast stroma (22) and converted to PEP by dikinase (see text for discussion).

Figure 5. Pyruvate (A) and PEP (B) levels in Z. mays whole leaves and MC during dark-inactivation of dikinase. For details, see the legend to Figure 4. Any missing error bars were smaller than the corresponding square or circle.

Period were estimated (Fig. 4A). The maximum level was when all of the MC pyruvate was in the chloroplast. The calculation of the minimum level was based on the reported kinetic properties \( V_{max} \) and \( K_m \) (pyruvate) of the pyruvate carrier in the mesophyll chloroplast envelope of the C4 plant Panicum miliaceum (22) and the activity of dikinase, which could range from near-zero to the maximum activity determined under optimal assay conditions (Fig. 3A). The concentration of pyruvate in the chloroplast at any given time is assumed to be equal to an initial amount plus the amount transported into the stroma minus the amount converted to PEP by dikinase. Iterative calculations allowed for changes in rates over time. The initial amount in the mesophyll chloroplast prior to illumination was assumed to be equal to the amount in the MC (22). The rate of transport was calculated from \( V_{max} = 47 \) nmol/mg Chl·s, which is a fivefold increase over the reported value (22) to account for an increase in rate as the temperature is increased from 4°C to 25 to 30°C (a fivefold increase in rate corresponds to an activation energy for transport of 13 kcal/mol). The concentration of pyruvate in the mesophyll cytoplasm, which must be known in order to calculate the rate of transport, was estimated from the difference between the mesophyll chloroplast and MC concentrations, assuming equal volumes of the cytoplasm and chloroplast.

**ATP and ADP Levels**

MC and whole leaf concentrations of ATP and ADP increased and decreased, respectively, approximately twofold within the first minute after the light was turned on (Fig. 6).
To explain the decrease in ATP concentration in the MC from 1 to 5 min of illumination we propose that there is an increase in metabolic flux, for example, a rapid conversion of ATP to AMP by the light-activated dikinase (Fig. 3). The accompanying increase in [ADP] that would one expect due to the high activity of adenylate kinase in the C₃-mesophyll chloroplast (14) would be within the standard deviations shown in Figure 6. Unfortunately, the lower levels of AMP present prevented its quantitative measurement by the method used here. The opposite trends in [ATP] and [ADP] occur upon darkening, with ATP decreasing and ADP increasing (Fig. 7). Again, during the first 3 min of darkness there appears to be a burst of MC ADP as ATP is rapidly metabolized before adenylate levels reach a new steady state.

**DISCUSSION**

The regulatory threonyl phosphorylation of DK (Fig. 1) is a monocyclic cascade (25) in which the covalent modification and demodification system is assumed to be a continuous process. This presumably allows the extent of activation of dikinase to be attuned to the metabolic needs of the C₃ carbon-fixation pathway. As the cascade is currently defined (Fig. 1), the most likely means of altering the activation state of the target enzyme, dikinase, is to change the concentrations of the metabolites involved in the regulatory cycle. Besides the obvious choices of the nonprotein substrates ADP and Pi, pyruvate and PEP must also be considered because they can alter the concentration of the requisite form of the protein substrate, DK-HisP, used by the regulatory protein during the inactivation/phosphorylation reaction (Fig. 1). This role of a target enzyme's substrate serving as an effector in a cascade deserves attention in other systems besides that of dikinase because it is likely that, in general, regulatory proteins will use a particular form of the target enzyme (free enzyme, enzyme-substrate complex, or, as in the case of dikinase, an enzyme intermediate [Fig. 1]) as the substrate. In all cases the protein substrate concentration will be affected by its own substrates, products, and/or effectors.

A possible role of pyruvate in decreasing the extent of inactivation (phosphorylation) of dikinase has been explored both with purified DK in a reconstituted system (4, 8) and with isolated intact mesophyll chloroplasts from Z. mays (8). As expected, the addition of pyruvate markedly decreased the extent of inactivation (phosphorylation) of dikinase, presumably by reacting with the requisite DK-HisP form of the enzyme to yield PEP and DK (Fig. 1). These in vitro observations do not, however, demonstrate the necessity of a role for pyruvate in vivo. The results presented here show that pyruvate is not responsible for the light activation of dikinase in Z. mays Mc in vivo. During the initial 3 min of illumination required to fully activate the enzyme (Fig. 3A), the level of pyruvate in the whole leaf dropped dramatically (Fig. 4A), consistent with some previously reported observations (11, 19), but not others (8). The reason for this discrepancy with the maize whole-leaf study of Burnell et al. (8) is not known, but may be due to the low light intensity (80 W·m⁻²) used by these workers for light activation. Pyruvate levels in the MC, while slightly higher than in the whole leaf on a Chl basis, show a similarly dramatic downward trend during the light-activation process (Fig. 4A). The concentrations of pyruvate in the stroma of the mesophyll chloroplast are of greatest interest since this is where the dikinase and regulatory protein are located. Unfortunately, these levels cannot be measured in vivo using aqueous fractionation techniques (cf. 29, 30). Instead, maximum and minimum values were calculated (Fig. 4A). The maximum amount of pyruvate possible in the mesophyll chloroplast is, of course, all of the pyruvate measured in the MC. The theoretical minimum value was calculated based on the estimated rate of pyruvate transport into the chloroplast by the light-activated pyruvate carrier (16, 17, 22) and the rate at which pyruvate is converted to PEP by dikinase. In order to minimize the pyruvate concentration in the mesophyll chloroplast, dikinase activity was maximized for a particular activation state as determined by assaying the enzyme at each time point under optimum conditions. In either extreme case, the levels of pyruvate in the mesophyll chloroplast decrease dramatically throughout the light activation of dikinase (Fig. 4A). It is therefore highly unlikely that the actual level of pyruvate in the mesophyll chloroplast stroma increases. If pyruvate were to prevent inactivation of dikinase in the light in vivo, its concentration would have to increase. Concomitant with the marked decrease in pyruvate,
PEP concentrations show a burst over the first few minutes of illumination in both the whole leaf (Fig. 4B) (8, 11) and the MC (Fig. 4B). These opposing changes in pyruvate and PEP levels are most readily explained by a rapid increase in dikinase activity compared with the rates of pyruvate synthesis and transport, and PEP utilization. While the level of PEP in the mesophyll chloroplast stroma is not known under the conditions used here, one would expect a rapid exchange out of the chloroplast via the C₄-phosphate translocator (9, 16). In this regard, recently published work on the level of various metabolites in nonaqueously purified maize mesophyll chloroplasts indicates that only about 35% of the PEP in a leaf illuminated at 600 to 700 μE/m²·s is in the mesophyll chloroplast (29).

If pyruvate (or PEP) is involved in modulating the dark inactivation of dikinase in vivo, then there should be a decrease in the ratio of pyruvate to PEP during the initial stages of darkness, thus favoring the formation of DK-HisP (Fig. 1). Both whole leaf and MC show the exact opposite trend (Fig. 5) (8, 19), which is most readily interpreted as being due to a decrease in dikinase activity relative to the other steps in the C₄ pathway. The pyruvate concentration in the mesophyll chloroplast in the dark is the same as in the MC (22). It is also noteworthy that the PEP and pyruvate levels change rapidly upon darkening when compared with the rate of dark inactivation of dikinase (see Figs. 5 and 3B, respectively). This would suggest that despite the relatively low extent of inactivation and, hence, threonyl phosphorylation that occurs during the first 5 min of darkness, dikinase activity in the leaf is lowered by additional factors (i.e. dikinase in the mesophyll chloroplast is not operating under the optimum conditions at which it was assayed in Figure 3, but has its activity lowered by, perhaps, a change in stromal pH or an increase in the concentration of inhibitors).

A potentially more likely candidate for a metabolite that links the dikinase regulatory cascade to light is ADP. A 10-fold increase in dikinase activity in a maize mesophyll chloroplast stromal extract plus 2 mm Pi has been shown to require an increase in adenylate energy charge from 0.60 to 0.98 (20). The corresponding decrease in ADP concentration can be calculated from a graph of adenylate energy charge versus ADP/(ATP + ADP + AMP) (e.g. ref. 2). If one assumes that the adenylates in the C₃-mesophyll chloroplast stroma are at equilibrium due to the high levels of endogenous adenylate kinase (14) with an equilibrium constant of 2.8 (ADP/AMP·ATP) (26), then the above 63% increase in adenylate energy charge (20) would correspond to a 14-fold decrease in [ADP] (if Kₑ = 2.0, the [ADP] would decrease 10-fold). The decrease in [ADP] upon illumination of a Z. mays leaf would probably have to be even greater to activate dikinase because [Pi] is thought to decrease upon illumination, at least in C₃ plants (10, 13).

There is little precedent for such an order of magnitude change in ADP levels in mesophyll chloroplasts during dark/light transitions (26). The results in Figure 6 show only about a 2-fold decrease in the mean [ADP] in both the whole leaf and mesophyll cell upon illumination, which, even with the large standard deviation, is not of a sufficient magnitude to increase the observed activation state of dikinase 10-fold (Fig. 3A). The corresponding adenylate energy charges in the mesophyll cell are 0.7 in the dark and 0.9 in the light, assuming equilibrium due to adenylate kinase with a Kₑ of 2 to 3. The values for [ATP] and [ADP] after 15 min in the light are in good agreement with those previously reported for Z. mays (27), except for the difference in the mesophyll concentration of ADP (about 8 ± 6 nmol/mg Chl [Fig. 6], compared to 3 ± 1 nmol/mg Chl [27]). The decrease in [ADP] seen in the MC upon illumination (Fig. 6) is presumably a reflection of a decrease in its concentration in the mesophyll chloroplast, if, as in C₄ protoplasts, the lower concentration of cytoplasmic ADP does not change (26). However, direct parallels between the intracellular distribution of adenylates in C₃ and C₄ mesophyll cells may be unfounded because there is some evidence for a chloroplastic ATP translocator in C₄ plants that is not present in most C₃ species (16).

The metabolite-related data presented in this paper imply that the 10-fold changes in DK activity seen upon light-dark transitions of Z. mays leaves cannot be ascribed solely to the concomitant changes in the MC concentrations of pyruvate, PEP, or ADP. The direction of change in the pyruvate and PEP levels is the exact opposite of that necessary for light activation and dark inactivation of dikinase. Rather, the observed changes in the level of this substrate/product pair can be interpreted as being caused by the changes in dikinase activity. The increase in [ADP] upon darkening light-adapted leaves (Fig. 7), while changing in the right direction for favoring inactivation/threonyl phosphorylation of dikinase, appears too small to be responsible for the large decrease in dikinase activity (even when the large standard deviation is taken into account). Obviously, more detailed information on the mesophyll-chloroplast stromal levels of ADP and Pi would strengthen this argument. While the metabolites studied here certainly have a role in determining the activation state of dikinase, the lack of strong correlational results suggests that another, as yet unidentified effector(s) or mechanism(s) is the major link between light and the activation state of DK in vivo. Unfortunately, there is currently no supportive evidence for the differential regulation of the bifunctional dikinase regulatory protein by effectors, pH, [Mg++] or covalent modification (4, 7).

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LITERATURE CITED


* Along these lines, Usuda (30) has recently reported that there is no significant correlation in vivo between the activation-state of dikinase and the total (i.e. free plus bound) stromal levels of ADP, Pi, ATP/ADP or adenylate energy charge at various light intensities in nonaqueously purified maize mesophyll chloroplasts.


