PURIFICATION AND PROPERTIES OF PHOSPHOENOLPYRUVATE CARBOXYLASE FROM GREEN LEAVES OF MAIZE

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Received April 6th, 1978

Phosphoenolpyruvate carboxylate was isolated from green leaves of maize (*Zea mays* L.) by a procedure including fractionation with ammonium sulphate, chromatography on DEAE-cellulose and preparative electrophoresis on polyacrylamide gel. The specific activity of the electrophoretically homogeneous enzyme was 23 U/mg. Its molecular weight was about 405000, pH optimum was within the range 7·9 to 8·3, K_m for phosphoenolpyruvate was $1{\cdot}05 \cdot 10^{-3}$ and the apparent K_m for the magnesium ions was $8{\cdot}0 \cdot 10^{-4}$ M. The enzyme was inhibited by malate, aspartate, citrate, pyruvate, ATP and ADP and chloride ions. It was strongly activated by glycine and glucose 6-phosphate and to a lesser degree by glucose 1-phosphate and fructose 1,6-bisphosphate; no activation by orthophosphate and 3-phosphoglycerate was observed.

Phosphoenolpyruvate carboxylase (orthophosphate: oxaloacetate carboxy-lyase EC. 4.1.1.31) catalyzes the primary fotosynthetic fixation of CO_2 in certain species of higher plants (C_4-plants). Phosphoenolpyruvate is an acceptor of CO_2 and through its β-carboxylation oxaloacetate appears in the reaction[1]:

$$\text{Phosphoenolpyruvate} + CO_2 + H_2O \rightarrow \text{oxaloacetate} + \text{phosphate}.$$

The investigation of the properties of the enzyme, which plays a key role in the photosynthetizing cell is interesting mainly from the aspect of the possible regulation of its activity. So far most of the authors dealt only with enzyme extracts prepared from bundle sheath chloroplasts[2-6]. Separating techniques for the purification of the enzyme were rarely applied[7,8]. In this paper we propose a method for the isolation of electrophoretically homogeneous phosphoenolpyruvate carboxylase from plant material. The enzyme isolated from maize leaves is characterized in detail.

EXPERIMENTAL

Materials

Following agents were used in the work: NADH and malate dehydrogenase (Boehringer, GFR), human γ-globulin (Serva, GRF), phosphoenolpyruvate (Calbiochem, USA), Jack bean urease

(Fluka, Switzerland), $NaH^{14}CO_3$ (Radioaktive Präparate, GDR), Fast Violet B (Hoechst, GFR). All the other chemicals were of analytical grade.

Methods

Plant cultivation. The enzyme was prepared from the green leaves of three-week old maize plants (*Zea mays* L.), which were cultivated at 25°C in a greenhouse with natural light.

Determination of phosphoenolpyruvate carboxylase activity. The activity was determined on the one hand by measuring the incorporation of ^{14}C supplied as $NaH^{14}CO_3$ into maleate in the arrangement according to Evers and coworkers[9], on the other hand, spectrophotometrically by coupling the reaction with that catalyzed by malate dehydrogenase and measuring the decrease of NADH concentration.

1 ml of the reaction mixture contained 50 μmol of Tris-HCl pH 7·9, 10 μmol $NaHCO_3$, 5 μmol $MgCl_2$, 10 μmol 2-mercaptoethanol, 0·2 μmol NADH, 5 μg malate dehydrogenase and 5 μmol phosphoenolpyruvate. For radiometric determination the reaction mixture also contained 2·5 μCi $NaH^{14}CO_2$ (specific activity 0·1 μCi/μmol); the reaction was run at 25°C and stopped by adding 1 ml 1M-HCl. The Bray[10] solution was added and the radioactivity was measured in an Isocap 300 liquid scintillation counter (Nuclear-Chicago, USA).

The enzyme activity unit is defined as the amount of enzyme that catalyzes the carboxylation of 1 μmol phosphoenolpyruvate in one minute under standard conditions.

Isolation of phosphoenolpyruvate carboxylase. All the purification steps were performed, if not indicated otherwise, at 4°C. 50 g leaves were homogenized in a Warring blender with 100 ml 50 mM Tris-HCl buffer pH 7·7 containing 2 mM-EDTA, 5 mM 2-mercaptoethanol and 5 mM-$MgCl_2$. The homogenate was filtered through 4 layers of gauze and the filtrate was centrifuged 30 min at 15000*g*. The supernatant was fractioned by ammonium sulphate precipitation; the active portion precipitated between 30 and 60% saturation. The active sulphate fraction was desalted on a Sephadex G-25 column (2·5 . 30 cm) equilibrated with the buffer used for the extraction. The eluate (20 ml) was then applied on a DEAE-cellulose column (2 . 30 cm) equilibrated with the same buffer. Phosphoenolpyruvate carboxylase was eluted with 300 ml Tris-HCl buffer, pH 7·7, with a linearly increasing concentration of Tris from 50 to 400 mM. The active fractions were pooled, the concentration of the buffer was adjusted to the value of 20 mM (pH 7·5, 1 mM $MgCl_2$, 1 mM 2-mercaptoethanol) by ultrafiltration and the volume reduced to 10 ml. The preparative electrophoresis was performed on the apparatus "Prep. P.A.G.E." (Quickfit) according to Barthová and coworkers[11]. The buffers used contained 1 mM-$MgCl_2$ and 1 mM 2-mercaptoethanol, the temperature was kept at 10°C, and the separation process lasted 16 h (the height of the separation gel was 3 cm and the current used 80 mA).

Analytical electrophoresis. The homogeneity of phosphoenolpyruvate carboxylase was determined by discontinuous electrophoresis on polyacrylamide gel[12]. Acrylamide concentration was 2·5%, buffer pH 8·1 and the electrophoresis was run for 2 h a current of 3·5 mA per tube. Proteins were detected by Amidoblack 10 B, and the enzyme activity by Fast violet B (ref.[13]).

The approximate molecular weight was determined by comparing the elution volumes of phosphoenolpyruvate carboxylase and standards during gel filtration on a column of Sephadex G-200 (1 . 80 cm). γ-globulin (m.w. 157000), catalase from bovine liver (244 000) and urease from Jack bean (483000) were used as standards.

The concentration of proteins was determined according to Lowry and coworkers[14], with bovine serumalbumin as a standard.

RESULTS

In Table I the efficiency of the individual isolation steps is presented. The phosphoenolpyruvate carboxylase preparation obtained was electrophoretically homogeneous and its specific activity was usually between 20 to 23 units/mg of protein.

The crude enzyme extract as well as the sulphate fraction are labile; their activity decreases by 50–60% in 24 h at 4°C. The enzyme preparation obtained by chromatography on DEAE-cellulose is, under the stated conditions, stable for 5 days. The complete procedure described in this paper resulted in an enzyme preparation which did not lose its activity for 14 days when kept in 20% sucrose solution at −18°C.

The molecular weight of phosphoenolpyruvate carboxylase from maize leaves is 405 000 daltons as determined by gel chromatography. The pH optimum of the enzyme lies between 7·9–8·3. At pH 7·9 the enzyme followed Michaelis and Menten

TABLE I

Isolation of Phosphoenolpyruvate Carboxylase from the Green Leaves of Maize

Fraction	Proteins mg	Activity		Purification degree
		total U	specific U/mg	
Crude extract	407·5	273·1	0·58	1
30–60% Saturation with ammonium sulphate	92·6	135·7	1·47	2·5
Chromatography on DEAE-cellulose	25·2	116·4	4·62	7·9
Preparative electrophoresis	2·6	61·3	23·58	40·5

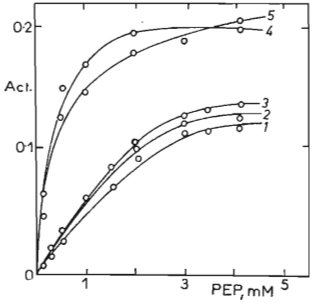

FIG. 1

The Effect of Glucose 1-Phosphate, Glucose 6-Phosphate, Fructose 1,6-Bisphosphate and Glycine on the Activity of Phosphoenol pyruvate Carboxylase from Maize Leaves

Ordinate: concentration of phosphoenolpyruvate (mM) Abscissa: enzyme activity (U), *1* without activator, *2* 15 mM fructose 1,6-bisphosphate, *3* 15 mM glucose 1-phosphate, *4* 15 mM glucose 6-phosphate, *5* 15 mM glycine .

kinetics and the K_m value for phosphoenolpyruvate was $1{\cdot}05 \cdot 10^{-3}$M. The enzyme requires Mg^{2+} for its activity; the apparent Michaelis constant for $MgCl_2$ is $8{\cdot}0 \cdot 10^{-4}$M. The study of the effect of some metabolites on the activity of phosphoenolpyruvate carboxylase revealed that glucose, orthophosphate and 3-phosphoglycerate do not in principle affect its activity, glucose 1-phosphate, fructose 1,6-bisphosphate were weak activators, whereas glucose 6-phosphate and glycine had a significant activating effect (Fig. 1). In contrast, the enzyme is inhibited by pyruvic, malic, citric and aspartic acids. Pyruvate is a competitive inhibitor with respect to phosphoenolpyruvate, the other effectors are non-competitive inhibitors. In addition, nucleotides ATP and ADP function as competitive inhibitors. Chloride ions also have an inhibitory effect on phosphoenolpyruvate carboxylase but the nature of the inhibition is uncompetitive (Table II).

DISCUSSION

A method for the isolation of phosphoenolpyruvate carboxylase was elaborated to include fractionation by ammonium sulphate, chromatography on DEAE-cellulose and finally electrophoresis on polyacrylamide gel. Electrophoresis was the most effective process of the whole procedure (Table I). The enzyme preparation obtained by this procedure was electrophoretically homogeneous and its specific activity was approx. 23 U/mg. The main advantage of the method we propose is its simplicity in comparison with procedures described by other authors[7,14]. For example, Uedan and Sugiyama[15] achieved ninefold purification of the enzyme

TABLE II

The Effect of Some Inhibitors in the Activity of Phosphoenolpyruvate Carboxylase from Maize Leaves

Inhibitor	Type of inhibition with respect to phosphoenolpyruvate	Constant of inhibition M
Pyruvate	competitive	$7{\cdot}5 \cdot 10^{-3}$
Citrate	noncompetitive	$3{\cdot}0 \cdot 10^{-3}$
Malate	noncompetitive	$6{\cdot}0 \cdot 10^{-3}$
Aspartate	noncompetitive	$1{\cdot}2 \cdot 10^{-2}$
ATP	competitive	$8{\cdot}0 \cdot 10^{-4}$
ADP	competitive	$3{\cdot}0 \cdot 10^{-3}$
Cl^-	uncompetitive	—

by a procedure that was two operations more complicated than our procedure, which resulted in a 40-fold purification of the enzyme with the same yield.

We studied some fundamental structural and kinetic properties of the isolated enzyme mainly with respect to its physiological function in a green cell. The molecular weight (405000), pH optimum and the apparent K_m for Mg^{2+} of the enzyme do not differ from those of the enzyme isolated by Uedan and Sugiyama[15].

The choice of metabolites the effect of which on phosphoenolpyruvate carboxylase was studied, was directed mainly by the position of the enzyme in pathways of photosynthetic fixation of carbon dioxide in plants. The other criteria used in the selection of metabolites were the possibilities of comparison with results of other workers, who had studied their effect mainly by working with very crude enzyme preparations. The results obtained indicate that phosphoenolpyruvate carboxylase from maize leaves is influenced by a set of metabolites. Glucose 6-phosphate may be considered to be a typical activator. It acts as an activator not only of the maize enzyme (Fig. 1) but also of enzymes from other C_4 plants[15-17].

According to Mukerji[7], glucose 6-phosphate is evidently an allosteric effector, as documented by the fact that its activation effect is not proportional to its concentration and that the enzyme, partially denaturated by heat, loses its ability to be activated by the compound.

It is noteworthy that whereas the enzyme we prepared was activated by glycine, some authors[18] claim an inhibitory effect of glycine on phosphoenolpyruvate carboxylase from dicotyledon C_4 plants. It is evident that further studies will be necessary for the elucidation of the physiological role of the above-mentioned substances. However, it seems clear that malate and aspartate probably play a role in the regulation of the reaction catalyzed by phosphoenolpyruvate carboxylase because they are products of reactions following the process of carboxylation[19,20]. Furthermore, the inhibitory effect of ATP and ADP may be explained by their ability to decrease the concentration of Mg^{2+} (forming a bridge between enzyme and phosphoenolpyruvate) in the active site of enzyme which results in a decrease of substrate binding to the enzyme.

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Translated by M. Štulíková.