iodoacetate, and iodoacetamide for the X and Y sites in yeast alcohol dehydrogenase, with the hope of establishing the respective role of the two sulfhydryl groups in dehydrogenase action.

References

Auricchio, F., and Bruni, C. B. (1969), *Biochim. Biophys. Acta 185*, 461.
Brown, W. E., and Wold, F. (1973), *Biochemistry 12*, 835.
Chin, C. C. Q., and Wold, F. (1972), *Anal. Biochem. 46*, 585.
Dickinson, F. M. (1970), *Biochem. J. 120*, 821.
Dickinson, F. M. (1972), *Biochem. J. 126*, 133.
Harris, J. I. (1964a), *Nature (London) 203*, 30.
Harris, J. I. (1964b), *in* Structure and Activity of Enzymes, Goodwin, T. W., Harris, J. I., and Hartley, B. S., Ed., New York, N. Y., Academic, p 97.
Hayes, J. E., and Velick, S. F. (1954), *J. Biol. Chem. 207*, 225.
Kagi, J. H. R., and Vallee, B. L. (1960), *J. Biol. Chem. 235*, 3188.
Pfleiderer, G., and Auricchio, F. (1964), *Biochem. Biophys. Res. Commun. 16*, 53.
Rabin, B. R., Cruz, J. R., Watts, D. C., and Whitehead, E. P. (1964), *Biochem. J. 90*, 539.
Theorell, H., and Yonetani, T. (1963), *Biochem. Z. 338*, 537.
Twu, J. S., and Wold, F. (1973), *Biochemistry 12*, 381.
van Eys, J., Ciotti, M. M., and Kaplan, N. O. (1957), *Biochim. Biophys. Acta 23*, 581.
Whitehead, E. P., and Rabin, B. R. (1964), *Biochem. J. 90*, 532.

Purification, Molecular, and Catalytic Properties of Pyruvate Phosphate Dikinase from the Maize Leaf†

Tatsuo Sugiyama

ABSTRACT: Pyruvate phosphate dikinase, which presumably is involved in photosynthetic C_4 dicarboxylic acid pathway in plant, has been purified to homogeneity from maize leaves. The enzyme has a sedimentation coefficient ($s_{20,w}$) of 8.86 S and a molecular weight, determined by sedimentation equilibrium, of 387,000 daltons. Dissociation of the dikinase and electrophoresis on sodium dodecyl sulfate–polyacrylamide gels yields a single stained protein band which corresponds to a subunit weight of 94,000 daltons. Thus it appears that the native enzyme is composed of four identical or similar polypeptide chains. In the absence of magnesium ion, the tetrameric enzyme molecule appears to reversibly dissociate into dimer having a molecular weight, assessed by Sephadex gel filtration, of approximately 195,000 daltons. The enzyme exhibits the property of cold lability, reversibly losing activity at 0°. The enzyme is able to catalyze a reversible conversion of pyruvate to P-enolpyruvate; the observed equilibrium constant of 204 at pH 7.24. At pH 7.5 and 22°, the native enzyme catalyzes the conversion of 2620 mol of pyruvate to P-enolpyruvate/min per mol of enzyme; under comparable conditions, the rate in the reverse (pyruvate formation) direction is 2420 mol/min per mol of enzyme. The enzyme is stimulated by ammonium ion; at pH 7.5, the activation constants being 0.25 and 5.0 mM for the direction toward P-enolpyruvate synthesis and pyruvate synthesis, respectively.

Pyruvate phosphate dikinase, previously named P-enolpyruvate synthase (Hatch and Slack, 1967, 1968), is a new type of enzyme catalyzing the following reaction: ATP + pyruvate + $P_i \rightleftharpoons$ AMP + P-enolpyruvate + PP_i. The dikinase was extracted originally from monocotyledonous tropical grasses such as maize, sugar cane, and *Sorghum* (Hatch and Slack, 1968). The enzyme has now been found in protozoa (Reeves, 1968), two different species of bacteria (Evans and Wood, 1968, 1971; Reeves *et al.*, 1968), and dicotyledonous plants (Johnson and Hatch, 1968). Recent studies in Wood's laboratory on the enzyme isolated from propionibacteria have produced evidence indicating a tri-(uni,uni) Ping-Pong mechanism involved in the enzyme reaction. This idea was further supported by a successful isolation of phosphoryl- and pyrophosphoryl-enzymes (Evans and Wood, 1968; Milner and Wood, 1972).

In the higher plant, this enzyme is believed to operate in the C_4 dicarboxylic acid pathway of photosynthesis (Hatch and Slack, 1966, 1968, 1969; Slack and Hatch, 1967; Johnson and Hatch, 1968). Moreover, the dikinase activity within the leaves of plants is somehow regulated photochemically. The decay rate of enzyme activity upon transferring the plants from light to darkness is characterized by a first-order reaction with a half-time of 15 min, which is rapidly reactivated upon reexposure of the leaves to light (Slack, 1968). In a subsequent paper, Hatch and Slack (1969) demonstrated that heat-labile component(s) present in crude plant extracts may participate in the regulation of dikinase activity in leaves. These findings and analytical data which reveal the same order of the dikinase activity as that of photosynthetic rates observed for tropical grasses (Hatch and Slack, 1970) support a thesis that this enzyme plays a pivotal role in the carbon dioxide fixation of the plants *via* the C_4 dicarboxylic acid pathway.

These observations on the plant dikinase in relation to its unique regulatory behavior led me to isolate the enzyme in a

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pure form in order to elucidate the molecular mechanism involved in its light-activation. The present paper reports the purification as well as certain molecular and catalytic properties of the maize leaf enzyme.

Experimental Section

Materials and Miscellaneous Methods. The following reagents are products of Boehringer Mannheim: AMP, ATP, NADH, pyruvate, P-enolpyruvate, alcohol dehydrogenase (EC 1.1.1.1), catalase (EC 1.11.1.6), glucose-6-phosphate dehydrogenase (EC 1.1:1.49), hexokinase (EC 2.7.1.1), lactate dehydrogenase (EC 1.1.1.27), malate dehydrogenase (EC 1.1.1.37), myokinase (EC 2.7.4.3), pyrophosphatase (EC 3.6.1.1), pyruvate kinase (EC 2.7.1.40), urease (EC 3.5.1.5), and serum albumin. Purified ribulose-1,5-diphosphate carboxylase (EC 4.1.1.39), which was a gift from Mr. Mikio Nishimura, was prepared as reported earlier (Sugiyama *et al.*, 1968). Commercially obtained AMP and ATP were purified by chromatography on Dowex 1 by a procedure described by Cohn (1957).

The means of purifying P-enolpyruvate carboxylase (EC 4.1.1.31) from maize leaf will be published elsewhere. The final preparation had a specific activity of 25 units/mg of protein and its acetate–membrane electrophoresis yielded a single stained protein band congruent with enzyme activity. This enzyme preparation was free of contamination by adenylate kinase, ATPase, pyrophosphatase, and pyruvate phosphate dikinase to less than 0.1% of P-enolpyruvate carboxylase activity.

Other methods of analyses and their authorities are: (1) P-enolpyruvate carboxylase (Lane *et al.*, 1969), (2) adenylate kinase and pyrophosphatase (Hatch *et al.*, 1969), (3) ATPase (Reeves *et al.*, 1968), (4) protein (Lowry *et al.*, 1951; Warburg and Christian, 1942), (5) ribulose-1,5-diphosphate carboxylase (Racker, 1962), and (6) enzymes used as markers for molecular weight determinations, Bergmeyer (1963).

Standard Assay Method for the Dikinase. The formation of P-enolpyruvate was assayed spectrophotometrically by measuring the decrease in NADH at 340 nm, pH 8.0, and 22°. Following a 3-min preliminary incubation of the dikinase in an assay mixture containing (in micromoles) Tris-HCl, pH 8.0 (100), $MgCl_2$ (10), EDTA (0.1), pyruvate (sodium salt, 1.25), dithiothreitol (5), NADH (disodium salt, 0.16), K_2HPO_4 (2.5), $NaHCO_3$ (50), 2 units of P-enolpyruvate carboxylase, and 3 units of malate dehydrogenase in a total volume of 1.0 ml. The reaction was initiated by the addition of 1.25 mM Na_2ATP. The initial rate is proportional to an enzyme concentration up to 50 munits. One unit of the dikinase catalyzes the formation of 1 μmol of P-enolpyruvate/min under these specified conditions.

Isolation and Purification of Dikinase from Leaves of Maize Plants. Extraction and ammonium sulfate (40–50% saturation) fractionation. Mature leaves obtained from 1-month-old maize plants (var. Golden Gross Bantam) grown outside were washed, then exposed to sunlight for at least 30 min, and subsequently homogenized. Laminar tissue (800 g) was diced, suspended in four volumes of chilled buffer (4°) consisting of 0.1 M Tris-HCl (pH 7.5), 10 mM $MgSO_4$, 2.5 mM pyruvate, 2 mM K_2HPO_4, 1 mM EDTA, 0.5% ascorbate, and 10 mM β-mercaptoethanol, and then homogenized in an atmosphere of N_2 using a Waring blender. Subsequent operations were carried out at 22–25° unless otherwise specified. Immediately after filtration through cheesecloth and centrifugation of the filtrates at 10,000*g* for 5 min, the supernatant was brought to 40% saturation with solid ammonium sulfate (243 g/l.). On standing 30 min, the precipitate was discarded and the resultant supernatant was brought to 50% saturation with additional solid ammonium sulfate. The suspension was centrifuged, then allowed to stand for 30 min after which the fresh precipitate was collected, and stored tightly stoppered at −20°.

DEAE-cellulose chromatography. The above precipitate (40–50% saturated ammonium sulfate cut) was dissolved in 47 ml of 50 mM Tris-HCl (pH 7.0) containing 5 mM $MgSO_4$, 2.5 mM pyruvate, 1 mM EDTA, and 10 mM β-mercaptoethanol, and resulting protein solution was passed through a column of Sephadex G-25 (4.0 × 60 cm) previously equilibrated with the same buffer. The eluate (2.23 g of protein in 106 ml) was brought to a final concentration of 5.0 mM dithiothreitol and then applied to a DEAE-cellulose columns (3.5 × 84 cm) previously equilibrated with the buffer described above. After washing the column with 640 ml of 0.12 M KCl in the buffer, the enzyme was eluted with a 3-l. linear KCl gradient (0.12–0.5 M). A single peak of the dikinase activity was obtained at a KCl concentration of about 0.13 M. Fractions containing activities were pooled, and the enzyme reprecipitated with solid ammonium sulfate (472 g/l.) and stored frozen.

Hydroxylapatite chromatography. The frozen precipitate obtained through purification with DEAE-cellulose was dissolved in 10 ml of 10 mM potassium phosphate buffer (pH 7.0) containing 5 mM $MgSO_4$, 0.5 mM EDTA, and 10 mM β-mercaptoethanol, and the solution was then passed through a column of Sephadex G-25 (2.5 × 26 cm), previously equilibrated with the same buffer. The eluate (464 mg of protein in 25 ml) was made to 5 mM dithiothreitol and then transferred to a hydroxylapatite column (2.8 × 21.5 cm) previously equilibrated as above. After washing the column with 140 ml of the equilibrating buffer, the dikinase was eluted by 40 mM potassium phosphate buffer (pH 7.0) containing 5 mM $MgSO_4$, 0.5 mM EDTA, and 10 mM β-mercaptoethanol. No additional activity of the dikinase was noted on eluting the column with 1 M potassium phosphate buffer (pH 7.0) containing Mg^{2+}, EDTA, and mercaptan. The pooled active fractions were brought to 70% saturation with solid ammonium sulfate and the precipitated enzyme was collected by centrifugation.

Gel filtration with Sephadex G-200. The above precipitate (273 mg of protein) was dissolved in 9 ml of 50 mM Tris-HCl (pH 7.0) containing 5 mM $MgSO_4$, 1 mM EDTA, 0.1 M KCl, and 2.5 mM dithiothreitol and applied to a column of Sephadex G-200 (2.5 × 90 cm) previously equilibrated with the above buffer. Elution was accomplished with the same buffer at a flow rate of 10 ml/hr. The dikinase emerges from the column coincident with the second protein peak after the preceding minor protein peak, the most active fractions were pooled, and after adding another 5 mM dithiothreitol, the protein was precipitated with 70% ammonium sulfate and stored frozen as described earlier.

Final purification of the dikinase after the method of Jakoby (1971). The above precipitate (135 mg of protein) obtained after gel filtration was suspended in 10 ml of 55% cold saturated (4°) ammonium sulfate solution containing 50 mM potassium phosphate buffer (pH 7.0), 10 mM $MgSO_4$, 0.1 mM EDTA, and 5 mM dithiothreitol, allowed to stand for 5 minutes at 4°, and then centrifuged for 5 min at 10,000*g* and 4°. After centrifugation the supernatant was decanted into a test tube at 24° leaving behind the solid residue. This process of resuspending and reprecipitating the residue with a less concentrated (53, 51, 48, 45, 42, 40, and 38%) ammonium sulfate solution was repeated. The suspended particles appearing in

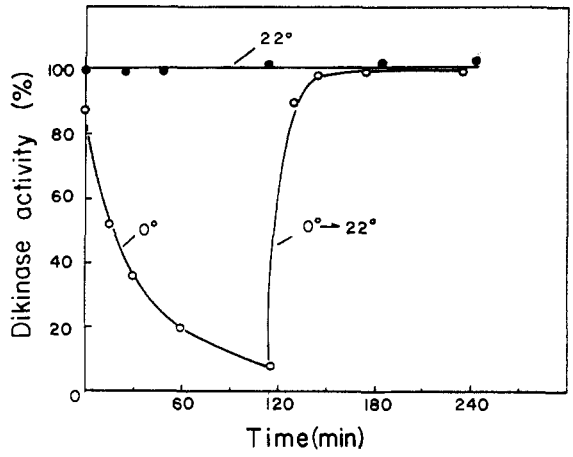

FIGURE 1: Reversible cold lability of the dikinase activity. Dikinase (810 μg) was incubated with 1.0 ml of a buffer consisting of 50 mM Tris-HCl (pH 7.0), 5 mM $MgCl_2$, 0.1 mM EDTA, and 2.5 mM dithiothreitol at 22°. For comparison, the same amount of enzyme was incubated 115 min in an ice bath with 1.0 ml of the above buffer. The chilled mixture was then transferred to a bath at 22° and while maintaining a constant pH during the experiment. Ten microliters was assayed for dikinase activity in the direction toward P-enolpyruvate synthesis as described in Methods section.

the ammonium sulfate solutions between 38 and 45% were collected by centrifugation at 24°. The remaining mother liquor at the end of the extraction was retreated with 70% saturated ammonium sulfate and the entire above process was repeated. The crystalline nature of the material in the precipitate, approximately 2 μ in largest axis, was established with a microscope equipped with a polarized light source.

Sedimentation Velocity Experiments of the Dissociated and Reconstituted Dikinase. The enzyme (7.1 mg/ml) was dialyzed against 50 mM Tris-HCl buffer (pH 7.0) containing 0.1 mM EDTA, 2.5 mM dithiothreitol, and 0.1 M KCl. After centrifugation of the dialysate, the pH of the enzyme solution (5.8 mg/ml) was raised up to 8.1 with NaOH and centrifuged again. The enzyme solution (4.9 mg/ml) after the second centrifugation was dialyzed against 50 mM Tris-HCl buffer (pH 7.0) containing 5 mM $MgCl_2$, 0.1 mM EDTA, 2.5 mM dithiothreitol, and 0.1 M KCl and subjected to centrifugation. Centrifugations were made at 20° and 51,200 rpm in a Hitachi UCA-1A analytical ultracentrifuge.

Equilibrium Study of the Dikinase. In the equilibrium experiment, a reaction mixture containing about 2.3 μmol each of P-enolpyruvate (monopotassium salt), AMP (sodium salt), and PP_i (tetrasodium salt), 10 μmol of $MgCl_2$ in a total volume of 2.4 ml, was adjusted to pH 7.24 with KOH. The control mixture had the same composition except that the enzyme was boiled 3 min prior to addition. The reaction was initiated by adding of 0.1 ml of the purified dikinase (0.7 mg) in 50 mM Tris-HCl buffer (pH 7.0) containing 5 mM $MgCl_2$, 0.1 mM EDTA, 2.5 mM dithiothreitol and 0.1 M KCl and acid consumption was monitored at a constant pH of 7.24 on the Radiometer pH-Stat. Reaction was followed for 46 min at 25° at the end of which the mixture was placed in a boiling-water bath for 3 min, and centrifuged, and the reactants and products were assayed as follows. Pyruvate was assayed by measuring NADH oxidation at 340 nm in a mixture containing 50 mM Tris-HCl (pH 7.0), 1 mM $MgCl_2$, 10 mM KCl, 0.16 mM NADH, and lactate dehydrogenase. P-enolpyruvate was assayed, after determining the residual pyruvate, by adding 1.0 mM ADP and pyruvate kinase. ATP was assayed by measuring NADP reduction at 340 nm in a mixture containing 50 mM Tris-HCl (pH 8.0), 4 mM glucose, 4 mM $MgCl_2$, 0.3 mM NADP, hexokinase, and glucose-6-P dehydrogenase. AMP was assayed by measuring NADH oxidation at 340 nm in a mixture containing 50 mM Tris-HCl (pH 7.6), 1 mM $MgCl_2$, 50 mM KCl, 0.16 mM NADH, 0.5 mM ATP, 0.4 mM P-enolpyruvate, lactate dehydrogenase, pyruvate kinase, and myokinase. P_i was assayed by the procedure of Lindberg and Ernster (1956). PP_i was assayed by measuring the increase in P_i after complete hydrolysis with pyrophosphatase in a mixture of 50 mM Tris-HCl (pH 7.0) and 1 mM $MgCl_2$.

TABLE I: Purification of Pyruvate Phosphate Dikinase from Maize Leaf.

Step	Total Act.[a] (Units)	Protein (mg)	Sp Act. (Units/mg of Protein)
Crude extract[d]	637	6123[b]	0.1
Ammonium sulfate fractionation (40–50%)	887	2233[b]	0.4
DEAE-cellulose	984	464[b]	2.1
Hydroxylapatite	686	273[b]	2.5
Sephadex G-200	556	135[b]	4.1
Jakoby's method	369	82[c]	4.5

[a] Assay in the direction of P-enolpyruvate formation. [b] Determined by the method of Lowry *et al.* (1951). [c] Determined by the method of Warburg and Christian (1942). [d] From 800 g of maize leaf.

Results

Purity and Physical Properties. The final dikinase preparation is purified about 45-fold from the crude homogenate stage by the procedure outlined (Table I). However, the extent of purification is overestimated since the dikinase activity in the earlier stages of the purification process, steps 1 and 2, gives underestimated values presumably due to a presence of interferring substance(s) for the dikinase reaction. Essentially no decrease in the enzyme activity of the final preparation was observed when stored for 3 months in 70% saturated ammonium sulfate solution containing 50 mM potassium phosphate buffer (pH 7.0), 5 mM $MgSO_4$, 0.1 mM EDTA, and 5 mM dithiothreitol at 4° under nitrogen.

The experiments with the partially purified dikinase indicate that the enzyme is cold labile which agrees with the findings of Hatch and Slack (1968). A time-course study of enzyme activity to examine cold lability of the purified enzyme by changing the incubation temperature (0 and 22°) revealed that the purified enzyme lost its activity at 0° but rapidly recovered its original activity at the elevated temperature (Figure 1).

Electrophoresis of the final enzyme samples on 6% gel, using a continuous buffering system of 18 mM glycine–lutidine (pH 8.3) in the presence of 5 mM $MgCl_2$, yielded a single stained protein band with a R_m value (mobility relative to the tracking dye) of 0.14. The stained protein band coincided exactly with a single peak of the dikinase activity on an identical gel obtained from the same run (Figure 2).

Subunit analysis of the dikinase utilizing sodium dodecyl sulfate–gel electrophoresis of the enzyme on 7% gel according to Weber and Osborn (1969) gave rise to a single stained pro-

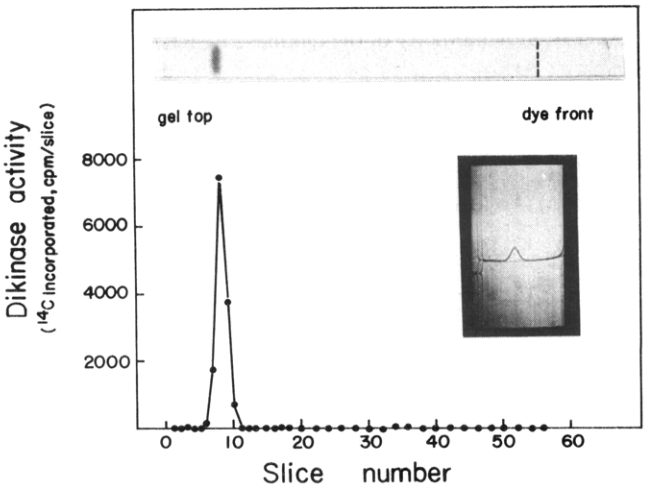

FIGURE 2: Polyacrylamide gel electrophoretic and sedimentation velocity patterns of the dikinase. Dikinase, approximately 20 μg, was applied to each 6% acrylamide gel prepared with a buffer consisting of 0.316 M glycine–KOH (pH 7.3) and 5 mM $MgCl_2$. Electrophoresis was conducted at 25° for 1.5 hr at a constant current of 4 mA/tube with a continuous buffering system of 18 mM glycine–2,6-lutidine (pH 8.3) and 5 mM $MgCl_2$. On completion, the gel was stained with Coomassie Brilliant Blue to locate proteins, while the adjacent one was sliced into segments, 1-mm thick. Each segment was eluted in 0.2 ml of 50 mM Tris-HCl buffer (pH 7.0) containing 5 mM $MgCl_2$ and 5 mM dithiothreitol for 20 hr at 24°. A 0.1-ml aliquot of each eluate was assayed for dikinase activity in the P-enolpyruvate formation direction using a mixture containing 50 μmol of Tris-HCl (pH 8.0), 5 μmol of $MgCl_2$, 0.05 μmol of EDTA, 1 μmol of dithiothreitol, 0.2 μmol of NADH, 1.25 μmol of K_2HPO_4, 5 μmol of [^{14}C]$NaHCO_3$ (0.4 μCi), and 2 units each of malate dehydrogenase and P-enolpyruvate carboxylase. Reaction time was 30 min at 25°, and acid-stable [^{14}C]malate produced was assayed by the method as described by Lane *et al.* (1969). For the sedimentation velocity experiment, the purified dikinase (3.6 mg/ml) dialyzed against 50 mM Tris-HCl buffer (pH 7.0) containing 5 mM $MgCl_2$, 0.1 mM EDTA, 2.5 mM dithiothreitol, and 0.1 M KCl was centrifuged at 51,200 rpm at 23.2° as described in Methods section. Photograph was taken at 51 min after reaching a maximum speed.

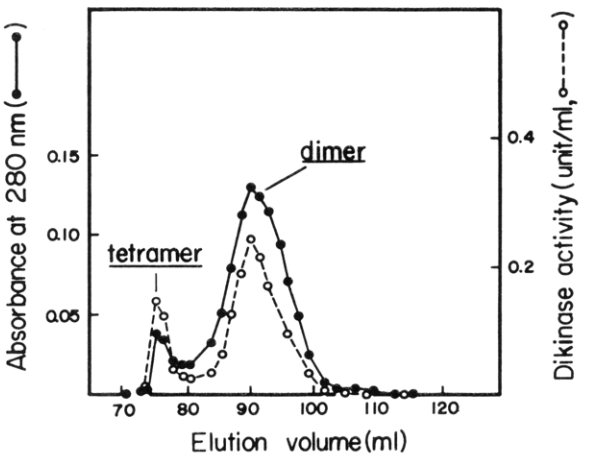

FIGURE 3: Gel filtration of the dissociated dikinase in the absence of Mg^{2+} on Sephadex G-200. Dikinase (1.4 mg) in 0.5 ml of 50 mM Tris-HCl buffer (pH 7.0) containing 5 mM $MgCl_2$, 0.5 mM EDTA, and 2.5 mM dithiothreitol was dialyzed against 50 mM Tris-HCl buffer (pH 8.0) containing 0.5 mM EDTA, 5 mM dithiothreitol, and 0.1 M KCl at 24°, and then applied to a column of Sephadex G-200 (1.6 × 88 cm) equlibrated with the dialysis buffer. Elution was accomplished with the same buffer at a flow rate of 4 ml/hr at 24°.

tein band with a mobility of 0.26; the molecular weight of the subunit was calculated to be 94,000 daltons by the calibration with protein markers of known molecular weight (pyruvate kinase, 57,000; catalase, 58,000; serum albumin, 68,000; and phosphorylase *a*, 94,000).

Sedimentation velocity experiments in the presence of 10 mM Mg^{2+} were carried out at pH 7.0 and 23.2°. The sedimentation coefficient of the enzyme is not dependent on protein concentration over the range of 2.4–7.4 mg of protein/ml and the enzyme molecule appears to be monodisperse having an $s_{20,w}$ of 8.86 S (Figure 2, inset). This and the results obtained from the experiments of electrophoresis on the native and dodecyl sulfate dissociated enzyme indicate that the final preparation is homogeneous.

The molecular weight of the native enzyme assessed by using a Sepharose 6B column calibrated with enzyme markers of known molecular weight (catalase, 232,000; phosphorylase *a*, 370,000; urease, 483,000; and ribulose-1,5-diphosphate carboxylase, 515,000) was estimated to be 370,000 daltons according to the method of Andrews (1970). Using the sedimentation equilibrium technique at protein concentrations of 0.55–1.1 mg/ml at 20° and 3533 rpm in a Hitachi UCA-1A ultracentrifuge equipped with Rayleigh interference optics and assuming a partial specific volume of 0.73, the molecular weight was estimated as 387,000 daltons. Thus the two approaches were in good agreement. In these determinations, linear plots of natural logarithm of protein concentration in fringes *vs.* r^2, according to the procedure described by Chervenka (1969), were obtained providing additional evidence for the homogeneity of the dikinase preparation with respect to molecular weight. Thus, the native maize leaf dikinase in the presence of Mg^{2+} appears to be a tetramer composed of identical or similar subunits of 94,000 daltons.

Dissociation and Association by Magnesium Ion. It has been demonstrated by Hatch and Slack (1968) that the partially purified dikinase from tropical grasses requires Mg^{2+} for its stability. Sedimentation velocity experiments to examine the Mg^{2+} requirement of the enzyme molecule were undertaken providing the first evidence to suggest that the dikinase dissociates in the absence of Mg^{2+}. The result indicated that the enzyme in the absence of Mg^{2+} appeared to dissociate into small heterogeneous components having an average $s_{20,w}$ of 5.75 S being accompanied by a decrease in the enzyme activity (22.3% of the native enzyme activity). Further dissociation of the enzyme molecule with an $s_{20,w}$ of 4.46 S occurred upon raising the pH to 8.1 but the enzyme still retained 19.8% of the native enzyme activity. After subsequent dialysis of the apparently dissociated enzyme against a buffer containing Mg^{2+} at pH 7.0, the enzyme molecule appeared to be partially reconstituted as the enzyme regained 69.1% of its native enzyme activity and attained an average $s_{20,w}$ of 7.6 S, although there still existed some dissociated component. The enzyme was subjected to a Sephadex G-200 gel filtration in the absence of Mg^{2+} at pH 7.0 to ascertain the dissociation of the enzyme molecule and assess the molecular weight of the dissociated component. The elution profile (Figure 3) indicates that an enzymatically active major component is preceded by a minor one of which K_{av} gives the same value as that of the native dikinase determined from a separate run at pH 7.0 in the presence of Mg^{2+}. A molecular weight of the major component was estimated to be 195,000 daltons using the calibrated gel column with catalase, alcohol dehydrogenase (150,000), hexokinase, and serum albumin. Regarding the remaining activity detectable in the dissociated fractions, it is inconclusive whether the activity is due to a reconstitution of the active molecular form (tetramer) from the dissociated and inactive form (dimer) or the dimer itself is capable of cat-

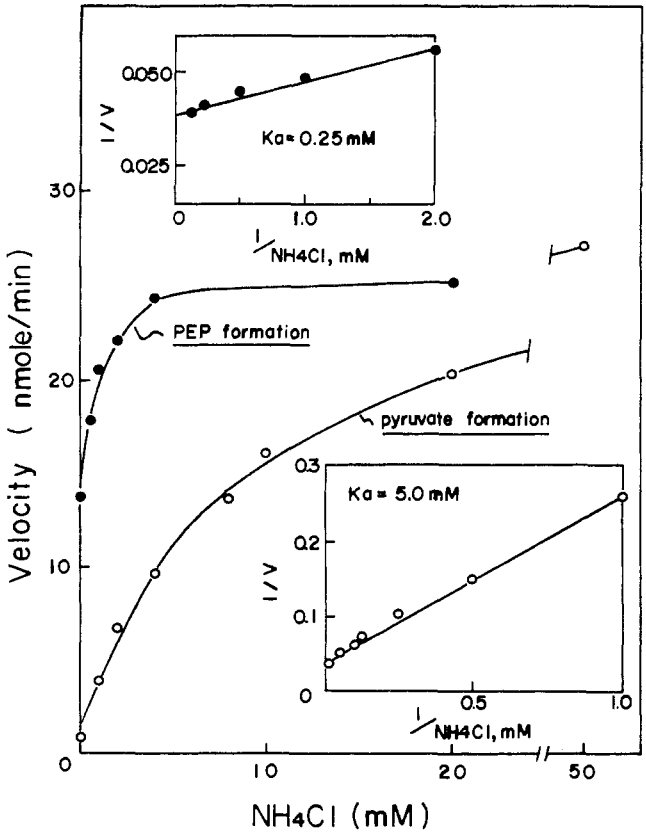

FIGURE 4: Activation of the dikinase by NH_4Cl. Reaction mixture for determining the rate of pyruvate formation contained (in micromoles): Tris-HCl (pH 7.5), 100; $MgCl_2$, 10; EDTA, 0.1; P-enolpyruvate (monosodium salt), 1.25; AMP (sodium salt), 0.5; PP_i (tetrasodium salt), 0.5; dithiothreitol, 2; NADH (disodium salt), 0.16; lactate dehydrogenase, 3 units; dikinase, 4 μg; and NH_4Cl, various amounts specified in a total volume of 1.0 ml. Reaction was initiated by the addition of P-enolpyruvate after 3-min preliminary incubation at 22°. For the determination of the rate of P-enolpyruvate formation, reaction mixture contained (in micromoles) Tris-HCl (pH 7.5), 100; $MgCl_2$, 10; EDTA, 0.1; pyruvate (monosodium salt), 1.25; $NaHCO_3$, 10; dithiothreitol, 2; NADH (disodium salt), 0.16; K_2HPO_4, 2.5; Na_2ATP, 1.25; P-enolpyruvate carboxylase, 2 units; malate dehydrogenase, 3 units; dikinase, 4 μg; and NH_4Cl, various amounts specified in a total volume of 1.0 ml. Following a 3-min preliminary incubation at 22°, the reaction was initiated by the addition of ATP. Assay of both directions was conducted at 22° for at least 4 min.

alyzing the reaction since the enzyme reaction exclusively requires Mg^{2+}.

Equilibrium Studies. Study of stoichiometry on the dikinase was made in the presence of AMP, P-enolpyruvate, and pyrophosphate resulting in equilibrium being attained from the reverse direction. When the substrates were incubated with an excess amount of the enzyme at a constant pH of 7.24 in an unbuffered solution and acid consumption is monitored on the pH-Stat, the consumption of P-enolpyruvate, AMP, and pyrophosphate is correspondingly accompanied by the production of equimolar amounts of pyruvate, ATP, and inorganic phosphate after reaching an equilibrium (Table II). Considering the consumption of two equivalent amounts of acid during the reaction, the quantitative results support the following equation: ATP + pyruvate + $P_i \rightleftharpoons$ AMP + P-enolpyruvate + PP_i + $2H^+$. Based on the equation, the K_{obsd} is calculated to be 204. When the K value is recalculated with respect to hydrogen ion concentration using the equation, log K' = log $K_{obsd}/(H^+)^2$, the value of log K' obtained

TABLE II: Stoichiometry of the Dikinase Reaction.[a]

	Amount (μmol/Reaction Mixture)					
Time (min)	P-enolpyruvate	AMP	PP_i	Pyruvate	ATP	P_i[b]
0	2.29	2.45	2.32	0.00	0.00	0.03
46	0.34	0.48	0.28	2.06	2.05	2.20
Net change	−1.95	−1.97	−2.04	+2.06	+2.05	+2.17

[a] Equilibrium was attained at 25° at a constant pH of 7.24. Details are described in Methods section. Acid consumption was 4.32 μmol/reaction mixture. [b] The solution of sodium pyrophosphate contained 0.7% of inorganic phosphate.

agrees reasonably well with that reported for bacterial dikinase by Reeves *et al.* (1968).

Kinetic Parameters. Maize leaf dikinase, like that of bacterial origin reported by Reeves *et al.* (1968) and Evans and Wood (1971), is activated by ammonium ion. As is shown in Figure 4, at a saturated concentration of NH_4Cl, the rate of P-enolpyruvate formation catalyzed by the dikinase, is only activated 1.8-fold having a K_a of 0.25 mM while the rate of pyruvate formation is enhanced 20-fold with a K_a of 5.0 mM. With regard to the difference in the K_a values for NH_4Cl determined for the forward and reverse reactions, further experimentation is necessary to offer an explanation. At a concentration of 34 mM, approximately 80 and 50%, respectively, of activation effect of NH_4Cl on the dikinase can be achieved by KCl and NaCl.

The apparent K_m values for ATP, pyruvate, and phosphate in the direction toward P-enolpyruvate formation and pyrophosphate and P-enolpyruvate in the direction toward pyruvate formation were determined at pH 7.5 by modifying the standard assay method so that the substrate determined was present at rate-limiting concentrations (Table III). In all cases, the relation of initial velocity and substrate concentration followed a typical Michaelis–Menten kinetics under the conditions described. The K_m value for AMP is too low to be determined by spectrophotometric assay method employed. At pH 7.5 and 22°, the maximum velocity of the reaction toward P-enolpyruvate formation is about same as that of the reverse direction (pyruvate formation). From their corresponding V_{max}'s and a molecular weight for the dikinase of 387,000 daltons, the molecular activities of the two directions are calculated to be 2620 mol of P-enolpyruvate and 2420 mol of pyruvate, which are synthesized per mol of enzyme.

Discussion

The purified dikinase from maize leaf reversibly catalyzes the interconversion of pyruvate and P-enolpyruvate at pH 7.24, having an observed equilibrium constant of 204 for the reaction towards pyruvate synthesis. The value for log K', 16.8, is closely approximate that, 17.3 at pH 7.22, reported on the enzyme of *Bacteroides symbiosus* by Reeves *et al.* (1968). However, the apparent equilibrium of the dikinase *in vivo* would be favored for the direction toward P-enolpyruvate synthesis by coupling with P-enolpyruvate carboxylase, pyrophosphatase, and adenylate kinase which have been demonstrated to exist in considerable excess of that required for

TABLE III: Kinetic Data for the Dikinase from Maize Leaf.[a]

Direction of P-enolpyruvate Formation	Direction of Pyruvate Formation
K_m (pyruvate), 250 μM	K_m (P-enolpyruvate), 140 μM
K_m (ATP), 15 μM	K_m (AMP), less than 10 μM
K_m (P_i), 1500 μM	K_m (PP_i), 40 μM
K_a (NH_4Cl),[b] 250 μM	K_a (NH_4Cl),[b] 5000 μM
V_{max}, 1.61 μmol/min per unit of enzyme	V_{max}, 1.49 μmol/min per unit of enzyme
Molecular activity, 2620 mol/min per mol of enzyme	Molecular activity, 2420 mol/min per mol of enzyme

[a] Standard reaction mixture for the determination of the apparent K_m values in the direction toward P-enolpyruvate formation contained the following components (in micromoles): Tris-HCl (pH 7.5), 100; $MgCl_2$, 10; EDTA, 0.1; dithiothreitol, 2; $NaHCO_3$, 10; NH_4Cl, 25; NADH, 0.16; pyruvate, 2.5; ATP, 1.25; K_2HPO_4, 15; P-enolpyruvate carboxylase, 2 units; malate dehydrogenase, 3 units; and dikinase, 2–6 μg (specific activity of 4.2 units/mg of protein) in a total volume of 1.0 ml. Varied concentrations (in micromolar) of each substrate determined were 25–1250 for pyruvate, 10–100 for ATP, and 200–4000 for P_i. For the determination of the apparent K_m values in the direction towards pyruvate formation, standard reaction mixture contained the following components (in micromoles): Tris-HCl (pH 7.5), 100; $MgCl_2$, 10; EDTA, 0.1; dithiothreitol, 2; NH_4Cl, 50; P-enolpyruvate, 1.25; AMP, 1; PP_i, 1; NADH, 0.16; lactate dehydrogenase, 3 units; and dikinase, 2–6 μg (specific activity of 4.2 units/mg of protein) in a total volume of 1.0 ml. Varied concentrations (in micromolar) of each substrate determined were 20–250 and 10–100 for P-enolpyruvate and PP_i, respectively. Assay for both directions was made at 22° following 3-min preliminary incubation. [b] See Figure 4.

maximum photosynthetic rates in leaves of tropical grasses (Hatch and Slack, 1970).

Mg^{2+}-dependent formation of an active complex from two protein subunits has been reported by Brown *et al.* (1967) on ribonucleoside diphosphate reductase from *Escherichia coli*. In this study, the maize leaf dikinase appears to be a tetramer in the presence of Mg^{2+} and dissociate into a less active dimer in its absence. Although the enzyme activity is detectable in the dimer fraction after separation on gel filtration, no conclusive evidence was found indicating that the dimer itself is capable of catalyzing the reaction. From a phylogenetic view point it is interesting to note that the propionibacterial dikinase, apparently exhibiting no Mg^{2+} requirement for its stability, has a molecular weight of approximately 150,000 daltons (Milner and Wood, 1972), which is close to that of the dimer of the maize leaf enzyme.

The property of cold lability or sensitivity observed in a crude preparation of dikinase (Hatch and Slack, 1968) was confirmed on the purified enzyme. Pyruvate carboxylase from chicken liver mitochondria known as a cold-labile enzyme undergoes the reversible dissociation of the native enzyme molecule into subunits by lowering temperature (Irias *et al.*, 1969). The conformational change in the dikinase molecule in relation to temperature change needs further investigation.

The dikinase in leaves of tropical grasses has been known to be rapidly inactivated by darkness and reactivated on illumination (Slack, 1968; Hatch and Slack, 1969). During the course of extracting the dikinase from maize leaf, it is observed that the enzyme activity depends upon the intensity of the incident light upon the leaf prior to extraction. Exposure of leaves to 7000 lux resulted in enzyme activity at the step of the ammonium sulfate fractionation of about 25% of that treated with 40,000 lux. These changes in activity suggest that the dikinase may be a rate-limiting step for photosynthetic carbon dioxide fixation *via* the C_4 dicarboxylic acid pathway in tropical grasses in response to diurnal as well as nocturnal change in light intensity. As part of this study, dikinase was assayed in dark and 5000 lux but no appreciable difference in activity was noted indicating that the enzyme *per se* is not light activated.

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References

Andrews, P. (1970), *Methods Biochem. Anal. 18*, 1.
Bergmeyer, H. U., Ed. (1963), Methods of Enzymatic Analysis, New York, N. Y., Academic.
Brown, N. C., Larsson, A., and Reichard, P. (1967), *J. Biol. Chem. 242*, 4272.
Chervenka, C. H. (1969), *in* A Manual of Methods for the Analytical Ultracentrifuge, New York, N. Y., Spinco Division of Beckman Instruments, Inc., p 42.
Cohn, W. E. (1957), *Methods Enzymol. 3*, 248.
Evans, H. J., and Wood, H. G. (1968), *Proc. Nat. Acad. Sci. U. S. 61*, 1448.
Evans, H. J., and Wood, H. G. (1971), *Biochemistry 10*, 721.
Hatch, M. D., and Slack, C. R. (1966), *Biochem. J. 101*, 103.
Hatch, M. D., and Slack, C. R. (1967), *Arch. Biochem. Biophys. 120*, 224.
Hatch, M. D., and Slack, C. R. (1968), *Biochem. J. 106*, 141.
Hatch, M. D., and Slack, C. R. (1969), *Biochem. J. 112*, 549.
Hatch, M. D., and Slack, C. R. (1970), *Progr. Phytochem. 2*, 54.
Hatch, M. D., Slack, C. R., and Bull, T. A. (1969), *Phytochemistry 8*, 697.
Irias, J. J., Olmsted, M. R., and Utter, M. F. (1969), *Biochemistry 8*, 5136.
Jakoby, W. B. (1971), *Methods Enzymol. 22*, 248.
Johnson, H. S., and Hatch, M. D. (1968), *Phytochemistry 7*, 375.
Lane, M. D., Maruyama, H., and Easterday, R. L. (1969), *Methods Enzymol. 13*, 277.
Lindberg, O., and Ernster, L. (1956), *Method Biochem. Anal. 3*, 7.
Lowry, O. H., Rosenbrough, N. J., Farr, A. L., and Randall, J. (1951), *J. Biol. Chem. 73*, 265.
Milner, Y., and Wood, H. G. (1972), *Proc. Nat. Acad. Sci. U. S. 69*, 2463.
Racker, E. (1962), *Methods Enzymol. 5*, 266.
Reeves, R. E. (1968), *J. Biol. Chem. 243*, 3202.
Reeves, R. E., Menzies, R. A., and Hsu, D. S. (1968), *J. Biol. Chem. 243*, 5486.
Slack, C. R. (1968), *Biochem. Biophys. Res. Commun. 30*, 493.

Slack, C. R., and Hatch, M. D. (1967), *Biochem. J. 103*, 660.
Sugiyama, T., Nakayama, N., Ogawa, M., Akazawa, T., and Oda, T. (1968), *Arch. Biochem. Biophys. 125*, 98.
Warburg, O., and Christian, W. (1942), *Biochem. Z. 310*, 384.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244*, 4406.

Purification and Characterization of the 4-Aminobutyrate–2-Ketoglutarate Transaminase from Mouse Brain†

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ABSTRACT: 4-Aminobutyrate-2-ketoglutarate transaminase (EC 2.6.1.19) from mouse brain has been purified 1200-fold over a brain homogenate using ammonium sulfate fractionation and various column procedures. The specific activity of the purified enzyme was 5.0 units/mg and it appeared homogeneous in polyacrylamide gel electrophoresis and high speed sedimentation equilibrium ultracentrifuge runs from which a partial specific volume of 0.742 and a molecular weight of 109,000 were calculated. The pH optimum for the enzyme was 8.05 and the K_m values for γ-aminobutyrate and α-ketoglutarate were estimated to be 1.1 and 0.25 mM, respectively. Of the keto acids tested only α-ketoglutarate was an amino group acceptor. However, of a series of amino acids tested, β-alanine, δ-aminovaleric acid, and β-aminoisobutyric acid were effective amino donors in the reaction catalyzed by the enzyme.

During recent years much information has accumulated concerning the dual function of γ-aminobutyric acid as a metabolite and inhibitory transmitter in invertebrate peripheral nerves and the vertebrate central nervous system (Otsuka *et al.*, 1966; Krnjevic and Schwartz, 1967; Roberts and Kuriyama, 1968; Baxter, 1970). Accordingly it has become increasingly important to obtain more detailed knowledge about the two enzymes directly involved in the metabolism of γ-aminobutyric acid, *i.e.*, the glutamic acid decarboxylase (EC 4.1.1.15) and the 4-aminobutyrate-2-ketoglutarate transaminase (EC 2.6.1.19). Although the latter enzyme has been purified from brain to some extent before (Waksman and Roberts, 1965; Sytinsky and Vasilijev, 1970) little is known with certainty about its properties. The decarboxylase has recently been obtained as a homogeneous protein in this laboratory (Wu *et al.*, 1973) and it seemed therefore appropriate also to undertake the purification of the transaminase.

Furthermore, a highly purified enzyme is a prerequisite for the production of a pure antibody by which it may be possible to localize the enzyme in the brain by the immunohistochemical technique described by Nakane and Pierce (1967). This method might give a specific and precise localization of the transaminase, which is essential to fully understand the mechanism of γ-aminobutyric acid as a neurotransmitter.

This article describes the purification procedures and some properties of the transaminase from mouse brain.

Experimental Procedures

Materials

Whole brains from Swiss albino mice were used as the source for the enzyme. The mice were obtained from Horton Laboratories (Oakland, Calif.). Ammonium sulfate was special enzyme grade from Schwarz/Mann (Orangeburg, N. Y.). 3-Acetylpyridine-NAD$^+$ was purchased from P-L Biochemicals (Milwaukee, Wis.) and glutamate dehydrogenase, type II, and aldehyde dehydrogenase from Sigma Chemical Co. (St. Louis, Mo.). Sephadex G-200 and DEAE-Sephadex were purchased from Pharmacia Fine Chemicals (Piscataway, N. J.) and calcium phosphate gel from Bio-Rad Laboratories (Richmond, Calif.). Pyridoxal phosphate was donated by Calbiochem (La Jolla, Calif.). All other chemicals were of purest grade available from regular commercial sources.

Methods

Standard Activity Assay. A widely used method for determination of γ-aminobutyrate transaminase activity is the radioassay (*cf.* Waksman and Roberts, 1963) in which ^{14}C-labeled α-ketoglutarate and cold γ-aminobutyrate are used as the substrates. The latter method is useful for measuring low activities because of its sensitivity, but it is impractical to employ as a standard assay method in purification procedures during which many column fractions must be assayed. A rapid spectrophotometric method was developed which in principle is similar to previously used methods (*cf.* Baxter, 1970).

The transaminase reaction was performed in a 100 mM Tris-HCl buffer, pH 8.0, containing 20 μM pyridoxal phosphate, 100 μM AET,[1] 50 mM γ-aminobutyric acid, and 10 mM α-ketoglutaric acid. This buffer plus enzyme was incubated in a water bath with shaking for 30 min at 37°. Under the above conditions the reaction is linear with time for 1 hr. The reaction was stopped by the addition of aminooxyacetic acid to a final concentration of 100 μM, and the tubes were immediately transferred to an ice bath. Aminooxyacetic acid is known to be a very potent inhibitor of the γ-aminobutyric acid transaminase (Wallach, 1961). Blanks run either without enzyme or

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[1] Abbreviation used is: 2-aminoethylisothiouronium bromide hydrobromide, AET.