Inhibition of Photorespiration and Increase of Net Photosynthesis in Isolated Maize Bundle Sheath Cells Treated with Glutamate or Aspartate

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ABSTRACT

Net photosynthetic 14CO2 fixation by isolated maize (Zea mays) bundle sheath strands was stimulated 20 to 35% by the inclusion of L-glutamate or L-aspartate in the reaction mixture. Maximal stimulation occurred at a 7.5 mM concentration of either amino acid. Since the photosynthetic rate and the glutamate-dependent stimulation in the rate were equally sensitive to a photosynthetic electron transport inhibitor, 3-(p-chlorophenyl)-1,1-dimethylurea, it was concluded that glutamate did not stimulate CO2 fixation by supplying needed NADPH (NADH) through glutamate dehydrogenase. Treatment of the bundle sheath strands with glutamate inhibited glycolate synthesis by 59%. Photorespiration in this tissue, measured as the O2 inhibition of CO2 fixation (the Warburg effect), was inhibited by treatment with glutamate. The stimulation in net photosynthetic CO2 fixation probably results from the decrease in photorespiratory CO2 loss. This metabolic regulation of the rate of glycolate synthesis and photorespiration observed with isolated bundle sheath strands could account for the inability to detect rapid photorespiration in the mature intact maize leaf.

In mature maize leaves CO2 fixation occurs in the mesophyll cells which have C3 photosynthesis and the bundle sheath cells which have C4 photosynthesis. Isolated maize bundle sheath strands exhibit the photorespiratory properties that are typical of all C4 plants (11, 19). Glycolate is synthesized and metabolized in the isolated tissue at measurable rates (2, 4) and net photosynthetic CO2 fixation is reversibly inhibited (3) by elevated O2 concentrations (the Warburg effect). In the intact leaf, photorespiration is either severely limited or nonexistent. Glycolate synthesis occurs in maize at less than 10% of the rate in tobacco and may involve a different mechanism (20). As a result, net photosynthetic CO2 fixation in corn is not inhibited by atmospheric levels of O2 (6).

Since maize bundle sheath strands have the full complement of enzymes needed to synthesize and metabolize glycolate, yet photorespiration does not occur in intact corn leaves, the enzymes involved may be inhibited in situ. High CO2 levels and low O2 levels have been suggested as possible mechanisms for inhibiting glycolate synthesis by the bundle sheath tissue in the intact corn leaf (8).

We have reported earlier that certain metabolites normally found in plant tissue, including glutamate and aspartate, could severely inhibit the rate of glycolate synthesis in tobacco leaf discs (16) and suggested that this mechanism of regulation could help account for the low photorespiration in C4 species. This paper extends these findings to isolated maize bundle sheath strands and demonstrates that glycolate synthesis in this tissue is inhibited by these amino acids at concentrations similar to those found in the bundle sheath cells (7) as well as by low O2 and high CO2 levels.

MATERIALS AND METHODS

Maize plants were grown in a greenhouse for 3 weeks and bundle sheath strands were isolated by a procedure adapted from Hatch and Kagawa (9). Primary and secondary corn leaves (9 g, midribs removed) were held below the surface of 50 ml of the cold grinding solution (20 mM HEPES-KOH [pH 6.7], 0.3 mM sorbitol, 2 mM MgCl2, 2 mM KH2PO4, and 2 mM sodium isosaccharinate) and sliced transversely into 1- to 2-mm sections with a razor blade. The tissue was then homogenized in 30 ml of the grinding solution for 15 sec in a Monel head of a Waring Blender at full line voltage. The slurry was quickly filtered through four layers of cheesecloth and the corn bundle sheath tissue placed in an enzyme mixture containing 0.2 g of cellulysin (Calbiochem), 0.2 g of hemicellulase (Sigma), and 0.2 g of pectinase (Sigma) in 20 ml of the grinding solution. After 30 min at room temperature the bundle sheath tissue was removed by filtration through four layers of cheesecloth and blended in 30 ml of the grinding solution for 5 sec. The bundle sheath tissue was then filtered once more through four layers of cheesecloth and rinsed thoroughly with 100 ml of the assay solution and resuspended in 20 ml of the same solution.

Bundle sheath strands prepared by this method were essentially free of mesophyll contamination as determined by light microscopy and showed little P-enolpyruvate carboxylase activity, an enzyme found in the mesophyll. The tissue was stored at 0 C and used within 30 min.

Photosynthesis and glycolate synthesis were measured in 10-ml Fernbach flasks (Belco Corp.) containing a final volume of 2 ml of the assay solution which was composed of 8 mM HEPES-KOH (pH 7.3), 0.25 mM sorbitol, 1.6 mM MgCl2, 1.6 mM K2HPO4, and 5 mM ribose-5-P. Bundle sheath tissue containing 40 to 70 μg of Chl was placed in the flask in an illuminated (340 μE m−2 sec−1, 400–700 nm) water bath (30 C) and stirred at 120 cycles/sec. After 5 min 20 μl of 0.1 M NaH14CO3 (1 μCi/μmol) for CO2 fixation experiments or 0.2 ml of 0.1 M α-hydroxy-2-pyridine methanesulfonic acid (pH 7.3), to inhibit glycolate oxidase and thus measure glycolate synthesis, was added. Following an additional 15-min period in the light with either NaH14CO3 or the sulfonate, the flasks were removed to a hot plate and 1.0 ml of ethanol added. After the solution boiled (about 15 sec), 5 ml of acetone was added to each flask. The Chl contained in the bundle sheaths was determined after centrifuging the strands from the solution.

Incorporation of 14CO2 was measured by liquid scintillation counting of samples (0.5–1.0 ml) which were first acidified with
of NADP-malic enzyme. Due to the faulty PSII activity (and therefore presumably NADPH production) of isolated maize bundle sheath chloroplasts (1, 12, 13), any reaction which supplies NADPH to the tissue should stimulate photosynthetic CO₂ fixation. Although glutamate dehydrogenase is normally assumed to favor glutamate production, the reaction is reversible and could also lead to NADPH (NADH) formation.

In order to assess the possibility that an increase in NADPH (NADH) produced by glutamate dehydrogenase was responsible for the stimulation in CO₂ fixation observed with added glutamate, the sensitivities of both the basal photosynthetic rate and the glutamate-dependent stimulation in this rate to increasing concentrations of a photosynthetic electron transport inhibitor, CMU, were compared (17). The two activities (both the basal rate and the stimulation) were inhibited in a similar manner with increasing CMU concentrations (Fig. 2). This contrasts with the stimulation in CO₂ fixation by isolated maize bundle sheath tissue caused by malate, a compound whose dehydrogenation is linked to NADPH production, which is much less sensitive than the basal CO₂ fixation rate of the tissue to photosynthetic electron transport inhibitors (18). Since only NADPH production by photosynthetic electron transport would be expected to be sensitive to CMU, this experiment probably indicates that glutamate (unlike malate) is not stimulating net photosynthetic CO₂ fixation by supplying NADPH through the activity of glutamate dehydrogenase. This is further supported by the observation of Rathnam and Edwards (17) with another NADP-malic enzyme plant, Digitaria sanguinalis, that aspartate could not supply reducing equivalents for CO₂ fixation.

Glutamate (7.5 mM) inhibited glycolate accumulation in the presence of α-hydroxy-2-pyridinemethanesulfonic acid (Table 1). Bundle sheath strands in the standard reaction mixture synthesized glycolate at a rate of 2.07 μmol/mg of Chl·hr while those in the reaction mixture with 7.5 mM glutamate synthesized glycolate at 0.84 μmol/mg of Chl·hr. Glutamate treatment inhibited glycolate accumulation in the presence of the sulfonate by 59%. The rate of photosynthetic CO₂ fixation was two to five times greater than glycolate synthesis in this tissue on a molar basis. This is considerably different from the approximately equal rates seen with tobacco leaf discs (15, 16). The lower relative rates of glycolate synthesis found with bundle sheath strands probably result from the limited uptake of the ionized sulfonate at the pH (7.3) of the assay. These rates of glycolate synthesis are similar to those reported earlier by others (2, 4).

Additional support for the hypothesis that glutamate and aspartate are stimulating photosynthesis in isolated maize bundle sheath strands by inhibiting glycolate synthesis and therefore photosrespiration comes from an investigation of the effect of these two amino acids on the O₂ sensitivity of net photosynthesis (the Warburg effect). The Warburg effect is a measure of photosrespi-

**FIG. 1.** Effect of glutamate, aspartate, and glyoxylate on the net rate of photosynthetic ¹⁴C fixation by bundle sheath strands isolated from corn. Bundle sheath tissue was isolated and photosynthesis measured as described under "Materials and Methods." Each reaction vessel contained 1 mM NaH¹⁴CO₃ and 5 mM ribose-5-P. Atmosphere in the flasks was 21% O₂/79% N₂. Control rates were: 4.6 μmol of CO₂/mg of Chl·hr for the glutamate experiment (■); 5.4 for the aspartate experiment (○); and 7.2 for the glyoxylate experiment (▲).

0.2 ml of 10 N acetic acid and then dried in a 90 C oven. In order to measure glycolate accumulation, the acetone extract and the supernatant from the two 5-ml water washes of the homogenized bundle sheath strands were combined and the glycolate fraction isolated by column chromatography on Dowex 1-acetate (16). Glycolate was determined colorimetrically. In all cases where glycolate synthesis was being measured a correction was made for the color yield obtained with an identical sample of the bundle sheath preparation without the sulfonate.

In the experiments where the atmosphere over the reaction mixture was controlled, the flasks were flushed at 150 ml/min with the mixture described for the entire light period through a serum stopper fitted with two hypodermic needles.

**RESULTS AND CONCLUSIONS**

Glutamate and aspartate were added to isolated maize bundle sheath strands to see if, as with tobacco leaf discs (16), glycolate synthesis and photosrespiration would be inhibited and net ¹⁴CO₂ fixation stimulated. Stimulation of the rate of net photosynthetic ¹⁴CO₂ fixation up to a maximum of 20 to 35% was indeed observed for either metabolite (Fig. 1). Both amino acids showed maximal stimulation at about 7.5 mM with less stimulation or even inhibition of the photosynthetic rate at higher concentrations. These similarities suggest that the two amino acids may affect net photosynthesis in the bundle sheath tissue by the same mechanism. This similarity in mode of action was also noted for the effects of glutamate and aspartate on glycolate synthesis and net photosynthesis in tobacco leaf discs (16). Glyoxylate, a metabolite that inhibits glycolate synthesis and stimulates net photosynthesis in tobacco leaf discs (14, 15), did not increase the net photosynthetic rate of isolated corn bundle sheaths (Fig. 1). In order to avoid inhibiting photosrespiration in these tissues, rate-limiting concentrations of NaH¹⁴CO₃ were employed. When saturating NaH¹⁴CO₃ (10 mM) was used, the photosynthetic rate ranged between 30 and 40 μmol/mg of Chl·hr.

Malate was reported to stimulate net photosynthetic ¹⁴CO₂ fixation in isolated maize bundle sheath tissue about 3-fold (9). The reason for at least the majority of this much larger stimulation was believed to be the production of NADPH through the action

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1 Abbreviation: CMU: 3-(p-chlorophenyl)-1,1-dimethylurea.
Glycolate accumulation in the presence of 10 mM α-hydroxy-2-pyridinemethane sulfonic acid was assayed as described in MATERIALS AND METHODS. The atmosphere in the reaction vessels was 21% O_2:79% N_2. The glutamate concentration in the tissue was determined by ninhydrin analysis of the glutamate fraction from a Dowex 1-acetate column (14). The samples were treated in the same manner as those used to measure glycolate synthesis except no sulfamate was added and the strands were washed 3 times with 10 ml water before killing. The glutamate concentration supplied initially was 7.5 mM and the results shown are the means of three independent experiments.

<table>
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<th>Glutamate in Tissue</th>
<th>Rate of Glycolate Synthesis</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>mM</td>
<td>µmol/mg Chl-hr</td>
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<tr>
<td>H_2O</td>
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<tr>
<td>Glutamate</td>
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Table II. The effect of glutamate on the O_2 sensitivity of CO_2 fixation by isolated maize bundle sheath strands.

Photosynthetic CO_2 fixation was measured as described in MATERIALS AND METHODS. The final NaHCO_3 concentration was 1 mM. All samples contained 5 mM ribose-5-phosphate, and where indicated the flasks contained 7.5 mM glutamate. The samples were flushed as indicated with either 100% O_2 or 3% O_2:97% N_2 at a rate of 150 ml/min. The results shown are the means of four independent experiments.

<table>
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<tr>
<th>Treatment</th>
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<th>Stimulation 100% O_2</th>
<th>3% O_2</th>
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<tr>
<td></td>
<td>µmol/mg Chl-hr</td>
<td>%</td>
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</tr>
<tr>
<td>H_2O</td>
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<td>Glutamate effect</td>
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DISCUSSION

Treatment with either glutamate or aspartate inhibited glycolate synthesis in isolated maize bundle sheath strands. This inhibition caused a decrease in the amount of photorespiration as measured by the Warburg effect (14). When the O_2 concentration in the atmosphere was lowered from 100% to 3% the photosynthetic rates of untreated bundle sheath strands increased 70% from 2.3 to 3.9 µmol of CO_2 fixed/mg of Chl-hr (Table II). Decreasing the O_2 concentration with bundle sheath strands treated with glutamate resulted in only a 43% increase from 2.8 to 4.0 µmol of CO_2 fixed/mg of Chl-hr. The glutamate treatment lessened the Warburg effect, indicating decreased photorespiration.

Three mechanisms currently exist to explain the in situ inhibition of the enzyme(s) responsible for glycolate synthesis by bundle sheath cells in intact mature maize leaves. One hypothesis suggests that the relative activities for the fixation and transport of CO_2 into the bundle sheath tissues, compared with the rates of fixation within this tissue, result in greatly elevated CO_2 concentrations within the bundle sheath cells. In order to remove the O_2 sensitivity of CO_2 fixation completely it has been estimated that the CO_2 concentration within the bundle sheath chloroplast must be at least 61 µM (5). This compares with the 0.6 mM total CO_2 or 13 µM free CO_2 measured in maize bundle sheath tissue (10). A second possibility is that the decreased O_2 production observed with isolated bundle sheath strands and chloroplasts (1, 12) represents a lesion which occurs in the PSI activity of these cells in intact tissue and may result in decreased O_2 concentration within the tissue. Lower O_2 levels would result in slower glycolate synthesis and therefore a decreased photorespiration (19). Finally, glycolate synthesis and photorespiration in bundle sheath strands may be inhibited by the presence of suitable concentrations of the amino acids, glutamate and aspartate. An analysis of the aspartate concentration in isolated maize bundle sheath tissue before and after a 15-min exposure to 7.5 mM aspartate revealed that this treatment increased the aspartate concentration within the tissue from approximately 0.2 to 0.6 mM. The higher value is similar to the aspartate pool size measured in 14C02 pulse-chase experiments with maize (7). Should the isolated strands function as the tissue does in situ, this concentration of aspartate could account for the lack of photorespiration found with mature intact maize leaves. Any of these mechanisms either alone or in combination with the others may explain the slow photorespiration in intact maize leaves. It remains to be determined whether glutamate and aspartate, or some metabolic product(s) of these amino acids, exert the inhibitory effect in vivo.

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