PHOSPHOGLYCOLATE PRODUCTION CATALYZED BY RIBULOSE DIPHOSPHATE CARBOXYLASE

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SUMMARY

An oxygen-dependent production of phosphoglycolate is catalyzed by purified soybean ribulose diphosphate carboxylase and by crude extracts of soybean and corn leaves. It is suggested that the phosphoglycolate produced in this reaction is the source of glycolate metabolized in photorespiration.

Oxygen is a potent inhibitor of net CO_2 fixation by soybean leaves and it has been proposed that the nature of this inhibition is twofold: an inhibition of photosynthesis and a stimulation of photorespiration (1,2). Recently it was shown that the oxygen inhibition of photosynthesis results from the competitive inhibition of ribulose diphosphate carboxylase by oxygen with respect to CO_2. Furthermore, it was suggested that oxygen might stimulate photorespiration by substituting for CO_2 in the carboxylase reaction to yield phosphoglycolate, a presumed photorespiratory intermediate, and 3-phosphoglycerate, the normal product of the carboxylase reaction (3). The experiments described here demonstrate that ribulose diphosphate carboxylase does catalyze the oxidation of ribulose diphosphate to phosphoglycolate.

METHODS

The assay procedure was carried out in two stages. Phosphoglycolate is produced from ribulose diphosphate (RuDP) and oxygen in the

presence of RuDP carboxylase in the first stage, and in the second stage this phosphoglycolate is converted to the phenylhydrazone of glyoxylic acid. In stage 1, 1.4 ml reaction mixtures containing 0.2 mM RuDP, 10 mM $MgCl_2$, 50 mM Tris or Tricine, pH 8.0, and 0.1 mM EDTA were flushed with N_2, O_2, or CO_2-free air for 15 min in flasks sealed with serum caps in a shaker bath at 30°. Purified or crude enzyme (0.1 ml) was then added to the flasks with a syringe. After 15 min the reaction was stopped by heating for 30 sec in a boiling water bath, followed by cooling in ice. In stage 2 of the assay, partially purified soybean phosphoglycolate phosphatase and glycolate oxidase in 0.9 ml of 20 mM Tricine, pH 8.0, 4 mM $MgCl_2$, and 30 units of catalase were added to a 1.0 ml aliquot from stage 1, and the mixture incubated for 10 min at 30°, followed by 1 min in a boiling water bath. The solution was centrifuged, 1.6 μmoles of phenylhydrazine hydrochloride added, and after 20 min at 40°, the optical density measured at 324 nm. The recovery of phosphoglycolate internal standards, added in amounts similar to that produced in stage 1 was 75%.

For chromatography studies, 2,4-dinitrophenylhydrazones were prepared by adding 0.10 ml of 0.2% 2,4-dinitrophenylhydrazine in 2N HCl instead of phenylhydrazine. The assay mixtures were extracted three times with ethyl acetate. The ethyl acetate was removed by evaporation under reduced pressure at 30°, the residue dissolved in benzene, applied to silica gel thin-layer plates, and the chromatograms developed with benzene:tetrahydrofuran:glacial acetic acid (60:36:4) (4).

Purified ribulose diphosphate carboxylase was prepared from soybean leaves following the procedure of Paulsen and Lane (5). Crude extracts of soybean and corn were prepared by grinding 500 mg (fresh weight) of tissue in 5 ml of 50 mM Tricine, pH 8.0, 10 mM $MgCl_2$, and 0.1 mM EDTA for 2 min in a motor-driven Ten Broeck homogenizer, followed by 15 min centrifugation at 35,000 x g.

Table I. Purified soybean RuDP carboxylase dependent phenylhydrazone production.

Gas phase during stage 1.	Glyoxylate phenylhydrazone produced in stage 2. (nmoles/min stage 1/mg protein)
Nitrogen	0
CO_2-free air	1.4
Oxygen	4.2
Nitrogen (Boiled enzyme)	0
Oxygen (Boiled enzyme)	0

$^{14}CO_2$ incorporation at 20 mM $NaHCO_3$ in air was 62 nmoles/min/mg protein.

RESULTS

The requirement for oxygen and active RuDP carboxylase in producing a phenylhydrazone in the reaction sequence described above is shown in Table I. No phenylhydrazone is produced in stage 2 of the reaction sequence when stage 1 is carried out under nitrogen or in the presence of enzyme heated for 30 sec in a boiling water bath. The amount of phenylhydrazone produced is three times higher when stage 1 of the reaction is performed under 100% oxygen than when it is run under 21% oxygen. Since oxygen inhibition of photosynthesis and oxygen stimulation of photorespiration in the soybean are not yet saturated at 100% oxygen, it is likely that this reaction is not saturated, and greater amounts of phenylhydrazone may be obtained if stage 1 is run at oxygen pressures greater than one atmosphere.

The requirement for catalase, glycolate oxidase, and phosphatase activity for phenylhydrazone formation in stage 2 of the assay is shown in Table II. No phenylhydrazone was produced when catalase or glycolate oxidase were omitted from the reaction mixture. When phosphatase was omitted, some phenylhydrazone was produced due to phosphatase contamination of glycolate oxidase. Phosphoglycolate phosphatase requires a divalent ion for activity (6), and when $MgCl_2$ was omitted from the stage

Table II. Enzyme requirements for phenylhydrazone formation during stage 2 of the assay.

Stage 2 reaction mixture	Phenylhydrazone formed (nmoles/ml stage 1)
complete	21.3
-catalase, -glycolate oxidase, -phosphatase	0
-catalase	0
-glycolate oxidase	0
-phosphatase	10.3
-$MgCl_2$, -phosphatase, +5mM EDTA	1.4

Stage 1 of the assay was carried out under oxygen

2 reaction mixture and 5 mM EDTA was added to complex the Mg^{++} carried over from stage 1 of the assay, almost no phenylhydrazone was formed. EDTA did not affect glycolate oxidase or catalase activity.

For further identification of the product formed in the reaction sequence, 2,4-dinitrophenylhydrazine derivatives were prepared and applied to silica gel thin-layer chromatography plates. The derivative produced migrated with the 2,4-dinitrophenylhydrazine derivative of authentic glyoxylic acid. From this chromatographic behavior, together with the data in Table II, it is concluded that the phenylhydrazone precursor produced from RuDP and oxygen in stage I of the reaction sequence is phosphoglycolate.

Crude extracts of soybean and corn also catalyze the production of the phenylhydrazone precursor in stage 1, and the amount of phenylhydrazone produced is again a function of the oxygen concentration (Table II). Thus RuDP carboxylase from corn, a species characterized as possessing little if any photorespiration (6), is also able to catalyze phosphoglycolate production.

Table III. Crude soybean and corn RuDP carboxylase dependent phenylhydrazone production.

Gas phase during stage 1.	Glyoxylate phenylhydrazone produced in stage 2. (nmoles/min stage 1)	
	Soybean	Corn
Nitrogen	0	0
CO_2-free air	2.1	0.6
Oxygen	3.4	3.0

DISCUSSION

It is well established that glycolate metabolism is involved in the process of photorespiration (8), but the specific source of this glycolate has not yet been established. Various laboratories have provided evidence to suggest that glycolate is derived from one or more sugar phosphate of the Calvin cycle, either by oxidation of the thiamine pyrophosphate-glycolaldehyde moiety of the photosynthetic transketolase reactions (9), by oxidation of a pentose phosphate (10), or by oxidation of RuDP (11,12). The evidence given here shows that RuDP can be oxidized to phosphoglycolate by oxygen, and that this oxidation is catalyzed by RuDP carboxylase. Since the rate of photorespiration is regulated by the same step which regulates the light-saturated rate of photosynthesis (3), and since this rate-limiting step is likely RuDP carboxylase (3,13), we conclude that RuDP is the source of glycolate metabolized in photorespiration, via phosphoglycolate. The chloroplast contains sufficient phosphoglycolate phosphatase activity to hydrolyze all the phosphoglycolate to glycolate (14). The glycolate is then transported out of the chloroplast into the peroxisomes, where photorespiratory metabolism occurs (8).

The catalysis of phosphoglycolate production by crude corn RuDP carboxylase indicates that this property may be found in RuDP carboxy-

lases from all plant species, whether or not they photorespire. In species which do not exhibit photorespiration, such as corn, CO_2 is initially fixed by PEP carboxylase in the mesophyll chloroplasts, and the product of this reaction, oxalacetate, is reduced to malate. The malate is transported to the bundle sheath chloroplasts and decarboxylated to pyruvate and CO_2 by "malic" enzyme. The CO_2 is refixed by RuDP carboxylase and flows through the Calvin cycle to carbohydrate (15). The PEP carboxylase functions as a CO_2 pump, increasing the CO_2 concentration in the bundle sheath chloroplasts (16). Since oxygen and CO_2 compete for the active site on RuDP carboxylase (3), an increased CO_2 concentration at RuDP carboxylase will reduce the rate of RuDP oxidation and thus reduce the rate of photorespiratory metabolism. If any phosphoglycolate is produced during corn photosynthesis and metabolized to CO_2 by the photorespiratory pathway, the CO_2 will be recaptured by PEP carboxylase as it passes out through the mesophyll (17).

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