ON THE ACTIVITY OF RIBULOSEDIPHOSPHATE CARBOXYLASE WITH CO_2 AND O_2 FROM LEAF EXTRACTS OF ZEA MAYS

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SUMMARY: Ribulosediphosphate carboxylase, partially purified from corn leaves, demonstrates a low $K_m(CO_2)$ of 19 μM if stabilized with ribose-5-phosphate during extraction. It also exhibits a ribulosediphosphate dependent uptake of oxygen, similar to that observed with spinach carboxylase. The low $K_m(CO_2)$ is similar to the apparent $K_m(CO_2)$ for photosynthesis by intact corn tissue and requires reconsideration of the hypothesis that CO_2 is concentrated in the bundle sheath cell by the C_4 pathway during photosynthesis.

One of the unique features of photosynthetic CO_2 assimilation in C_4 plants is that CO_2 is fixed by phosphopyruvate carboxylase in the mesophyll cells and shuttled to the bundle sheath cells as malate or aspartate (1,2). These compounds are decarboxylated, a step which has been proposed to concentrate CO_2 in the bundle sheath cell (3). CO_2 is then assimilated by ribulosediphosphate (RuDP) carboxylase to glycerate 3-P and on to sugars by the Calvin cycle. However, purified RuDP carboxylase exhibits a $K_m(CO_2)$ of approximately 450 μM (4), considerably higher than the 10 μM CO_2 in solutions in equilibrium with air at 25 C. Without concentrating CO_2 in the bundle sheath cell, CO_2 fixation by the RuDP carboxylase would apparently be quite ineffectual (3).

We wish to report that the RuDP carboxylase activity of partially purified extracts of Zea mays leaves can demonstrate a significantly lower $K_m(CO_2)$ of 19 μM at 25 C. The approach used is a modification of one we recently reported for C_3 plants (spinach, tobacco) where upon lysis of intact chloroplasts, the RuDP carboxylase exhibited a $K_m(CO_2)$ of 12 μM (5). The partially purified extracts from corn also show a significant RuDP dependent uptake of O_2, demonstrating that an oxygenase activity is associated with corn RuDP carboxylase.

Abbreviations: RuDP, ribulose-1,5-diphosphate; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; Bicine, N,N-bis[2-hydroxyethyl]glycine.

METHODS: RuDP carboxylase was prepared from 3 to 4 week old corn leaves (Zea mays, var. Golden Bantam) by a modification of the procedure of Andrews and Hatch (6). Approximately 2 gm of corn leaves with main ribs removed were ground with a mortar and pestle in 5.6 ml of 0.10 M HEPES, pH 7.8, 50 mM mercaptoethanol, 2.6 mM ribose-5-P and 4.5 mM $MgCl_2$ at 4 C. The extract was filtered through Miracloth and centrifuged at 21,000 x g for 10 min to remove particulate matter. 2.0 ml of supernatant was placed on a 5.0 x 2.5 cm Sephadex G-100 column at 4 C equilibrated with 10 mM HEPES, pH 7.8, 2.0 mM Na isoascorbate, 2.6 mM ribose-5-P, and 4.5 mM $MgCl_2$ and eluted with the same medium. The first two ml of yellow-green eluate, which appeared immediately after the void volume, was collected and stored on ice for assay.

RuDP carboxylase was assayed by adding the enzyme preparation last to 0.5 ml of 0.16 M HEPES, pH 7.8, 25 mM $MgCl_2$, 3.0 mM dithioerythritol, 0.30 mM RuDP and 3.0 mM $NaHCO_3$ (3.0 μC/μMole) in serum capped 2 ml vessels at 25 C. 50 or 100 μl aliquots were removed at fifteen sec intervals during the first minute of reaction and acid-stable ^{14}C determined by planchet counting (5).

RuDP oxygenase was assayed by adding enzyme preparation last to 0.20 M Bicine, pH 8.6, containing 0.40 mM RuDP and 25 mM $MgCl_2$ and equilibrated with air. The initial rate of oxygen uptake was determined with an oxygen electrode in a water-jacketed vessel at 25 C. The observed rate was corrected for the background uptake without RuDP.

Protein concentration was determined by the method of Lowry et al (7) after washing of the precipitate in 10 percent trichloroacetic acid.

RESULTS AND DISCUSSION: The low $K_m(CO_2)$ form of spinach RuDP carboxylase can be stabilized for several hours at 0 C and pH 7.7 to 8.0 with ribose-5-P, ATP, $MgCl_2$ and mercaptoethanol or dithioerythritol (5). Extracts prepared by grinding corn leaves in this medium without ATP also yielded RuDP carboxylase having a low $K_m(CO_2)$. The corn extracts were purified 2-3 fold by gel filtration and retained a low $K_m(CO_2)$ for up to three hours when kept at 0 C.

During the first minute of fixation with RuDP, greater than 95 percent

TABLE I. CO_2 Fixation by Corn RuDP Carboxylase

Substrate Added	Time of Observation (min)	Rate Observed μmoles/mg prot/hr
none	0-1	0.1
plus 0.3 mM RuDP	0-1	13.5
plus 1.0 mM glycerate-3-P	0-1	0.2
plus 2.0 mM ribose-5-P and 2.0 mM ATP	0-1 5-10	4.1 10.5

Enzyme activity was assayed according to METHODS and the rate was determined over the time interval indicated. For comparison, the low $K_m(CO_2)$ carboxylase in crude extracts assayed after Miracloth filtration was 140 μmoles per mg chlorophyll per hr at 5 mM $NaHCO_3$ and pH 7.8.

of the CO_2 was incorporated into glycerate 3-P by the RuDP carboxylase (Table 1). The apparent $K_m(HCO_3^-)$ for the initial rate was 0.8 mM at pH 7.8, corresponding to a $K_m(CO_2)$ of 19 μM. Ribose-5-P and ATP supported fixation, but required up to five minutes to reach maximal rates.

In the presence of RuDP and less than 50 mM $NaHCO_3$, carboxylation proceeded linearly for two minutes and then declined. This loss of activity was associated with a change in the apparent $K_m(HCO_3^-)$. Following a 10 minute incubation of the extract at 25 C, the maximal velocity of the low $K_m(HCO_3^-)$ activity dropped from 19 μmoles per mg protein per hr to 1.1 μmoles per mg protein per hr (Fig. 1). A new activity appeared having a much higher $K_m(HCO_3^-)$ of about 25 mM (apparent $K_m(CO_2)$ = 650 μM).

The major rationale for proposing a CO_2 concentrating role for the C_4 shuttle pathway by malate-aspartate decarboxylation in the bundle sheath cells has been the apparent low affinity of the RuDP carboxylase for CO_2 (3). The RuDP carboxylase in maize is located only in the bundle sheath cells (8). Based on the characteristics and activities of phosphopyruvate carboxylase

and RuDP carboxylase in corn leaves, the rate of steady state CO_2 fixation will be limited by the RuDP carboxylase (3). Therefore, the apparent CO_2 affinity of the intact leaf should depend on the $K_m(CO_2)$ of the RuDP carboxylase and the magnitude of the CO_2 concentration gradient, assuming leaf resistances to CO_2 are minimal. The apparent $K_m(CO_2)$ for corn leaf segments was 260 to 325 ppm CO_2 (9) (about 10 to 13 μM CO_2 (10)), only slightly lower than the apparent $K_m(CO_2)$ of 19 μM we have observed for the corn RuDP carboxylase. Thus, it appears that during photosynthesis in air, CO_2 need not be concentrated more than two fold in the bundle sheath cells by the C_4 shuttle pathway.

This conclusion apparently contradicts the observation by Hatch (11) that the "CO_2" species (CO_2, HCO_3^-, $CO_3^=$) were concentrated ten fold in maize leaves in the light. Werden and Heldt (12) have shown that upon illumination isolated intact spinach chloroplasts accumulate bicarbonate in the stroma following the formation of a pH gradient. This accumulation does not increase CO_2 but only bicarbonate, in response to the pH change. The increase in "CO_2" species reported by Hatch (11) could well be explained by this observation.

The extracts of low $K_m(CO_2)$ RuDP carboxylase also exhibited substantial RuDP oxygenase activity. This activity was measured as the RuDP-dependent uptake of O_2, and has recently been shown to produce phosphoglycolate and glycerate-3-P (13). At air levels of O_2 (240 μM) and CO_2 (10 μM) and pH 8.6, the oxygenase activity was 1.9 μmoles O_2 per mg protein per hr. Under these same conditions and with the same enzyme preparation, CO_2 fixation was 7.8 μmoles CO_2 per mg protein per hr. The ratio of the carboxylase to oxygenase activity was 4.1, similar to the ratio of 3.4 observed for the low $K_m(CO_2)$ spinach carboxylase under the same conditions (unpublished results).

It appears that the bundle sheath cells from C_4 plants are capable of glycolate formation and metabolism similar to C_3 plants. Phosphoglycolate produced by the corn RuDP oxygenase would go to glycolate via phosphoglycolate phosphatase (14), providing the substrate for photorespiration. Indeed, the enzymes of the glycolate pathway are reported to be concentrated in the

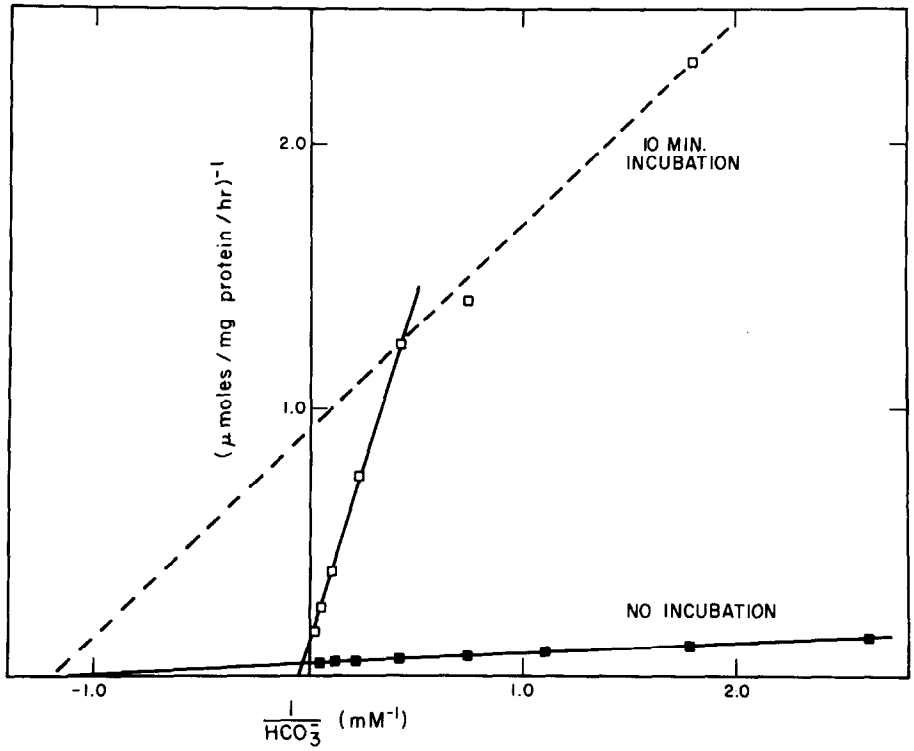

Figure 1 Effects of incubation of enzyme at 25 C in the absence of substrates. Carboxylase activity was assayed as described in METHODS. The medium was bubbled with N_2 for 30 min prior to addition of enzyme and $NaH^{14}CO_3$.(-■-); Enzyme was added last to assay medium and fixation measured during the first minute. (-□-); Enzyme was incubated with 0.16 M HEPES pH 7.8 and 3.0 mM dithioerythritol for ten minutes prior to initiation of carboxylation with 0.3 mM RuDP, 0.025 M $MgCl_2$ and $NaH^{14}CO_3$.

bundle sheath cells of corn and other C_4 plants (14,15,16). Why photorespiration is not observed with intact C_4 plants is yet to be established, but it may be due to the efficient refixation of CO_2 by the mesophyll cells (2).

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