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ACTIVITY AND PROPERTIES OF RIBULOSEDIPHOSPHATE CARBOXYLASE FROM PLANTS WITH THE C_4-DICARBOXYLIC ACID PATHWAY OF PHOTOSYNTHESIS

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Abstract—The properties of partially-purified ribulosediphosphate carboxylase from maize leaf were compared with those reported for the enzyme from Calvin cycle plants. The pH optimum for the enzyme was not altered by changing the Mg^{2+} concentration between 1 mM and 10 mM provided the CO_2 concentration was maintained constant. A sharp decline in activity on the alkaline side of the pH optimum, 7·8, was probably due in part to inhibition by bicarbonate. At least in the presence of Mg^{2+} concentrations above 10 mM, and at pH 7·8, concentrations of CO_2 plus bicarbonate of more than 20 mM were inhibitory. The concentration of CO_2 giving half maximum velocity also varied with the Mg^{2+} concentration being approximately 0·13 mM with 10 mM Mg^{2+}. The apparent K_m for ribulose 1,5-diphosphate, 17 μM, was much lower than that reported for the enzyme from Calvin cycle plants. The ribulosediphosphate carboxylase activity and fraction-1 protein content of leaves of several plants with the C_4-dicarboxylic acid pathway were much higher than those reported in earlier studies. This was attributed in part to the incomplete extraction of the enzyme and in part to the previous failure to use optimal assay conditions.

INTRODUCTION

THE PRIMARY carboxylation reaction of the C_4-dicarboxylic acid pathway is catalyzed by phosphopyruvate carboxylase.[1,2] There is evidence that the contribution of ribulosediphosphate (RuDP) carboxylase to the direct fixation of externally-derived CO_2 must be insignificant in plants with this pathway.[3,4] Earlier reports of low RuDP carboxylase activities in C_4-dicarboxylic acid pathway plants[5,6] supported this conclusion but in recent studies[7] much higher activities were obtained. Furthermore, radiotracer studies have implicated RuDP as an intermediate in the pathway[3] and the activity of the enzymes responsible for the conversion of ribose 5-phosphate to RuDP are comparable to those

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reported for Calvin cycle plants.[1,5,8] In spite of the interest in the role of RuDP carboxylase in plants with the C_4-dicarboxylic acid pathway, there is little comparative information about the properties of the enzyme from this source and from plants with the Calvin cycle. This paper describes such studies and examines the reasons for the discrepancies in the reported activities of the enzyme.

RESULTS AND DISCUSSION

Assay of RuDP Carboxylase

With extracts from several plants with the C_4-dicarboxylic acid pathway (see Table 1) there was a lag of several minutes before linear rates were obtained if reactions were started with bicarbonate. This lag was avoided if RuDP was added last after incubating the enzyme with all other reactants for about 10 min. Similar behaviour was reported for RuDP carboxylase from plants with the Calvin cycle.[9] A point of practical interest was that with both the assays described in the Experimental there was a decline in activity after an essentially linear phase. This decline was a function of time rather than the extent to which the reaction had proceeded. The effect may have been due to RuDP or an inhibitor therein since inhibition by RuDP has been observed previously.[9]

For the assay of enzyme in crude extracts the spectrophotometric procedure, rather than the radiotracer method, was employed. With the later procedure additional $^{14}CO_2$ may be fixed by phosphopyruvate carboxylase if 3-phosphoglycerate is converted to phosphopyruvate. With the partially purified enzyme the results of the two assay procedures were in close agreement and the one employed depended upon the particular requirements of the study being conducted.

Purification

Maize leaf RuDP carboxylase was partially-purified as described in the Experimental to remove the enzymes which catalyse the conversion of 3-phosphoglycerate to phosphopyruvate and other enzymes that may interfere with studies of its properties. Even though there was little activity in the fractions precipitating before and after the 37–45% saturated ammonium sulphate fraction, this fraction contained only about 30% of the original activity. However, if the 30–50% saturated ammonium sulphate fraction was taken almost all the activity was recovered. We have no explanation for this observation.

Properties

Except where otherwise specified the partially-purified RuDP carboxylase from maize leaf was used for these studies. The pH optimum for the enzyme was not altered by changing the Mg^{2+} concentration from 1 mM to 10 mM provided the concentration of CO_2 was maintained constant at 2 mM (Fig. 1). This was achieved by varying the amount of bicarbonate added in accordance with the Henderson–Hasselbach equation.[10] Different results have been obtained with the spinach enzyme[11,12] but in these studies the CO_2 concentration was not kept constant. The fact that CO_2 is the substrate for RuDP carboxylase[13] was

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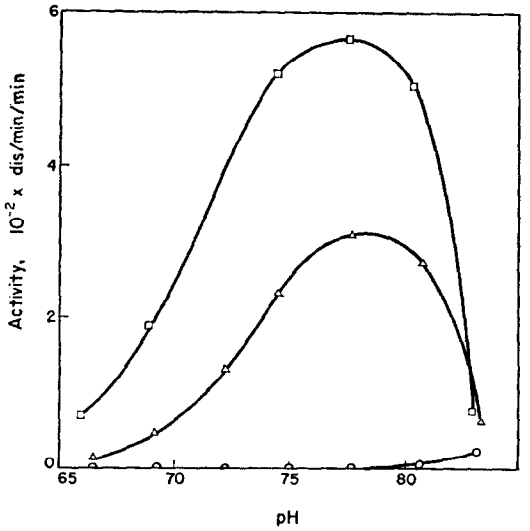

FIG. 1. DEPENDENCE OF RuDP CARBOXYLASE ACTIVITY ON pH AT DIFFERENT Mg^{2+} CONCENTRATIONS. Reactions were as described in the Experimental for the radiotracer procedure except that they were run in a 0·2 ml pipette at 24° to minimize loss of CO_2 at the lower pH values. CO_2 was maintained at 2 mM by varying the concentration of $NaHCO_3$ (see text). The buffer, HEPES–NaOH, was varied as shown and either no $MgCl_2$ (○), 1 mM $MgCl_2$ (△), or 10 mM $MgCl_2$ (□) was provided.

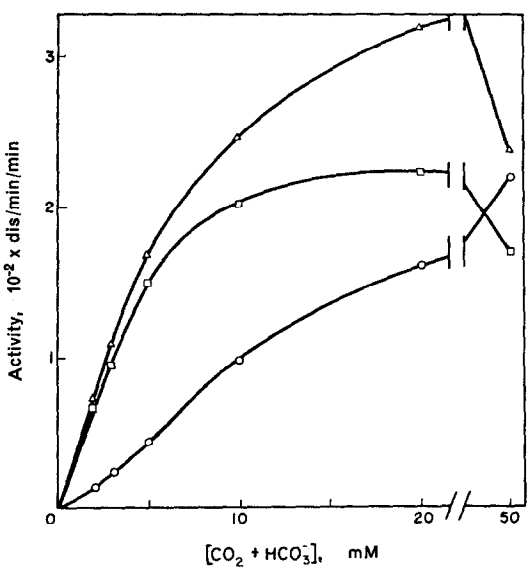

FIG. 2. EFFECT OF CO_2 PLUS HCO_3^- CONCENTRATION ON RuDP CARBOXYLASE ACTIVITY AT DIFFERENT Mg^{2+} CONCENTRATIONS.
Reaction mixtures were as described in the Experimental but the concentration of $NaHCO_3$ added was varied and either 1 mM $MgCl_2$ (○), 10 mM $MgCl_2$ (△), or 40 mM $MgCl_2$ (□) was provided.

not recognized at that time. Another feature of the pH-activity curve in Fig. 1 was the very rapid decline in activity as the pH was increased from 7·8 to 8·5. This contrasts with the spinach enzyme which shows only a small decline over this range.[14,15] However, the decline observed with the maize leaf enzyme would have been due in part to inhibition by bicarbonate (see below and Fig. 2).

With lower Mg^{2+} concentrations the plot of RuDP carboxylase activity against the concentration of CO_2 plus bicarbonate was clearly sigmoidal (Fig. 2). Sigmoidicity was also apparent from the Lineweaver-Burk plots of values at the higher Mg^{2+} concentrations. Similar observations have been made with the spinach enzyme.[16] The concentration of CO_2 plus bicarbonate giving half maximum velocity varied with the Mg^{2+} concentration, the lowest value being approximately 5 mM with 10 mM Mg^{2+}. This corresponds to a CO_2 concentration of 0·13 mM. Increasing the CO_2 plus bicarbonate concentration above 20 mM decreased activity, at least when Mg^{2+} concentration was 10 mM or more. It is clear that the bicarbonate concentration and pH previously employed for the assay of RuDP carboxylase from plants using the C_4-dicarboxylic acid pathway[4–6] would not have been optimal for activity.

A normal Michaelis–Menten response was observed when the concentration of RuDP was varied (Fig. 3). However, the apparent K_m of 17 μM was much lower than the value reported with RuDP carboxylase obtained from plants with the Calvin cycle[11,15,16] and from an alga.[17] Apparently this may be a feature of RuDP carboxylase from plants with the C_4-dicarboxylic acid pathway. With the enzyme obtained from all the species listed in Table 1 (except *Atriplex nummularia*, which was not tested) a concentration of 0·15 mM RuDP gave optimal activity.

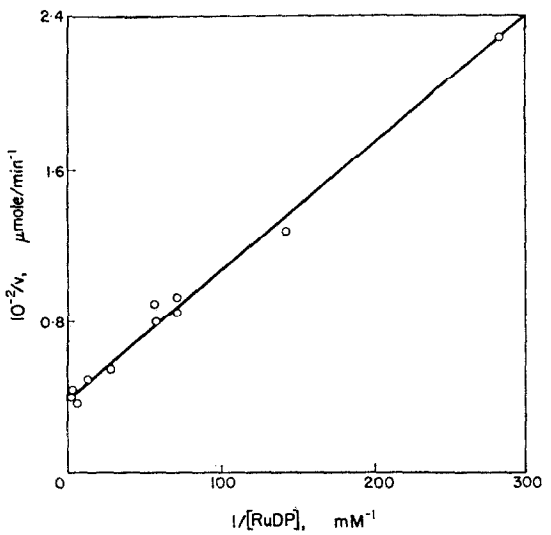

FIG. 3. LINEWEAVER–BURK PLOT OF RUDP CARBOXYLASE ACTIVITY AGAINST RUDP CONCENTRATION. Assays were conducted at 25° using the spectrophotometric assay procedure.

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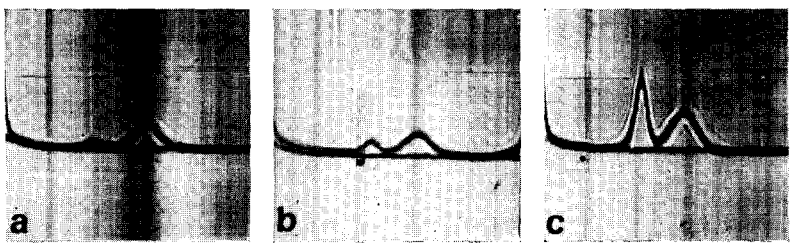

FIG. 4. FRACTION-1 PROTEIN DETERMINED BY ULTRACENTRIFUGATION OF LEAF EXTRACTS.

Details of the extraction and centrifugation procedures are described in the Experimental. The patterns shown are for (a) maize extracted in 2 vol. of media with a Servall blender, (b) maize extracted with 4 vol. of media in a mortar, (c) spinach extracted in 2 vol. of media in a mortar.

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Several photosynthetic intermediates were tested as possible activators or inhibitors of RuDP carboxylase. The existence of an activator could provide one explanation for the apparent anomalies associated with the operation of this enzyme in photosynthesis.[2] Malate, aspartate, pyruvate, $NADPH_2$, orthophosphate, 3-phosphoglycerate and several phosphorylated sugars were provided at concentrations of up to 2 mM but none stimulated activity. Inhibitions of between 20% and 35% were observed with D-ribose 5-phosphate, 3-phosphoglycerate, dihydroxyacetone phosphate and fructose 1,6-diphosphate at a concentration of 2 mM. Some of these effects may have physiological significance. The effect of DL-glyceraldehyde 3-phosphate, which inhibited 25% at 0·4 mM and 55% at 2 mM, could be significant if the L-isomer is the effective species.

RuDP Carboxylase and Fraction-1 Protein in Different Species

We have already intimated that the assay procedure for RuDP carboxylase employed previously in our laboratory would have underestimated the activity of this enzyme. In addition to the factors of pH and inhibition by high bicarbonate concentrations already mentioned, the concentration of CO_2, the substrate for the enzyme, would not have been saturating at pH 8·3. The present studies indicate that an additional factor contributing to the low RuDP carboxylase activities reported earlier was the incomplete extraction of the enzyme. This confirms the conclusions of Bjorkman and Gauhl[7] who found activities in plants using the C_4-dicarboxylic acid pathway which were almost comparable to those found in plants of the Calvin cycle.

Using the extraction procedure originally employed in our laboratory,[5,6] blending in a Servall Omni-mix, maize leaf extracts were found to contain 0·8–0·9 units of RuDP carboxylase/mg chlorophyll. In contrast there was 2·5 units/mg chlorophyll in extracts obtained by grinding leaves in a mortar (see Experimental). We now know that RuDP carboxylase in plants of the C_4-dicarboxylic acid pathway is associated exclusively with the bundle sheath chloroplasts.[8,18] Microscopic examination of the residue remaining after extraction by blending revealed large numbers of unbroken bundle sheath cells associated with strands of vascular tissue. Few intact mesophyll cells were observed and in maize these cells contain the majority of the chloroplasts. In contrast, the residue from mortar-grinding contained relatively few intact bundle sheath cells. With these two procedures similar differences were observed in the extractability of fraction-1 protein,[19] now assumed to be identical with RuDP carboxylase[20–22] (Fig. 4). Extraction by blending gave 0·56 mg of fraction-1 protein/mg chlorophyll whereas mortar-grinding gave a value of 1·4. The value for a spinach leaf extract, included for comparison, was 5·8. The observation on fraction-1 protein content reduces the possibility that the difference in RuDP carboxylase activity was due to differential inactivation. The same activities were obtained when either Tris-HCl or HEPES–NaOH were used as the buffer for both extraction and assay.

Several plants in which the C_4-dicarboxylic acid pathway is operative[1] were examined for their RuDP carboxylase and fraction-1 protein contents (Table 1). Extracts were prepared by grinding in a mortar and activities were much higher than those recorded in earlier studies.[5,6] They were comparable to those reported by Bjorkman and Gauhl[7] and close

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to the maximum photosynthetic rates for plants with the C_4-dicarboxylic acid pathway.[5,6] Intermediate values for RuDP carboxylase activity were obtained for maize and *Amaranthus* in an early study in which mortar-grinding was used.[4] The difference between the latter activities and those of Table 1 were presumably attributable to differences in the assay conditions employed.

The fraction-1 protein content of extracts, expressed as a percentage of the total protein, was also higher than previously observed.[5] There was a correlation between the content of fraction-1 protein and RuDP carboxylase activity but the ratio of the two was not constant between species (Table 1). However, with all species including spinach the sedimentation coefficient for the fraction-1 protein peak was 17 $\pm$ 1S. Fraction-1 protein was undetectable in extracts of *Atriplex nummularia* and the RuDP carboxylase activity was also low. Even when leaves of this species were frozen and powdered in liquid nitrogen in a percussion mortar prior to grinding with buffer, there was little increase in the amount of fraction-1 protein extracted. Furthermore, the residual material still contained large numbers of apparently intact bundle sheath cells. Higher RuDP carboxylase activity has been obtained for some other *Atriplex* species with the C_4-dicarboxylic acid pathway.[7]

There is evidence that RuDP carboxylase contributes insignificantly to the direct fixation of externally derived CO_2 in plants of the C_4-dicarboxylic acid pathway.[1,2] For maize at least there is direct evidence that essentially all the $^{14}CO_2$ appearing in the C-1 of 3-phosphoglycerate enters via the C-4 of C_4-dicarboxylic acids.[3] However, RuDP has been implicated as an intermediate in this pathway.[3] Two possible mechanisms have been considered to explain how carbon is transferred from the C-4 of dicarboxylic acids to 3-phosphoglycerate, one involving a transcarboxylation directly to an acceptor and the other decarboxylation followed by refixation of the released CO_2.[8,23] There are several observations that apparently conflict with the latter proposition and these are discussed in recent reviews.[1,2] It is now clear that one of these, the apparent inadequacy of RuDP carboxylase activity to account for refixation of released CO_2, must be discounted. Possibly none of the other observations can be considered as compelling evidence against the operation of a process involving decarboxylation and refixation of the CO_2 released. Further evidence

TABLE 1. RuDP CARBOXYLASE AND FRACTION-1 PROTEIN CONTENT OF LEAF EXTRACTS*

Species	RuDP carboxylase (μmole/min/mg chlorophyll)	Fraction-1 protein (mg/mg chlorophyll)
Maize (*Zea mays*)	2·5	1·4
Sugarcane (*Saccharum*, hybrid)	2·0	0·7
Chloris gayana	2·3	0·6
Eragrostis brownii	2·4	1·1
Amaranthus edulis	4·4	1·5
Atriplex nummularia	0·5	<0·3

* The spectrophotometric assay procedure was used. Details of the extraction procedure (mortar-grinding) and the assay procedures are provided in the Experimental.

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(Andrews, Hatch, Johnson and Slack, unpublished results) and interpretations relating to this question will appear in a forthcoming publication.

EXPERIMENTAL

Plants were grown in a glasshouse at 28° except for the species of *Eragrostis* and *Chloris* which were field-grown.

Extraction of Enzymes

For most studies a weighed sample (about 1·5 g) of leaf was ground vigorously for 60 sec in a mortar at 0° with acid-washed sand and 4 vol. (w/v) of either 50 mM HEPES–NaOH buffer or Tris-HCl buffer, pH 7·8 containing 5 mM dithiothreitol and 2 mM EDTA. After straining through fine muslin the filtrate was treated on a small Sephadex G-25 column previously equilibrated with the above buffer solution to free the preparations of small mol. wt. materials. For spectrophotometric assays extracts were centrifuged at 11,000 *g* for 10 min. Where specified an alternative procedure was to blend leaves with 4 vol. of the above buffer solution in a Servall Omnimix for 2 min at 0°. The resulting homogenate was then treated as described above.

Assay for RuDP Carboxylase

Two procedures were employed. Acid-stable radioactivity was measured after incubating the enzyme with [^{14}C] HCO_3^- using the procedure for counting described by Paulsen and Lane.[16] Reactions contained 100 mM Tris-HCl or HEPES–NaOH buffer, pH 7·8, 10 mM $MgCl_2$, 5 mM dithiothreitol, 25 mM [^{14}C] $NaHCO_3$ (0·7 μc/μmole) and 0·7 mM RuDP in a total vol. of 0·25 ml. At pH 7·8 the addition of 25 mM $NaHCO_3$ would give a CO_2 concentration of approximately 0·75 mM. Alternatively, a spectrophotometric procedure[24] was used with reaction mixtures containing enzyme, 55 mM HEPES–NaOH, pH 7·8, 10 mM $MgCl_2$, 0·5 mM dithiothreitol, 0·2 mM EDTA, 2 mM ATP, 0·25 mM $NADH_2$, 25 mM $NaHCO_3$, 0·18 mM RuDP, 100 μg of bovine serum albumin, 10 μg of phosphoglycerate kinase and 50 μg of glyceraldehyde phosphate dehydrogenase. Enzymes were freed of $(NH_4)_2SO_4$ prior to use. Assays were conducted in a Cary 14 spectrophotometer and rates were linear until a change of about 0·1 absorbance units. Reactants were preincubated without RuDP for about 8 min. A unit of enzyme activity is defined as the amount of enzyme that catalyses the fixation of 1 μmole of CO_2 in 1 min at 30°.

Chlorophyll was measured[25] in extracts obtained by filtering through fine muslin. Protein was determined by the procedure of Warburg and Christian.[26]

Purification of RuDP Carboxylase

Maize leaves (300 g) were blended at 0° with 0·1 mm dia. glass beads and 1 l. of 0·15 M Tris–acetate buffer, pH 7·8, containing 100 mM 2-mercaptoethanol, 2 mM EDTA and 7 mM magnesium acetate in a high speed Waring Blendor. To the extract obtained by centrifuging at 11,000 *g* for 10 min saturated $(NH_4)_2SO_4$ (pH 7·3, 4°) was added to obtain the protein precipitating between 37 and 45% saturation. This protein was dissolved in 30 ml of 50 mM Tris–acetate buffer, pH 7·7, containing 1 mM dithiothreitol, 1 mM EDTA and 6 mM magnesium acetate and applied to a 5 × 63 cm column of Sephadex G-200 previously equilibrated with the same buffer solution at 4°. The enzyme emerged just after the first peak of u.v.-absorbing material. Active fractions were stored at 4° after adding 1·5 vol. of saturated solution of $(NH_4)_2SO_4$. As required protein was recovered by centrifugation and then dissolved in buffer solutions containing 5 mM dithiothreitol and 1 mM EDTA.

Determination of Fraction-1 Protein

Extracts were prepared by the procedures described above except that centrifugation was for 20 min at 25,000 *g*. Samples were then centrifuged at 20° in a Spinco model E analytical ultracentrifuge at 56,000 rev/min until fraction-1 protein was separated from other material. Protein concentrations were determined from the areas of individual peaks using the value of $1{\cdot}85 \times 10^{-4}$ ml/mg for the specific refraction increment.

3-Phosphoglycerate to Phosphopyruvate Assay

Phosphopyruvate formation was measured by the oxidation of $NADH_2$ at 340 mμ in systems that included ADP, pyruvate kinase and lactate dehydrogenase.

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