

Purification and Characterization of Phosphoenolpyruvate Carboxylase from Maize Leaves¹

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ABSTRACT

Phosphoenolpyruvate carboxylase has been purified to homogeneity from maize (*Zea mays* L. var. Golden Cross Bantam T51) leaves. The ratio of specific activities in crude extracts and the purified enzyme suggests that the enzyme is a major soluble protein in the tissue. The enzyme has a sedimentation coefficient ($s_{20,w}$) of 12.3S and a molecular weight, determined by sedimentation equilibrium, of 400,000 daltons. Dissociation of the enzyme and electrophoresis on dodecyl sulfate polyacrylamide gels yields a single stained band which corresponds to a subunit weight of 99,000 daltons. Thus it appears that the native enzyme is composed of four identical or similar polypeptide chains.

The enzyme yields cooperative rate-concentration plots (Hill number of 2) with phosphoenolpyruvate as the variable substrate at pH 7. This cooperativity disappears in the presence of an activator, glucose-6-P, or by raising the pH of the assay mixture to 8. Glycerol (20%, v/v) exerts a similar effect. The enzyme is also activated in the presence of glycine which causes an increase in V_{max} without significant effect on the apparent K_m for phosphoenolpyruvate and Hill number. The apparent K_m for HCO_3^- is 0.02 mM, and the activation constant for Mg^{2+} is 1.54 mM at pH 7. There is an abrupt discontinuity in Arrhenius plots and an associated increase in activation energy below 10.8 C.

Phosphoenolpyruvate carboxylase [orthophosphate:oxaloacetate carboxylase (phosphorylating), EC 4.1.1.31] plays an important role in functional carboxylation of PEP² to form oxaloacetate in various organisms including algae and higher plants. Recent comparative studies of PEP carboxylase from higher plants have revealed a diversity of metabolic roles in different species. In leaves of C_4 plants, the enzyme functions as the catalyst for production of photosynthetic intermediates, malate, or aspartate when coupled with malate dehydrogenase or aspartate aminotransferase (6). These metabolites, on the other hand, are produced as photosynthetic products in the leaves of C_3 plants (1). Ting and Osmond (19) provided evidence showing differences in the kinetic parameters of this enzyme as well as distinguishable physical properties between the enzymes of C_4 and C_3 species.

In addition to the distinct metabolic roles of PEP carboxylase in C_3 and C_4 plants, the enzyme from C_4 plants has received considerable attention because of the sigmoidal nature of its saturation curve with respect to PEP (3, 19) and the activating effect of glucose-6-P (3, 20). Enzyme activity in C_4 plants was shown to respond to prolonged changes in light intensity during growth (7).

Despite these important features, the molecular properties of PEP carboxylase of C_4 plants have not been well documented. The preparation of PEP carboxylase in a pure form from maize leaves and a description of some kinetic and physical properties of the enzyme are reported in this paper.

MATERIALS AND METHODS

Enzyme was prepared from fully expanded leaves of 1-month-old maize plants (*Zea mays* L. var. Golden Cross Bantam T51) grown in a naturally illuminated greenhouse at temperature between 20 and 35 C.

The following reagents are products of Boehringer Mannheim: nucleotides, sugar phosphates, NADH, and malate dehydrogenase (EC 1.1.1.37). Anion exchangers and hydroxylapatite are products of Whatman Biochemicals and Seikagaku Kogyo, Tokyo, respectively.

Standard Assay Method for Carboxylase. The coupled formation of malate dehydrogenase was assayed spectrophotometrically at 22 C and pH 8 according to Lane *et al.* (9). Following a 3-min preincubation of the carboxylase in a 0.9 ml assay mixture containing (in μ mol): tris-HCl, pH 8 (100); $MgCl_2$ (10); dithiothreitol (2); NADH (disodium salt, 0.2); $NaHCO_3$ (10); and 5 units of malate dehydrogenase, the reaction was initiated by addition of 0.1 ml of 50 mM PEP (potassium salt). In some cases, enzyme activity was assayed by measuring the incorporation of $H^{14}CO_3^-$ into acid-stable ^{14}C -malate according to Lane *et al.* (9). This assay mixture contained the same components described above except for the use of $NaH^{14}CO_3$ (2.5 μ Ci) in place of $NaHCO_3$. A unit of enzyme is defined as the amount of enzyme which carboxylates 1 μ mol of PEP/min under standard assay conditions.

Analytical Ultracentrifugation. The enzyme was dialyzed against 50 mM tris-HCl buffer (pH 7) containing 0.1 mM EDTA, 5 mM 2-mercaptoethanol, and 0.1 M KCl. For the determination of sedimentation velocity, the dialysate (7 mg/ml) was centrifuged at 20 C and 40,269 rpm in a Hitachi UCA-1A analytical ultracentrifuge. For the determination of mol wt, sedimentation equilibrium was attained by centrifuging the dialysate (0.2-0.7 mg/ml) at 8,292 rpm and 20 C in an analytical ultracentrifuge equipped with Rayleigh interference optics.

Other Methods of Analyses. Polyacrylamide disk gel electrophoresis was conducted by the method of Davis (4). For the detection of enzyme activity, gel after electrophoresis was sliced into segments (1 mm thick) and each segment was eluted in 0.2 ml of a buffer consisting of 50 mM tris-HCl (pH 7) containing 0.1 mM EDTA, 1 mM dithiothreitol, and 20% glycerol (v/v) for 13 hr at 4 C. A 0.1-ml aliquot of each eluate was assayed for the carboxylase activity using $H^{14}CO_3^-$ as described earlier. Subunit analysis was carried out by the method of Weber and Osborn (22). Protein was determined according to the method of Lowry *et al.* (10). Oxaloacetate, inorganic phosphate, PEP, pyruvate,

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² Abbreviation: PEP: phosphoenolpyruvate.

and orthophosphate dikinase were determined as described previously (18) as were adenylate kinase, pyrophosphatase (7), and ATPase (16).

RESULTS

Extraction and Purification of Enzyme. All procedures from extraction to DEAE-cellulose chromatography were conducted at 22 to 25 C unless otherwise noted. Mature leaves were exposed to sunlight for at least 30 min prior to homogenization. Laminar tissue (950 g) was diced, suspended in 4 volumes of chilled buffer (4 C) consisting of 0.1 M tris-HCl (pH 7.5), 10 mM MgSO₄, 2.5 mM pyruvate, 2 mM KH₂PO₄, 1 mM EDTA, 0.5% ascorbate (w/v), and 10 mM 2-mercaptoethanol as described previously for pyruvate orthophosphate dikinase (18), and then homogenized in an atmosphere of N₂ using a Waring Blender. Immediately following filtration through cheesecloth (four layers) and centrifugation of the filtrates at 10,000g for 5 min, the supernatant was brought to 50% saturation with solid ammonium sulfate. The suspension was centrifuged, then allowed to stand for 30 min after which time the fresh precipitate was collected and stored tightly stoppered at -20 C.

The above precipitate was dissolved in 50 ml of 50 mM tris-HCl (pH 7) containing 5 mM MgSO₄, 2.5 mM pyruvate, 1 mM EDTA, and 10 mM 2-mercaptoethanol, and the resulting protein solution was passed through a column of Sephadex G-25 (4 × 60 cm) previously equilibrated with the same buffer. The eluate (2.4 g of protein in 120 ml) was adjusted to a final concentration of 5 mM dithiothreitol and then applied to a DEAE-cellulose column (3.5 × 85 cm) previously equilibrated with the buffer described above. The enzyme was eluted with 650 ml of buffer containing 0.12 M KCl, collected, and precipitated with ammonium sulfate.

Subsequent operations were carried out at 4 to 6 C unless otherwise specified. The precipitate was dissolved in 20 ml of 50 mM tris-HCl (pH 7) containing 0.1 mM EDTA and 5 mM 2-mercaptoethanol, and the solution was passed through a column of Sephadex G-25 (2.5 × 40 cm) equilibrated with the above buffer. The eluate (1,050 mg of protein in 26 ml) was applied to a column of DE-32 (3.0 × 25 cm) previously equilibrated as above. The carboxylase was eluted with a 700-ml linear KCl gradient (0-0.15 M) as a single peak of activity. Active fractions were collected, and the enzyme was stored frozen as above.

The precipitate obtained after purification with DE-32 was dissolved in 8 ml of 50 mM K phosphate buffer (pH 7) containing 0.1 mM EDTA and 5 mM 2-mercaptoethanol, and the solution was desalted by passing through Sephadex G-25 (2.5 × 27 cm) previously equilibrated with the above buffer, and the enzyme was eluted with 0.1 M K phosphate buffer (pH 7) containing EDTA and mercaptan. After washing the column with the equilibrating buffer, the active fractions were collected and pooled, and the enzyme was precipitated as above.

The precipitate was dissolved in 5 ml of 50 mM K phosphate (pH 7) containing 0.1 mM EDTA, 0.1 M KCl, and 5 mM 2-mercaptoethanol and applied to a column of Sephadex G-200 (2.5 × 90 cm) equilibrated with the above buffer. Elution was accomplished with the same buffer at a flow rate of 10 ml/hr. The carboxylase emerges from the column coincident with the major peak preceding a minor, small mol wt protein peak. The most active fractions were pooled, and after adding 5 mM dithiothreitol, the protein was precipitated with 70% saturated ammonium sulfate and stored frozen until used as described earlier.

Purity and Physical Properties. The results of the enzyme purification are given in Table I. The specific activity of the final enzyme preparation was 20 to 25 units/mg protein, which is comparable to that reported by Miziorko *et al.* (13) for the purified spinach enzyme. Based on this value, the carboxylase constitutes about 10% of the soluble protein in crude extracts.

The final preparation was free of contamination by adenylate kinase, ATPase, pyrophosphatase, and pyruvate orthophosphate dikinase.

There was no significant loss of enzyme activity in the final preparation for several months when the 70% saturated ammonium sulfate precipitate was stored frozen at -20 C. However, the activity of the enzyme in solution was rather labile and about half of the initial activity was lost after 3 days (pH 7, 4 C). Loss of activity was partially prevented by the addition of glycerol (20%, v/v) to the enzyme solution.

Polyacrylamide gel electrophoresis of the final enzyme samples on 5.6% gel indicated a single stained protein band with R_m value (mobility relative to the tracking dye) of 0.26. The stained protein band coincided exactly with a single peak of carboxylase activity obtained from an identical gel. The enzyme forms aggregates upon aging, and the aggregation seems to depend upon enzyme concentration.

Subunit analysis of the enzyme obtained by SDS gel electrophoresis on 7% gel yielded a single stained protein band with a mobility of 0.20 (Fig. 1). The molecular weight of the peptide was calculated to be 99,000 daltons by calibration with protein markers of known mol wt (pyruvate kinase, 57,000; serum albumin, 68,000; and phosphorylase *a*, 94,000).

Sedimentation velocity experiments were carried out at pH 7 and 20 C. The enzyme molecule appears to be monodisperse having a *s*_{20,w} of 12.3S, which agrees well with that (13S) determined by sucrose density gradient centrifugation according to Martin and Ames (11) when using catalase as a marker. These results and those obtained from analyses of the native and dissociated subunit on gel electrophoresis proved that the final preparation is homogeneous.

The mol wt of the native enzyme was estimated to be 400,000 daltons using the sedimentation equilibrium technique at 20 C and assuming a partial specific volume of 0.73. In this determi-

Table I. Purification of Phosphoenolpyruvate Carboxylase of Maize

| Step | Total Activity | Protein | Specific Activity | Yield |
|-----------------------------|----------------|---------|-------------------|-------|
| | units | mg | units/mg protein | % |
| Extract (950 g leaf tissue) | 17,640 | 6,515 | 2.7 | 100 |
| Ammonium sulfate fraction | 6,944 | 2,360 | 2.9 | 39.4 |
| DEAE-cellulose | 10,666 | 1,050 | 10.2 | 60.5 |
| DE-32 | 7,852 | 458 | 17.1 | 44.5 |
| Hydroxylapatite | 6,324 | 280 | 22.6 | 35.9 |
| Sephadex G-200 | 5,206 | 210 | 24.8 | 29.5 |

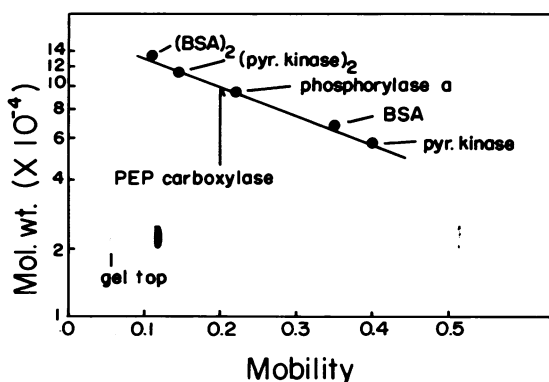


FIG. 1. SDS polyacrylamide gel electrophoresis of the carboxylase. The enzyme, approximately 10 μ g, was dissociated with SDS and 2-mercaptoethanol and then subjected to gel electrophoresis. (BSA)₂ and (pyr. kinase)₂ represent the dimers of their corresponding subunits.

nation, the mol wt is not dependent on protein concentration over the range of 0.2 to 0.7 mg of protein/ml. Linear plots of the logarithm of protein concentration versus r^2 were obtained and they provided additional evidence for the homogeneity of the enzyme preparation with respect to mol wt (Fig. 2). Thus, the native maize leaf PEP carboxylase appears to be a tetramer composed of identical or similar subunits of 99,000 daltons.

Kinetic Properties. The stoichiometry of the carboxylase reaction was determined using coupled reactions with malate dehydrogenase. The results showed that the molar ratio of oxaloacetate and inorganic phosphate formed to PEP utilized was 1:1:1. Maize leaf PEP carboxylase exhibits optimum activity at pH between 8 and 8.5 in the presence of tris-HCl buffer. The velocity at pH 7 was approximately 40% of the optimum activity.

Initial velocities were measured as a function of PEP at saturating levels of Mg^{2+} and HCO_3^- . At pH 7, the saturation curve was found to be sigmoidal (Fig. 3), and the Hill coefficient calculated by using the empirical Hill equation and apparent K_m were 1.80 and 2.60 mM, respectively (Table II). The tricyclohexylammonium salt of PEP was used with no significant effect. By raising the pH to 8, however, the sigmoidal property disappeared; this was accompanied by a decrease in both the Hill coefficient (1.20) and apparent K_m (1.16 mM). A similar trend, but not as significant as seen at alkaline pH, was observed by addition of a rather high concentration of glycerol (20%, v/v). As mentioned earlier, glycerol acts as a stabilizer.

Saturation curves with respect to HCO_3^- and Mg^{2+} were determined at pH 7 by modifying the standard assay method so that the substrate (or activator) was present at rate-limiting concentrations. In both cases, the relation of the initial velocity and substrate (or activator) concentration followed typical Michaelis-Menten kinetics under the conditions described. The apparent K_m for HCO_3^- and activation constant for Mg^{2+} were 0.02 mM and 1.54 mM, respectively. From the V_{max} and a mol wt for the carboxylase of 400,000 daltons, the molecular activity of the enzyme was calculated as 9,920 mol of PEP carboxylated/mol enzyme.

The effect of some metabolites on the initial velocity of the enzyme was tested at two different concentrations of PEP which give half-maximal and maximal velocity. Not remarkably effective metabolites tested were fructose phosphates, glucose-1-P, pyruvate, alanine, aspartate, Pi, and adenosine nucleotides including AMP, ADP, and ATP. Glucose-6-P was found to be a marked activating ligand. This was noted in other investigations wherein crude enzyme extracted from C_4 (3, 20) and Crassulacean acid metabolism plants (20) was used. Glycine, a specific

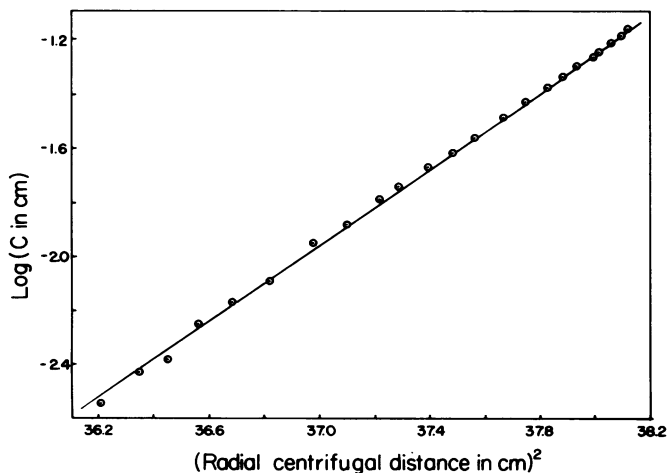


Fig. 2. Plot of concentration versus the square of the radius of rotation. Protein concentration was 0.7 mg/ml.

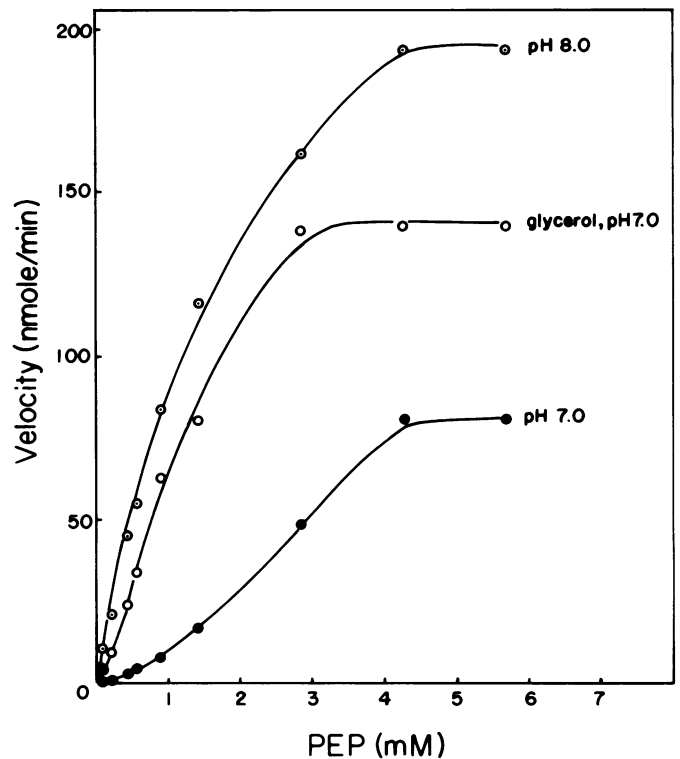


Fig. 3. Saturation curves for PEP showing the kinetic behavior of the carboxylase. Assays were conducted spectrophotometrically using approximately 8 μ g of the enzyme. Tris-HCl buffer (50 mM) was used to adjust pH.

Table II. Kinetic Parameters for Phosphoenolpyruvate Carboxylase of Maize Leaf with Respect to PEP

| Addition | pH | Hill Coefficient | K_m (mM) | V_{max} (munits) |
|-------------------|-----|------------------|------------|--------------------|
| none | 7.0 | 1.80 | 2.60 | 80.6 |
| none | 8.0 | 1.20 | 1.16 | 193.6 |
| glycerol(20% v/v) | 7.0 | 1.45 | 1.25 | 139.8 |
| glucose-6-P(5 mM) | 7.0 | 1.28 | 0.60 | 245.2 |
| glycine(5 mM) | 7.0 | 1.80 | 1.62 | 188.7 |

activator of monocotyledonous C_4 plant PEP carboxylase (14), also exhibited a significant effect on the purified enzyme. It caused an increase in the maximal velocity and slight decrease in apparent K_m with no effect on the Hill coefficient (Table II and Fig. 4). In the presence of a saturating concentration of glucose-6-P, there was a decrease in both the apparent K_m for PEP from 2.60 to 0.60 mM and Hill coefficient from 1.80 to 1.28.

It was demonstrated previously that crude extracts from cold-sensitive C_4 species, such as maize and millet, exhibit an abrupt discontinuity in the Arrhenius plot, and an associated increase in the activation energy, below 12 C (15). Initial velocities were determined as a function of temperature in the range of 4 and 25 C using standard assay mixture. As illustrated in Figure 5, there is a break in the Arrhenius plot at 10.8 C, indicating a significant change in the activation energy from 16.4 kcal/mol above 10.8 C to 32.5 kcal/mol below 10.8 C.

DISCUSSION

Properties of PEP carboxylase from maize were investigated to elucidate hopefully the role of the enzyme in metabolism of C_4

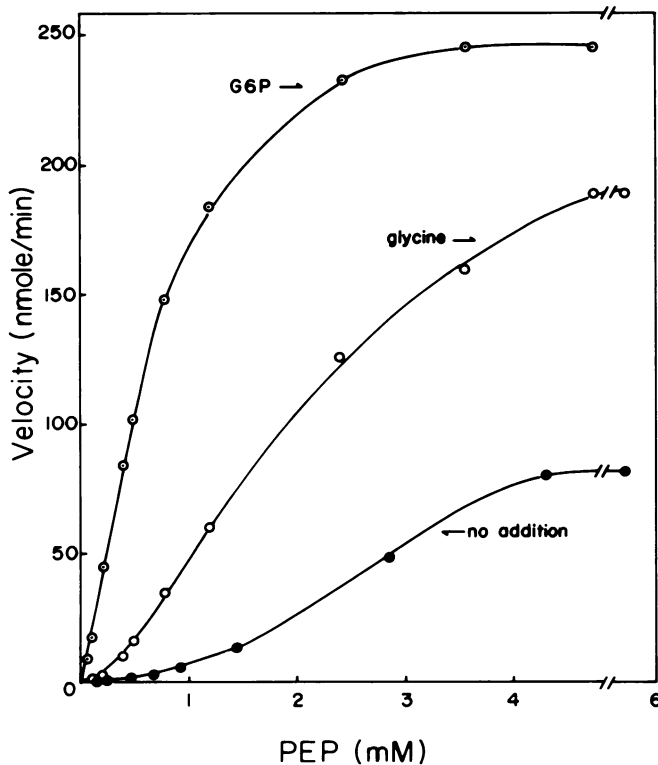


FIG. 4. Activation of the carboxylase by glucose-6-P and glycine. Assays were conducted spectrophotometrically at pH 7 using 8 μ g of the enzyme.

photosynthetic tissue. The procedure developed for purifying the enzyme yielded a homogeneous preparation that was quite stable to storage in a frozen state after purification. It should be noted that the enzyme is a major soluble protein in the leaf tissue. Considering its location in mesophyll cells (2, 5, 8), the content in these particular cells must be higher than estimated (10% of soluble protein in the whole leaf tissue). The high content and cellular location of the enzyme in the leaf favor the view that the C_4 pathway functions as an antenna for trapping CO_2 .

Although nearly homogeneous preparations of this enzyme have been obtained from C_3 plant species such as peanut cotyledon (12) and spinach (13), this is the first report of a pure PEP carboxylase from a C_4 plant source. Absolute comparisons of the properties of the enzymes from different sources is not possible at present since both the methods of analysis and assay conditions were not the same. Nevertheless, all of the enzymes have high mol wt which range between 350,000 and 560,000 daltons. The spinach and maize enzymes are tetramers composed of monomeric subunits of equal mol wt similar to the bacterial enzyme (17).

Kinetic analyses of the CO_2 fixation reaction catalyzed by the carboxylase of maize leaf showed that the saturation curve for PEP at neutral pH deviated from the classical Michaelis-Menten type. The interaction coefficient (n value) was calculated to be approximately 2, which may suggest a homotropic interaction of PEP, similar to the enzyme isolated from Enterobacteriaceae (21). Inclusion of glucose-6-P or increase in pH appeared to normalize the originally sigmoidal saturation curve. This was accompanied by a decrease in the apparent K_m as well as an increase in maximal velocity. Another activator, glycine, caused an increase in maximal velocity and a slight shift in apparent K_m with no effect on the n value. The possible physiological role of these activators or factors in plant metabolism must await future investigations.

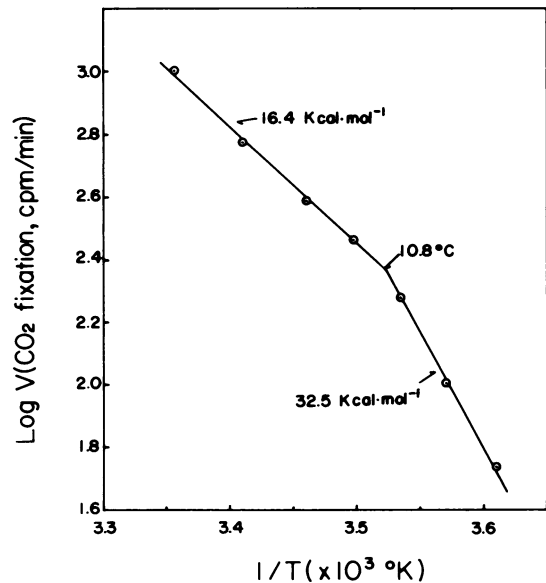


FIG. 5. Arrhenius plot for the carboxylase. Assays were conducted radiochemically at pH 7.5 using 2 μ g of the enzyme.

Kinetic behavior of PEP carboxylase at low temperature has received considerable attention since the initial report by Phillips and McWilliam (15) that the enzyme from cold-sensitive C_4 plants exhibited a significant increase in activation energy below 12 C; in contrast, the enzyme from cold-resistant plants does not show any change in the temperature range between 2 and 15 C. They also demonstrated that treatment of crude extracts with 0.5% Triton-X eliminated such discontinuity and resulted in a higher but constant activation energy over the temperature range. This finding suggested a possible interaction of the enzyme with lipid membranes in crude extracts. No effect of Triton X-100 (0.5%, w/v) was observed in our study on the activation energy of the purified enzyme at temperatures between 4 and 25 C (not shown). This strongly suggests that the kinetic behavior of the enzyme at low temperature is an inherent property of the maize PEP carboxylase.

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