Evidence for a Transcarboxylase Reaction in Maize Chloroplast Extracts

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The authors have suggested (Can. J. Bot. **49,** 631 (1971) that the –COOH group of the C_4-P carbonyl compound of the thermostable P-enolpyruvate acid carboxylase reaction may transcarboxylase with a pentose phosphate as acceptor. We now have considerable evidence supporting this consideration. In an assay system (0.8 ml) containing sonicated chloroplast extract in 0.1 M Tris-HCl, pH 6.3; and PEP, 0.1 µmol; Mg^{2+}, 0.5 µmol; NADH, 0.25 µmol; sugar phosphate, 0.5 µmol. The amount of $^{14}CO_2$ fixation is considerably enhanced by either ribose-5-phosphate, fructose-1,6-bisphosphate or ribulose-1,5-bisphosphate in the presence of PEP. The products of the reaction include malate as a product of β-carboxylation, and glycerate or 3-phosphate glycerate, their proportion being determined by the acceptor sugar phosphate. The results provide evidence for a "transcarboxylase" presented in the crude extract of Maize chloroplasts.

Introduction

Since the discovery of a new pathway of photosynthetic CO_2 fixation in 1965, much of the works has been dealed with the biochemical mechanism of carbon assimilation in C_4 plants. The leaves of C_4 plants have two distinctive pathways of photosynthetic CO_2 fixation: the C_4-dicarboxylic acid pathway and the reductive pentose phosphate pathway, which include phosphoenolpyruvate (PEP) carboxylase and ribulose-1,5-bisphosphate (RuBP) carboxylase as carboxylating steps respectively. Leaf anatomy of C_4 plants is characterized by the presence of two types of chlorophyllous cells, namely the mesophyll and bundle sheath cells. The coordinate operation of both cell types is suggested in current theory of photosynthesis in C_4 plants for the entire process of CO_2 assimilation. According to this contemporary concept the primary CO_2 assimilation occurs in the mesophyll cells of C_4 plants by PEP carboxylase and the product (OAA) is reduced to malate (C_4) or aminated to aspartate, which is then transported to the bundle sheath cells where it is decarboxylated and the $^{14}CO_2$ is then re-fixed by RuBP carboxylase to form two molecules of 3-PGA and subsequently sugars and starch as in the reductive pentose phosphate cycle [1]. It can be understood that this "transport scheme" which is considerably more complicated than the Calvin-cycle. This together with the questionable rapidity of "diffusion" mechanism is contrary to what one would expect of so called "efficient plants". Moreover, the fact that PEP carboxylase (EC 4.1.1.31) has not been shown to be localized in chloroplasts is serious drawbacks to its involvement in primary photosynthetic CO_2 fixation since the parenchyma bundle sheath cells of C_4 plants fix CO_2 in considerably great proportion than mesophyll cells [2]. However, it is interesting to note that, to account for the formation of 3-PGA in C_4 plants, Hatch and Slack had made a similar interesting suggestion that the primary product, oxalacetate transferred its recently acquired $^{14}CO_2$, by β-carboxylation, to RuBP by a transcarboxylation reaction [3]. Moreover, we have reported that the presence of a thermostable PEP acid carboxylase in Maize chloroplasts on the basis of its substrate specific and utilization of its product from $^{14}CO_2$ fixation by Maize chloroplasts [4]. We also considered that there must be some type of transcarboxylation as previously envisioned by Hatch and Slack between the product of the acid carboxylase and a C_5-P acceptor in order to account for the appearance of either 3-PGA, glycerate or malate as first product of photosynthesis in certain plants [5–7]. Considerable evidence have now been accumulated that such a transcarboxylase reaction does occur in Maize chloroplasts.

Materials and Methods

Plant material

Two week-old primary and secondary leaves of *Zea mays* L. var Earliking were harvested from greenhouse grown plants.

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Protein determination

Protein was either determined spectrophotometrically at 280 nm or by the procedure of Lowry *et al.* [8] using bovine serum albumin as standard.

Isolation of bundle sheath and mesophyll chloroplasts

Chloroplasts were isolated from leaves using the laceration technique of Mache and Waygood [9], and modified by Waygood *et al.* (unpublished data) as follows. About 10 g of 12 day-old maize were harvested from leaves grown in the green-house, on a sunny day when the bundle sheath chloroplasts were loaded with starch. The leaves were placed in a glass through containing 25 eml solution (H) (pH 5.5) of Shepard *et al.* [10] in the absence of Cleland reagent and BSA. The leaves were gently lacerated parallel to the veins with three or five small closely spaced scalpels (Irex Surgical Instruments, Toronto). The lacerated leaves were removed and the suspension filtered through five layers of Kleenex tissue (E. B. Eddy Co., Ottawa), and then centrifuged for 50 sec at $2000 \times g$. The chloroplasts were then suspended to 0.1 M Tris-HCl buffer, pH 7.0 containing 10 mM 2-mercaptoethanol and 10^{-4} M EDTA before sonication.

Preparation of crude enzyme extract

The chloroplasts were sonicated for 3 min in a Biosonik (Brownwill) operated at full power under the temperature range 0–5 C. The suspension was then centrifuged for 30 min at $30000 \times g$. The clear supernatant was used as the enzyme solution for all studies. The preparation showed the usual thermostable activity of P-enolpyruvate acid carboxylase at pH 5.5 and pH 7.0, but negligible activity of P-enolpyruvate carboxylase (EC 4.1.1.31) at either pH 7.0 or pH 8.2. Ribulose-1,5-bisphosphate carboxylase was presented in the enzyme preparation, but showed negligible activity under the assay conditions.

Assay of "transcarboxylase" reaction by $NaH^{14}CO_3$ fixation

The enzyme assay system employed to study the activity of enzyme preparation at pH 6.3 was as follows: The reaction system of 0.8 ml final volume contained (μ mol): PEP, 0.1; R5P or sugar phosphates, 0.5; NADH, 0.25; Mg^{2+}, 0.5; ADP, 0.2; cyclic-3′,5′-AMP, 0.5; 1.6×10^6 cpm $NaH^{14}CO_3$ and an unknown concentration of HCO_3^- with 0.4 ml enzyme (ca. 0.3–0.5 mg protein). The reaction was started by the addition of enzyme and incubated at room temperature for 20 min and then terminated by the addition of 0.2 ml 2 N HCl. The reaction mixture was dried in a stream of air over a steaming waterbath, and finally the dried residue was used either for measuring its radioactivity or for perfoming ion exchange chromatography on a Dowex AG 1-X-10 $HCOO^-$ column and verified with authentic labelled compounds.

Results and Discussion

Table I shows $NaH^{14}CO_3$ fixation by the complete system, omission of either R5P, PEP, Mg^{2+} or NADH showed a marked decrease in the rate of incorporation of $^{14}CO_2$ indicating the essential nature of these components of the system. Omission of either ADP or cyclic-3′,5′-AMP decreased the rate of reaction to certain extent indicating that they were perhaps activators. Using the complete system with R5P as the second substrate (–COOH acceptor) various cations were examed (Table II) for their effects on the reaction. The results show that Mn^{2+} ion is the most effective cation, and Mg^{2+} ion shows a relativley lower activity. The effect of sugar phosphates on the transcarboxylation reaction is shown in Table III. The most effective sugar phosphate appears to be R-1-P followed in descending order by R5P, FBP, RuBP, F6P and Ru5P. It is interesting to note that in some aspects these data are consistent with those of other investigators [11–18], who demonstrated that certain sugar phosphates increased the rate of $^{14}CO_2$ fixation by chloroplasts from different plants (it should be emphasized here that in all cases either R5P or FBP is the most effective

Table I. Assay system of transcarboxylation reaction from Maize chloroplast crude extract at pH 6.3.

Assay system	$^{14}CO_2$-Fixation 1×10^5 cpm/ 0.8 ml	% of $^{14}CO_2$ fixed
Complete	1.320	7.33
– R-5-P	0.196	1.08
– PEP	0.160	0.88
– Cyclic 3′,5′ AMP	0.880	4.88
– ADP	1.040	5.77
– Mg^{2+}	0.025	0.14
– NADH	0.330	1.83

Table II. Effect of divalent cations on transcarboxylation reaction at pH 6.3.

Cations added (2 μmol/0.8 ml)	Activity [%]
None	4
Mn^{2+}	100
Mg^{2+}	28
Zn^{2+}	10
Co^{2+}	3
Fe^{2+}	2
Ca^{2+}	2
Cd^{2+}	1
Ba^{2+}	1
Cu^{2+}	1
Ni^{2+}	1

substrate for enhancing CO_2 fixation by chloroplasts, which is contrary to the fact of RuBP as the substrate for the carboxylation by RuBP carboxylase reaction in the chloroplast. Moreover, from molecular structure point of view, it is very unlogical to consider that there is a membrane-barrier for the diffusion of RuBP, but not R5P or FBP). However, they interpreted this effect as evidence for the functioning of the pentose phosphate cycle in chloroplasts. The effect of sugar phosphate in augmenting CO_2 fixation into acid stable products in the presence of PEP and Maize chloroplast extract in this study can only be interpreted as providing evidence for a transcarboxylase reaction involving the carboxylation of PEP. Since these extracts lack the classical PEP carboxylase (EC 4.1.1.31) and RuBP carboxylase under our assay conditions at pH 6.3 (data to be published), the carboxylation of PEP can be attributed to the thermostable PEP acid carboxylase which produced C_4-P carbonyl compound as described by Pan and Waygood [4–6]. In addition, the

Table III. Effect of sugar phosphates on transcarboxylation reaction at pH 6.3.

Assay system	$^{14}CO_2$-Fixation 1×10^5 cpm/ 0.8 ml	% of $^{14}CO_2$ fixed
Complete with R-1-P	4.4	24.5
Complete with R-5-P	1.32	7.4
Complete with FBP	1.20	6.7
Complete with RuBP	0.96	5.4
Complete with F-6-P	0.73	4.1
Complete with M-1-P	0.67	3.8
Complete with Ru5P	0.60	3.4
Complete with Xu-1-P	0.29	1.6
Complete with F-1-P	0.14	0.8
Complete with G-6-P	0.05	0.3

Table IV. Variation of end product formation from transcarboxylation reaction in the presence of different sugar phosphates used as COO^- acceptor.

Assay conditions	Ratio of end product formation		
	Malate	3-PGA	Glycerate
Omission of sugar phosphate	3.5	0.25	1.7
R-5-P or F-6-P	1.5	0.75	3.5
RuBP or FBP	2.0	2.5	0.7

involvement of PEP in $^{14}CO_2$ fixation is evidence against the consideration of the functioning of the reductive pentose phosphate cycle in these chloroplast extracts. This study also shows that glycerate is the main end product when R5P or F6P used as $-COO^-$ acceptor in the transcarboxylation reaction, but 3-PGA is the major end product when either R-1-P, RuBP or FBP used as second substrate (Table IV). However, if the sugar phosphate is omitted in the reaction, malate is the main product which is derived from the carboxylation of PEP by the thermostable PEP acid carboxylase and enzyme system responsible for the formation of malate (manuscript in preparation). Thus, taken all evidence together, our present working hypothesis postulates that two substantive reactions occur in C_4 plants as follows:

1. P-enolpyruvate + CO_2 → C_4-P ···► malate
2. C_4-P + C_5-P or C_6-P → C_3 + C_3-P + C_3-P or C_4-P

One final point as indicated in Introduction that the only enzymatic mechanism which could satisfy the results demonstrated by a number of laboratories; i. e. in certain plants, glycerate or 3-phosphohydroxypyruvate is the early stable product of photosynthesis [7, 19]; R5P or FBP is the most effective sugar phosphate for enhancing CO_2 fixation by chloroplasts [11–18]; the stimulation of CO_2 fixation by R5P for isolated mesophyll cells of Digitaria pentzii in which the activity of RuBP carboxylase in negligible [19]; and more importantly the isotopic discrimination data of Whelan *et al.* [20], would be a transcarboxylation reaction in C_4 plants.

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