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Purification and Properties of Pyruvate Phosphate Dikinase from Propionic Acid Bacteria*

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ABSTRACT: Pyruvate phosphate dikinase has been purified 300-fold from *Propionibacterium shermanii* grown with glycerol and 85-fold from the organism grown with lactate as the substrates. The enzyme is induced by a factor of 6–10 by growth with lactate as compared to glycerol. The dikinase has optimal activity in the pH range of 6.5–7.0, and requires Mg^{2+} and a monovalent cation (NH_4^+ or K^+) for maximal activity.

The apparent K_m values observed for the formation of P-enolpyruvate from pyruvate are: pyruvate, 1.0×10^{-4} M; ATP, 4.3×10^{-5} M; P_i, 1.0×10^{-3} M; free Mg^{2+}, 4.0×10^{-3} M; and NH_4^+, 2.0×10^{-3} M; and for the formation of pyruvate from P-enolpyruvate: P-enolpyruvate, 3.6×10^{-5} M; AMP, 1.5×10^{-5} M; PP_i, 1.2×10^{-4} M; Mg^{2+}, 2.4×10^{-3} M; and NH_4^+, 2.1×10^{-3} M. Under the conditions tested the reaction was very specific for the adenine nucleotides in both directions. The guanine nucleotides inhibit 8% in the forward reaction and 50% in the reverse reaction. Co^{2+} and Mn^{2+} will substitute for Mg^{2+} but with reduced velocities. Ca^{2+}, Fe^{2+}, and Cu^{2+} inhibit the reaction. The $s_{20,w}$ value of the enzyme is ~6.9 S. The enzyme is cold labile but is stabilized in 1 M sucrose.

Pyruvate phosphate dikinase catalyzes the reaction shown subsequently in eq 1. The enzyme has been observed in propionic acid bacteria by Evans and Wood (1968a,b), in the leaves of tropical grasses by Hatch and Slack (1968), and in the parasitic amoeba, *Entamoeba histolytica*, by Reeves (1968). The enzyme has subsequently been reported to occur in another plant, *Amaranthus palmeri* (Slack, 1968), and another bacterium, *Bacteroides symbiosus* (Reeves *et al.*, 1968).

Hatch and Slack proposed that the enzyme functions in tropical grasses to convert pyruvate to P-enolpyruvate, and that CO_2 is fixed by combination with the P-enolpyruvate. However, the opposite conversion, P-enolypyruvate to pyruvate, appears to occur in *E. histolytica* and *B. symbiosus*, since these organisms lack pyruvate kinase (Reeves, 1968; Reeves *et al.*, 1968).

The mechanism of the reaction has been investigated by Evans and Wood (1968b) and it has been proposed to involve the following sequence.

$$\text{enzyme} + \text{ATP} \rightleftarrows \text{enzyme}\text{–}\overset{\beta\gamma}{\text{P}}\text{P} + \text{AMP}^1 \quad (1a)$$

$$\text{enzyme}\text{–}\overset{\beta\gamma}{\text{P}}\text{P} + \text{P}_i \rightleftarrows \text{enzyme}\text{–}\overset{\beta}{\text{P}} + \overset{\gamma}{\text{P}}\text{P}_i \quad (1b)$$

$$\text{enzyme}\text{–}\overset{\beta}{\text{P}} + \text{pyruvate} \rightleftarrows \text{enzyme} + \overset{\beta}{\text{P}}\text{-enolpyruvate} \quad (1c)$$

$$\text{sum: pyruvate} + \text{ATP} + \text{P}_i \rightleftarrows \text{P-enolpyruvate} + \text{AMP} + \text{PP}_i \quad (1)$$

Evidence for the scheme includes isolation of a ^{32}P-labeled protein after incubation of the enzyme with [^{32}P]P-enolpyruvate. [^{14}C]Pyruvate exchange into P-enolpyruvate occurs by reaction 1c without addition of nucleotides or Mg^{2+}, and [^{14}C]AMP exchange occurs with ATP by reaction 1a without an observed requirement for pyruvate or PP_i. For the exchange of [^{32}P]P_i into PP_i there was a requirement for either ATP or P-enolpyruvate to provide the enzyme–PP_i or enzyme–P for reaction 1b. [^{32}P]P-enolpyruvate labeled the β-phosphate and [^{32}P]PP_i the γ-phosphate of ATP as

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[1] Abbreviations used are definined in *Biochemistry 5*, 1445 (1966).

is required for this sequence. Thus far, however, the enzyme–PP_i has not been detected. There may not be a pyrophosphate intermediate but rather two phosphates bonded to the enzyme at two different sites and one of them may be quite labile.

Andrews and Hatch (1969) investigated the mechanism of the enzyme from the leaves of sugar cane, and indicated that the reaction proceeds in two steps rather than three.

$$\text{enzyme} + \text{ATP} + P_i \rightleftharpoons \text{enzyme–P} + \text{AMP} + PP_i \quad (1d)$$

$$\text{enzyme–P} + \text{pyruvate} \rightleftharpoons \text{enzyme} + \text{P-enolpyruvate} \quad (1c)$$

$$\text{sum: pyruvate} + \text{ATP} + P_i \rightleftharpoons \text{P-enolpyruvate} + \text{AMP} + PP_i \quad (1)$$

As evidence against the previously given mechanism (reactions 1a–c) and for reaction 1d, Andrews and Hatch did not observe a P-enolpyruvate-dependent exchange of [^{32}P]P_i into PP_i, but found an apparent requirement for AMP as well as ATP for the exchange. In addition, they found that the [^{14}C]AMP exchange into ATP required both P_i and PP_i. The results of the exchange studies reported previously (Evans and Wood, 1968b) are consistent with this model, with the exception that a P-enolpyruvate-dependent exchange of [^{32}P]P_i into PP_i was observed with the enzyme from *P. shermanii*. This exception is difficult to explain by the latter mechanism. The exchanges observed with the enzyme from *P. shermanii* were slow, but would probably be stimulated by the addition of NH_4^+, since the exchange studies were performed before the requirement for NH_4^+ was demonstrated. It is possible that the mechanism of the enzyme from plants is different from that of the enzyme from bacteria.

The present report deals with the purification and properties of the enzyme from *Propionibacterium shermanii*.

Materials

AMP, ATP, trisodium P-enolpyruvate, and β-DPNH were purchased from Sigma Chemical Co.; sodium pyruvate and dithioerythritol from Calbiochem; imidazole from Eastman Organic Chemicals; monobasic and dibasic potassium phosphate, KCl, $MgCl_2$, NH_4Cl, and sodium pyrophosphate from Baker Chemical Co.; lactic acid from Clinton Corn Processing Co.; yeast hydrolysate from Yeast Products, Inc.; $(NH_4)_2SO_4$, enzyme grade, from Mann Research Laboratories; bovine serum albumin (fraction V) from Armour Pharmaceutical Co.; and ADP from Pabst Laboratories. Malate dehydrogenase was from Boehringer Mannheim Corp., or was purified from propionic acid bacteria (Allen *et al.*, 1964). Lactate dehydrogenase, pyruvate kinase, hexokinase, myokinase, and glucose 6-phosphate dehydrogenase were from Boehringer Mannheim. Inorganic pyrophosphatase was a product of Worthington Biochemical Corp. P-enolpyruvate carboxytransphosphorylase was purified from propionic acid bacteria (Lochmüller *et al.*, 1966; Wood *et al.*, 1969a).

DEAE-cellulose (type 40), TEAE-cellulose, and cellulose phosphate were obtained from Carl Schleicher & Schuell Co. Before use all the celluloses were washed successively with 0.5 N NaOH, distilled water, 0.1 N HCl, distilled water, and 0.05 M potassium phosphate buffer (pH 6.8). Fine particles were removed by centrifugation at 1000 rpm for 5 min in an International PR-2 centrifuge. The cellulose was thoroughly equilibrated with the starting buffer by five times repeated suspension in the buffer followed by filtration, until the pH of the wash was the same as the starting buffer. Sephadex G-50 and DEAE-Sephadex A-50 were obtained from Pharmacia Fine Chemicals, Inc. Before use they were swelled, washed, and equilibrated as recommended in the product brochures.

Methods

Pyruvate phosphate dikinase may be assayed by measuring P-enolpyruvate formation from pyruvate or pyruvate formation from P-enolpyruvate. Following purification this was done by linkage with other enzymes but in the initial stages of the purification the dikinase activity was measured by a nonlinked assay. The incubation mixture contained, in micromoles per milliliter: potassium phosphate buffer (pH 7.9), 20; $MgCl_2$, 12; ATP, 4; sodium pyruvate, 4; EDTA, 0.1; 2-mercaptoethanol, 2; and in units per milliliter, inorganic pyrophosphatase, 8; and the dikinase. The final volume was 0.5 ml. After 10-min incubation at 25°, the reaction was terminated by adding 0.3 ml of 10% trichloroacetic acid and after centrifugation, the supernatant solution was neutralized with Tris base. The P-enolpyruvate then was determined in a part of the solution by conversion into malate by the action of P-enolpyruvate carboxytransphosphorylase (reaction 3 of Discussion) and malate dehydrogenase, and the oxidation of DPNH was monitored at 340 mμ in a Beckman DU spectrophotometer equipped with a Gilford recorder. This assay contained in micromoles per milliliter: potassium phosphate buffer (pH 6.8), 2.7; $MgCl_2$, 9.4; $KHCO_3$, 30; β-DPNH, 0.13; $CoCl_2$, 0.1; and in units per milliliter, P-enolpyruvate carboxytransphosphorylase, 0.1, and malate dehydrogenase, 0.5.

The linked assay in the direction of formation of P-enolpyruvate involved coupling directly with P-enolpyruvate carboxytransphosphorylase (reactions 1 and 3 of Discussion) and malate dehydrogenase, and determining the rate of DPNH oxidation at 340 mμ. The temperature was 25° and the mixture contained, in micromoles per milliliter: potassium phosphate buffer (pH 6.8), 10; $MgCl_2$, 22; sodium pyruvate, 2; ATP, 2; $KHCO_3$, 30; NH_4Cl, 25; $CoCl_2$, 0.1; β-DPNH, 0.17; and in units per milliliter, P-enolpyruvate carboxytransphosphorylase, 0.3; malate dehydrogenase, 15, and the dikinase. A mixture containing all components except enzymes, ATP, and $MgCl_2$ was freshly prepared each day and gassed with 100% CO_2 to pH 6.7. All assays were performed with two cuvets, one of which contained all components of the reaction, and another in which ATP was omitted. The latter was necessary because of the presence of contaminating enzymes which oxidized DPNH.

In the direction of pyruvate formation the enzyme was coupled with lactate dehydrogenase. The following concentrations in micromoles per milliliter were optimal: imidazole-Cl buffer (pH 6.7), 50; $MgCl_2$, 20; NH_4Cl, 25; P-enolpyruvate, 2; AMP, 2; PP_i, 2; β-DPNH, 0.17; and in units per milliliter, lactate dihydrogenase, 1.0, and the dikinase. A mixture containing all the components except the enzymes and PP_i is stable for 2 days if kept on ice. The assay was performed at 25° with two cuvets, one cuvet containing the complete reaction mixture and the other all but PP_i. The control value was subtracted from that observed when PP_i was included.

Protein concentration was determined by the ultraviolet absorption method (Warburg and Christian, 1941) in the steps of the purification subsequent to the cellulose phosphate column. Prior to this purification the protein was determined by the microbiuret method (Zamenhof, 1957), using bovine serum albumin as a reference standard. All specific activities

are expressed in the tables as micromoles of P-enolpyruvate formed per minute per milligram of protein.

For determination of the stoichiometry of the reaction P-enolpyruvate and ADP were determined by the use of pyruvate kinase and lactate dehydrogenase. AMP was determined in the same cuvet as ADP by the addition of myokinase. ATP was determined with hexokinase and glucose 6-phosphate dehydrogenase. P_i was determined by the method of Gomori (1942) scaled down by a factor of ten. The P_i samples were read at 660 mμ against a blank containing no P_i. PP_i was determined in the same way as P_i after incubation with 0.5 unit of pyrophosphatase and 5 mM $MgCl_2$ for 20 min at 25°.

Growth of Bacteria. P. shermanii (strain 19W) was grown on a glycerol-bicarbonate-phosphate medium (Lochmüller *et al.*, 1966), or on a lactate medium which contained, per liter of medium, 65 ml of 60% sodium lactate, 2.7 g of KH_2PO_4, 13.35 g of K_2HPO_4, 3.5 g of yeast hydrolysate, and 1.0 ml of vitamin solution. The vitamin solution contained, per 100 ml of solution, 75 mg of calcium pantothenate, 75 mg of thiamine-HCl, 50 mg of D-biotin, and 1.0 g of $Co(NO_3)_2 \cdot 6H_2O$. The bacteria were adapted to growth on lactate by three or four transfers in this medium after an original inoculation from the glycerol medium. Fermentation was carried out in 15 l. of medium in 20-l. carboys at 30°, with daily shaking to avoid clumping of the cells. Large scale fermentations were in a 550-l. vessel at the New England Enzyme Center, Boston, Mass. The cells were harvested by centrifugation in Sharples centrifuges 19 days after inoculation when grown in carboys and 7 days after inoculation when grown in the large fermenter. The yield of bacteria was approximately 3.8 g/l. of medium.

TABLE I: Purification of Pyruvate Phosphate Dikinase from *P. shermanii* Grown with Glycerol as the Substrate.

Method of Fractionation	Total Units (μmoles/min)	Sp Act.[c] (unit/mg)	Purification	Yield (%)
Crude extract		0.002	1	
Batch elution from DEAE-cellulose (0.3 M eluate)		0.003	1.5	
Elution from cellulose phosphate (0.05 M eluate)	35	0.006	3	100[a]
Ammonium sulfate saturation (55–75%)	23.0	0.01	5	66
Elution from DEAE-cellulose	9.0	0.20	100	26[b]
Extraction with ammonium sulfate (60–55%)	5.2	0.60	300	15[b]

[a] The enzyme at this stage was obtained as a by-product of the purification of transcarboxylase (Wood *et al.*, 1969b). [b] One-half of the material from the ammonium sulfate step was used for the elution from DEAE-cellulose and extraction with ammonium sulfate. The values have been corrected to give the amount assuming all the material had been processed. [c] Activity was determined by the nonlinked assay described in the Methods section.

Results

Purification of Pyruvate Phosphate Dikinase from P. shermanii Grown on Glycerol Medium. The steps used to purify pyruvate phosphate dikinase from *P. shermanii* grown on glycerol are summarized in Table I. The first three steps of the purification are identical with those used in the initial steps of purification of oxalacetate transcarboxylase (Wood *et al.*, 1969b). These steps include breakage of the cells in an Eppenbach colloid mill, DEAE-cellulose adsorption and batch elution, and then elution from a cellulose phosphate column. The protein eluted with 0.05 M potassium phosphate (pH 6.8) was precipitated with ammonium sulfate and used as the source of the dikinase.

AMMONIUM SULFATE FRACTIONATION. The ammonium sulfate concentration of the protein solution was estimated from the conductivity as measured with a Barnstead purity meter and then solid ammonium sulfate was added to bring the solution to 35% saturation. After stirring for 1 hr the precipitate was removed by centrifugation at 20,000*g* for 30 min at 0°. The supernatant solution then was brought to 55% saturation, stirred, and centrifuged as before. The same procedure was used to prepare 55–75% fraction in which the majority of the enzyme occurred.

ELUTION FROM DEAE-CELLULOSE. The precipitate from the 55 to 75% fraction was dissolved in phosphate buffer and dialyzed at 4° for 3 hr against three changes of 1 l. of 0.20 M potassium phosphate buffer (pH 6.8) before placing it on a column of DEAE-cellulose equilibrated with the same buffer. The bed volume of the column was approximately 50 cm^3/g of protein. The column was washed with the equilibrating buffer until the OD_{280} fell below 0.1. The protein was then eluted with 200 ml of a linear gradient from 0.2 M potassium phosphate (pH 6.8) to 0.2 M potassium phosphate plus 0.2 M KCl (pH 6.8). The pyruvate phosphate dikinase appeared near the end of the gradient, and it was precipitated with ammonium sulfate at 90% saturation and collected by centrifugation.

EXTRACTION WITH AMMONIUM SULFATE: 70, 65, 60, 55, and 50% saturated ammonium sulfate solutions were prepared by diluting saturated ammonium sulfate to the appropriate concentration in 0.10 M potassium phosphate buffer (pH 6.8) containing 0.20 M KCl. The precipitate was successively extracted with each of the above solutions, using 1 ml of ammonium sulfate solution/10 mg of protein in the precipitate. The extractions were performed in a Nalgene centrifuge tube containing a magnetic stirring bar and the suspension was stirred for approximately 15 min and then centrifuged for 15 min at 20,000*g*. After the supernatant solution had been removed, the precipitate was resuspended in the next extraction solution, and the procedure was repeated. The pyruvate phosphate dikinase was associated primarily with the 55% extract which was then precipitated with ammonium sulfate at 75% saturation and sedimented at 20,000*g*.

Purification of Pyruvate Phosphate Dikinase from P. shermanii Grown on Lactate Medium. The amount of the enzyme in crude extracts from cells grown on lactate was found to be 6 to 10 times higher than that in extracts of glycerol-grown cells. The purification of this enzyme is summarized in Table II. The first four steps were essentially the same as those for cells grown in glycerol medium with the following modifications: 0.40 M potassium phosphate buffer (pH 6.8) was used in place of the 0.30 M phosphate for the final batch elution from DEAE-cellulose and the enzyme activity was found in the fraction from 35 to 55% saturated $(NH_4)_2SO_4$.

ELUTION FROM TEAE-CELLULOSE. The remaining steps in

TABLE II: Purification of Pyruvate Phosphate Dikinase from *P. shermanii* Grown with Lactate as the Substrate.

Method of Fractionation	Total Units[a] (μmoles/min)	Sp Act. (Units/mg	Purification	Yield[b] (%)
Crude extract	1435	0.02	1	
Batch elution from DEAE-cellulose (0.4 M eluate)	2820	0.05	2.5	
Elution from cellulose phosphate (0.05 M eluate)	3680	0.12	6	100
Ammonium sulfate saturation (35–55%)	2790	0.20	10	76
Elution from TEAE-cellulose	900	0.60[c]	30	24[d]
Elution from DEAE-Sephadex	520	1.70	85	14[d]

[a] Purification was from 2.0 kg (wet weight) of *P. shermanii* grown on lactate. The assay of pyruvate formation from P-enolpyruvate was found to be the most rapid and accurate method for locating the enzyme in the last two steps. The specific activity in the direction of P-enolpyruvate synthesis was then determined on the pooled enzyme. [b] Yield is based on the activity found after elution from cellulose phosphate, since the greatest number of units are found at that stage. [c] Thirty per cent of the activity was recovered from the TEAE-cellulose column, but the activity spontaneously increased during storage of the precipitate in 1 M sucrose. The highest specific activity observed after storage was 1.65. [d] Approximately one-third of the material from the ammonium sulfate step was used for the TEAE-cellulose and DEAE-Sephadex steps. The value has been converted to that which would be obtained assuming all the material was processed.

TABLE III: Stoichiometry of the Pyruvate Phosphate Dikinase Reaction.[a]

Compound	Zero Time (μmoles)	7 min (μmoles)	Net Change (μmoles)
Pyruvate	0	1.12	+1.12
P-enolpyruvate	1.84	0.59	−1.25
ATP	0	1.17	+1.17
AMP	1.79	0.64	−1.15
P_i	0.16	1.63	+1.47
PP_i	3.14	2.24	−0.90

[a] The incubation contained the following (in micromoles per milliliter): Tris-HCl buffer (pH 7.4), 50; $MgCl_2$, 5; P-enolpyruvate, 1.23; AMP, 1.19; PP_i, 2.10; β-DPNH, 1.0; and in units, lactate dehydrogenase, 0.8; and dikinase, 0.3 (in 1% bovine serum albumin). The final volume was 1.5 ml and the reaction was terminated after 7 min with trichloroacetic acid. Pyruvate was determined from the net change in optical density and the other components as described in the Methods section. No ADP was found before or after the incubation.

the purification were performed at room temperature. The precipitate from the 35 to 55% fraction was dissolved in a minimum volume of distilled water and dialyzed against 0.05 M potassium phosphate buffer (pH 6.8) until the conductivity as determined using a Barnstead meter was below that of 0.15 M $(NH_4)_2SO_4$. The solution was then placed on a column of TEAE-cellulose equilibrated with 0.10 M potassium phosphate buffer (pH 6.8), with a bed volume of approximately 60 cm^3/g of protein. The protein was eluted with 0.20 M potassium phosphate buffer (pH 6.8) until the protein concentration was approximately 0.01 mg/ml. Elution was then begun with 0.20 M potassium phosphate plus 0.20 M KCl (pH 6.8), and the fractions containing the dikinase were collected, assayed, pooled, and brought to 90% saturation with solid ammonium sulfate.

ELUTION FROM DEAE-SEPHADEX. The precipitated dikinase was dissolved in a minimum volume of distilled water and placed on a column of Sephadex G-50 (fine) equilibrated with 0.10 M potassium phosphate (pH 6.8) using 4-cm^3 bed volumes of Sephadex/ml of protein solution (approximately 80 mg/ml). The column was washed with the same buffer and the desalted protein was collected, pooled, and immediately placed on a DEAE-Sephadex A-50 column with a bed volume of approximately 200 cm^3/g of protein. The protein solution was eluted using a convex gradient from 0.15 M potassium phosphate (pH 6.8) to 0.2 M potassium phosphate plus 0.3 M KCl (pH 6.8), obtained with 500 ml of the former and twice the volume of the latter flowing into it. The fractions containing dikinase were pooled and precipitated with ammonium sulfate and sedimented at 20,000*g*. The protein was dissolved in 1.0 M sucrose and stored at −15°. When stored in this way, over a period of months the enzyme tended to spontaneously recover activity which had been lost during the fractionation on TEAE-cellulose.

Stoichiometry of the Pyruvate Phosphate Dikinase Reaction. The stoichiometry of the dikinase reaction is shown in Table III. It is evident that the amounts of P-enolpyruvate, AMP, and PP_i used were approximately equimolar with the amounts of pyruvate, ATP, and P_i formed during the reaction. The discrepancy in the values obtained for P_i and PP_i may reflect the hydrolysis of some PP_i during the determination of P_i.

Properties of Pyruvate Phosphate Dikinase. COLD LABILITY. One of the major problems encountered in the purification of the dikinase was the lability of the enzyme. Although the enzyme is relatively stable during the early stages of the purification, it does deteriorate when stored as the ammonium sulfate precipitate at 4° or colder. After the purification on DEAE- or TEAE-cellulose the enzyme becomes quite labile when cold. An example of the lability is presented in Table IV. The dikinase in 0.05 M potassium phosphate (pH 6.8) lost all enzymatic activity over a period of 17 hr at 0°. But an identical sample lost only 31% of its activity over the same period at 30°. Pyruvate, at 0.7 mM concentration, afforded some protection against cold deterioration. The activity lost by exposure to cold could not be regained by incubation of the enzyme at 30° for prolonged periods. For storage the ammonium sulfate precipitates of the enzyme were dissolved in 1.0 M sucrose and stored at −15°. Enzyme stored in this way retained full activity for at least 6 months. Samples for study of the enzyme were routinely prepared from the enzyme stored in sucrose by dilution in a solution of 1% bovine serum albumin containing 100 mM imidazole-Cl and 5 mM $MgCl_2$ at

TABLE IV: Effect of Temperature on the Stability of Pyruvate Phosphate Dikinase.[a]

Temp of Storage (°C)	Original Activity Remaining (%) 1 hr	4.5 hr	17 hr
30	94	83	69
0	85	50	0
0 (7×10^{-4} M pyruvate)	87	74	25

[a] Pyruvate phosphate dikinase from the extraction with ammonium sulfate (Table I) was passed through a Sephadex G-50 column equilibrated with 0.05 M potassium phosphate (pH 6.8), assayed by the nonlinked assay and divided into three samples for storage as described. After storage for the indicated time, the samples were warmed for 1 min at 30° and assayed. The stored samples contained 1.8 mg of protein/ml.

pH 6.7. Once prepared, the dilutions were kept at room temperature, and lost activity very gradually and could be used over a period of 2 days.

SEDIMENTATION PATTERN. The sedimentation behavior of pyruvate phosphate dikinase which was purified through the DEAE-Sephadex step (Table II) is shown in Figure 1. Enzyme which has been equilibrated with 0.05 M potassium phosphate buffer (pH 6.8) by passage through a Sephadex G-50 column gave two peaks with sedimentation coefficients of 6.7 and 4.6 S. The enzyme lost 86% of its activity during the preparation and centrifugation. Evidence that the major, faster sedimenting peak contained the enzyme was obtained by partial separation of the proteins of each peak using a partition cell (Yphantis and Waugh, 1956).

When the enzyme was equilibrated with 100 mM imidazole-Cl, 50 mM ammonium sulfate, 5 mM pyruvate, 5 mM $MgCl_2$ at pH 6.7, a major peak with a sedimentation coefficient of 6.9 S and a small amount of faster moving material were observed (Figure 1B). The faster moving material may indicate the presence of a contaminant or some aggregation of the major component. Assay of the enzyme before and after the centrifugation showed that 94% of the enzyme activity was retained.

ELECTROPHORESIS. Electrophoresis of the enzyme from the last step of Table II was performed on polyacrylamide gel at pH 7.4 by a method suggested by Williams and Reisfeld (1964), with 100 μg of the sucrose-stored enzyme. The electrophoresis was at room temperature for 2 hr, followed by staining with Amido Schwartz black for 1 hr. Excess dye was removed by electrophoresis overnight. One major band was observed but there was also a diffuse area of stained material which trailed behind the major band, possibly due to dissociation and reassociation occurring during the electrophoresis. Four minor bands were also visible and these all together were visually estimated to be no more than 5% of the total protein.

EFFECT OF pH ON THE REACTION. The effect of pH on the formation of P-enolpyruvate is shown in Figure 2 and on the formation of pyruvate in Figure 3. The optimal pH for the reaction is approximately pH 6.5–7.0 in either direction.

NUCLEOTIDE SPECIFICITY. The nucleotide specificity for the formation of P-enolpyruvate and of pyruvate is shown in Table V. Under the conditions of the assay for formation of P-enolpyruvate the requirement for ATP was specific, and

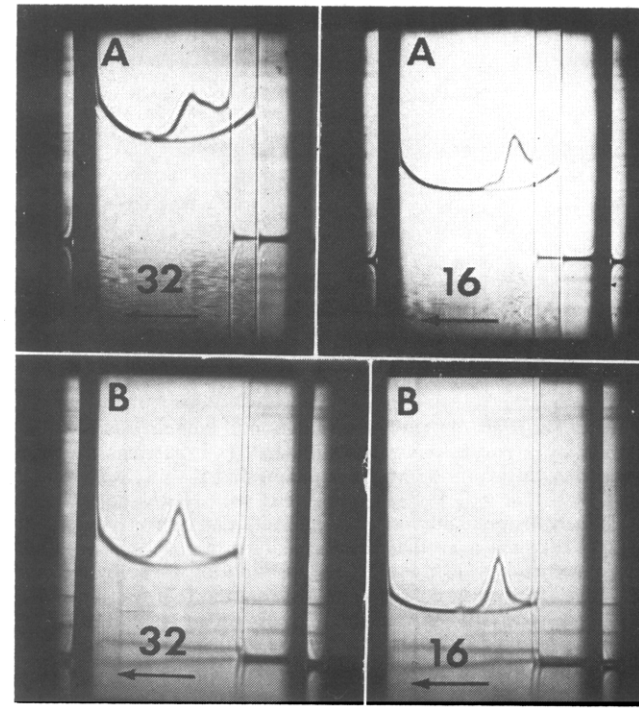

FIGURE 1: Sedimentation patterns of pyruvate phosphate dikinase. The preparation was from the last step of Table II. A total of 1.38 mg of protein/ml was used for part A which was prepared by passage through a Sephadex G-50 column to remove sucrose and to transfer the enzyme to 0.1 M potassium phosphate (pH 6.8). Sedimentation was in the direction of the arrows at 50,740 rpm and 19.2° in a 30-mm double-sector cell. The Schlieren photographs were taken at 16 and 32 min at phase-plate angles of 60 and 50°, respectively. The observed values are 6.7 and 4.6 S. At the end of the run the enzyme had 14% of the original activity. A total of 2.9 mg of protein/ml was used for part B which was prepared by passage through a Sephadex G-50 column equilibrated with 0.1 M imidazole-Cl (pH 6.7), 0.05 M ammonium sulfate, 0.005 M pyruvate, and 0.005 M $MgCl_2$. Sedimentation was in the direction of the arrows at 59,780 rpm and 21.4° in a 12-mm double-sector cell with the equilibration buffer in the other sector. The schlieren photographs were taken at 16 and 32 min at phase-plate angles of 55 and 40°, respectively. The observed value of the major peak is 6.9 S. At the end of the run the enzyme had 94% of its original activity.

TABLE V: Nucleotide Specificity of Pyruvate Phosphate Dikinase.[a]

Formation of P-enolpyruvate from Pyruvate			Formation of Pyruvate from P-enolpyruvate		
Nucleotide	Act. (%)	Inhibn (%)	Nucleotide	Act. (%)	Inhibn (%)
ATP	100		AMP	100	
GTP	0	8	GMP	0	50
CTP	0	0	CMP	0	6
UTP	0	0	UMP	0	0
TTP	0	0	TMP	0	0

[a] The activity was determined as described for the linked assays, except for substitution of the nucleotides shown in the table. The inhibition was determined using the indicated nucleotide in addition to ATP or AMP at equimolar concentration. The dikinase was from the TEAE-cellulose step of Table II.

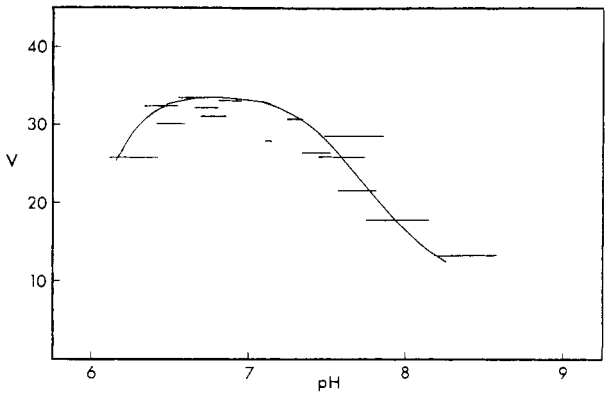

FIGURE 2: The effect of pH on the formation of P-enolpyruvate from pyruvate by pyruvate phosphate dikinase. The reactions were in 1.0 ml as described in the Methods except that the mix was gassed with CO_2 to the indicated pH range and the pH was determined immediately before addition to the cuvet. Each cuvet contained 2.6 μg of dikinase from the TEAE-cellulose step of Table II (0.95 specific activity by assay of the formation of P-enolpyruvate). After 2 min, the cuvet contents were transferred to a test tube and the pH was redetermined. The range in pH for the two determinations is represented by the horizontal line. Addition of P-enolpyruvate showed that P-enolpyruvate carboxytransphosphorylase and malate dehydrogenase were not limiting. V is expressed as change in optical density at 340 mμ per min $\times 10^3$.

when other nucleotides were added in equimolar concentration with ATP, GTP was found to inhibit approximately 8%, but the other nucleotides gave negligible inhibition. Likewise in the formation of pyruvate, AMP was specific, and when both AMP and a second nucleotide were added in equimolar concentration, GMP inhibited approximately 50%, CMP inhibited approximately 6%, and the other nucleotides were without effect.

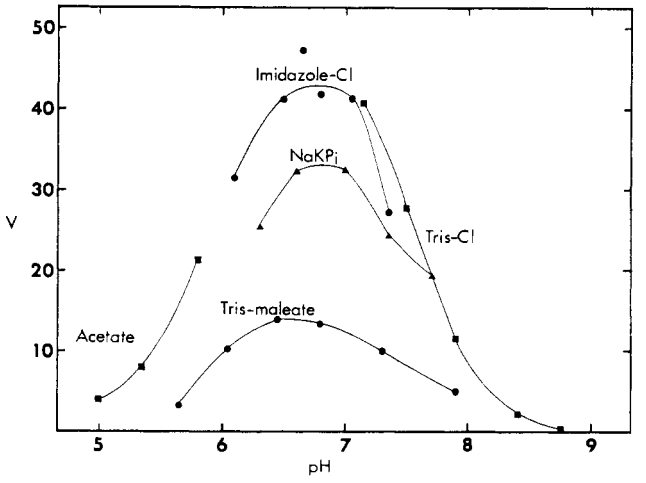

FIGURE 3: The effect of pH on the formation of pyruvate from P-enolpyruvate by pyruvate phosphate dikinase. The reactions were performed as described in the Methods section, except the following buffers were used in the pH range indicated (all at 0.05 ionic strength): acetate buffer, pH 5.0–5.8; Tris-maleate, pH 5.65–7.9; Tris-Cl, pH 7.15–8.75; sodium potassium phosphate, pH 6.3–7.7; and imidazole-Cl, pH 6.1–7.35. Each cuvet contained 0.86 μg of dikinase from the last step of Table II (1.7 specific activity by assay of P-enolpyruvate). Addition of pyruvate showed that lactate dehydrogenase was not limiting. V is expressed as change in optical density at 340 mμ per min $\times 10^3$.

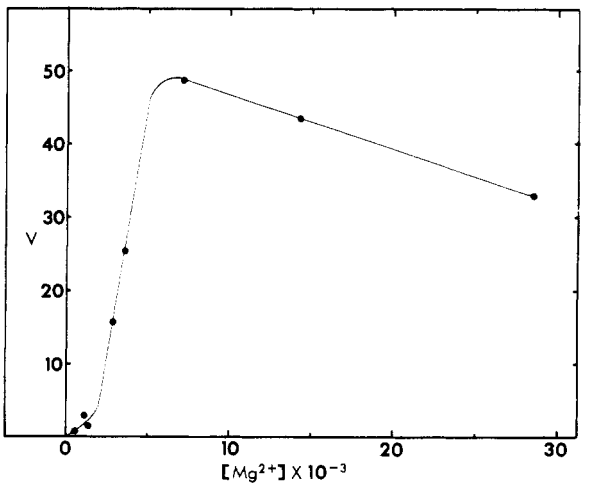

FIGURE 4: The effect of the concentration of Mg^{2+} on the formation of P-enolpyruvate from pyruvate by pyruvate phosphate dikinase. The assays were as described in the Methods section, except for variation of the concentration of Mg^{2+}. The dikinase was from the TEAE-cellulose step of Table II (0.95 specific activity). V is expressed as the change in optical density at 340 mμ per min $\times 10^3$.

K_m VALUES OF SUBSTRATES AND ACTIVATORS. The apparent K_m values for the substrates and activators of the dikinase reaction were determined both for the formation of P-enolpyruvate, and for the formation of pyruvate. The ratio of the concentration of substrate to velocity was plotted against the substrate concentration. This treatment of the data is recommended by Wilkinson (1961), and was found to give the most accurate determination of K_m and V_{max} with small errors in the observed velocity (Dowd and Riggs, 1965). The intercept of the line with the abscissa gives the $-K_m$ value and the intercept with the ordinate is the ratio K_m/V_{max}. The slope is equal to the reciprocal of V_{max}.

For formation of P-enolpyruvate the assays were as described in Methods, except for variation of the concentration of the substrate or activator. The dikinase was from the TEAE-cellulose step of Table II (0.95 specific activity). The apparent K_m for pyruvate was 1.0×10^{-4} M and the V_{max} 0.94 μmole/min per mg of protein. For ATP the K_m was 4.3×10^{-5} M, and the V_{max} 0.86, and for phosphate the K_m was 1.0×10^{-3} M and the V_{max} 0.97. The observed K_m for phosphate could have been that of the P-enolpyruvate carboxytransphosphorylase since it was used as one of the linking enzymes in the assay. Lochmüller *et al.* (1966) observed a K_m for P_i of 1.17×10^{-3} M with carboxytransphosphorylase. Evidence that this was not the case was obtained by varying the concentration of the carboxytransphosphorylase. If the K_m was that of the carboxytransphosphorylase and this enzyme became rate limiting rather than the dikinase, then an increase in the amount of carboxytransphosphorylase should increase the observed rate of reaction at the K_m value. This increase was not observed when the amount of carboxytransphosphorylase was tripled. The observed K_m is about the same as the apparent K_m of 5×10^{-4} M found for the dikinase from sugar cane leaves by Hatch and Slack (1968).

The effect of the concentration of Mg^{2+} on the velocity of the forward reaction is seen in Figure 4. The reaction was very slow below 1.8 mM, an increase in velocity was observed between 1.8 and 7 mM, and inhibition above 7 mM. The anomalous behavior below 1.8 mM can be explained if the reaction requires free Mg^{2+}, since essentially all the Mg^{2+} would be

bound to ATP until the Mg^{2+} concentration exceeded the ATP concentration, because of the high association constant of the Mg–ATP complex (Burton, 1959). The plot of the ratio of the concentration of free Mg^{2+} to velocity *vs.* the concentration of free Mg^{2+} gave an apparent K_m of 4.0×10^{-3} M, and a V_{max} of 1.3 μmoles/min per mg of protein. The association constant of 2.25×10^{-4} given by Burton was used to calculate the concentration of free Mg^{2+}. Although carboxytransphosphorylase also required Mg^{2+}, the apparent K_m for Mg^{2+} is 1.2×10^{-3} M (Lochmüller *et al.*, 1966) which is lower than that observed for the dikinase. The inhibition of the reaction at concentrations of Mg^{2+} above 7 mM may be due to a competitive inhibition of free Mg^{2+} for the site where Mg–ATP or Mg–phosphate binds to the enzyme.

REQUIREMENT OF MONOVALENT IONS. Pyruvate phosphate dikinase from *B. symbiosus* was reported by Reeves *et al.* (1968) to require NH_4^+ ion, and the observed apparent K_m was 2.5×10^{-3} M. A similar stimulation has been observed with the dikinase from *P. shermanii*. The apparent K_m was found to be 2.0×10^{-3} M, and the V_{max} was 0.78 μmole/min per mg of protein. Although K^+ would substitute for NH_4^+, the apparent K_m for K^+ was not determined.

The K_m determinations for the formation of pyruvate were performed as described in Methods except for variation of the concentration of the substrate or activator. The dikinase was from the last step of Table II (1.7 specific activity). The apparent K_m for P-enolpyruvate was 3.6×10^{-5} M and the V_{max} was 2.5 μmoles/min per mg of protein. The apparent K_m given by Reeves *et al.* (1968) for the dikinase from *B. symbiosus* is 6×10^{-5} M and Andrews and Hatch (1969) give a value of 1.1×10^{-4} M for the apparent K_m of the dikinase from the leaves of sugar cane. The apparent K_m for AMP was 1.5×10^{-5} M and V_{max} was 2.0; Reeves *et al.* (1968) reported a K_m of "less than 0.01 mM" and Andrews and Hatch (1969) reported no decrease in the reaction rate at 4×10^{-6} M. For PP_i the K_m was 1.2×10^{-4} M, compared to 1×10^{-4} M reported by Reeves *et al.* (1968) and 4.0×10^{-5} M reported by Andrews and Hatch (1969). The V_{max} was 3.3. For Mg^{2+} the K_m was 2.4×10^{-3} M and the anomalies with Mg^{2+} seen in Figure 4 for the formation of P-enolpyruvate were not observed. The V_{max} was 4.2. The K_m for NH_4^+ was 2.1×10^{-3} M and the V_{max} 3.5.

Within a factor of three the apparent K_m's of the dikinase from *P. shermanii* agree with those published for the dikinase from sugar cane (Hatch and Slack, 1968; Andrews and Hatch, 1969), and with those published for the dikinase from *B. symbiosus* (Reeves *et al.*, 1968). The fact that the values of K_m and V_{max} are dependent on the concentrations of the other substrates and activators may explain the differences between the values given in this report and those of the other investigators.

REQUIREMENT OF DIVALENT METAL. Various divalent metals were substituted for Mg^{2+} to determine if any could replace the requirement of Mg^{2+}. Only the formation of pyruvate was studied because the P-enolpyruvate carboxytransphosphorylase used in the assay for P-enolpyruvate formation also has a requirement for a divalent metal. The results are shown in Table VI. Of the metals tested, Mn^{2+} and Co^{2+}, and possibly Zn^{2+}, were able to activate the reaction, but not as well as Mg^{2+}. Precipitation occurred when higher concentrations of metals were tested. The apparent K_m for activation by Co^{2+} was 5×10^{-4} M, which is approximately fivefold lower than the apparent K_m of Mg^{2+}, but the V_{max} obtained with Co^{2+} was only 1.65 μmoles/min per mg of protein, which is considerably lower than the value with Mg^{2+}. Ca^{2+}, Cu^{2+}, and Fe^{2+} inhibited the dikinase reaction. Inhibition by Ca^{2+} is characteristic of a type II metal function, as described by Cohn (1963). In those cases reviewed by Cohn, the type II metal is bound to the enzyme and may form a bridge between the enzyme and substrate molecules.

TABLE VI: Requirement of Divalent Metals by Pyruvate Phosphate Dikinase.[a]

Cation	Concn (mM)	Act. (%)	Inhibn (%)
Mg^{2+}	25	100	
$Mg^{2+} + Ca^{2+}$	25 + 0.5	60	40
Ni^{2+}	8	0	
Co^{2+}	2.4	48	
$Co^{2+} + Ca^{2+}$	2.4 + 0.5	9	82
Mg^{2+}	5.9	100	
$Mg^{2+} + Cu^{2+}$	5.9 + 0.6	33	67
$Mg^{2+} + Fe^{2+}$	3.0 + 0.6	89	11
Mn^{2+}	5.9	12	
Zn^{2+}	3.0	4	

[a] The data in the upper portion of the table were obtained using the conditions as described in the text for assay of pyruvate formation with the indicated cations substituted for Mg^{2+}. The cations other than Mg^{2+} were present in the highest concentration which could be reached without formation of a precipitate. Mn^{2+}, Zn^{2+}, and Ba^{2+} all caused precipitates at 0.4 mM and therefore are not included in this section of the table. Each cuvet contained 1.7 μg of dikinase from the last step of Table II. The data in the lower part of the table were obtained using a modified assay. The mixtures contained in micromoles per milliliter: Tris-Cl (pH 7.4), 45; P-enolpyruvate, 3; PP_i, 3; AMP, 3; β-DPNH, 0.12; the indicated cations; and, in units per milliliter, lactate dehydrogenase, 2.4; and 1.4 μg of dikinase from the TEAE-cellulose step of Table II.

Discussion

The propionibacteria obviously catalyze the conversion of pyruvate into P-enolpyruvate since they grow on pyruvate and the mechanism of this conversion was the subject of these studies. When P-enolpyruvate carboxytransphosphorylase was discovered by Siu and Wood (1962), it appeared that P-enolpyruvate might be formed by the following reactions in analogy with the synthesis in liver by pyruvate carboxylase and P-enolpyruvate carboxykinase (Keech and Utter, 1963).

$$\text{pyruvate} + \text{ATP} + CO_2 \rightleftharpoons \text{oxalacetate} + \text{ADP} + P_i \quad (2)$$

$$\text{oxalacetate} + PP_i \rightleftharpoons \text{P-enolpyruvate} + CO_2 + P_i \quad (3)$$

$$\text{sum: pyruvate} + \text{ATP} + PP_i \rightleftharpoons \text{P-enolpyruvate} + \text{ADP} + 2\,P_i \quad (4)$$

In liver, P-enolpyruvate carboxykinase catalyzes a reaction similar to reaction 3 except it uses GTP instead of PP_i. Thus ATP plus GTP is required rather than ATP and PP_i. In support of reaction 4 synthesis of P-enolpyruvate from pyruvate was observed with ATP and PP_i by Wood *et al.* (1966) using pyruvate carboxylase from avian liver and carboxytransphos-

phorylase from propionibacteria. This led to a search for pyruvate carboxylase (reaction 2) in propionic acid bacteria. Synthesis of oxalacetate from pyruvate, ATP, and CO_2 was observed with extracts of propionibacteria, but there was a requirement for P_i which was not explained by reaction 2. Further study showed that the synthesis of oxalacetate from pyruvate was not *via* reaction 2 but occurred by the following reactions.

$$\text{pyruvate} + \text{ATP} + P_i \rightleftharpoons \text{P-enolpyruvate} + \text{AMP} + PP_i \quad (1)$$

$$\text{P-enolpyruvate} + CO_2 + P_i \rightleftharpoons \text{oxalacetate} + PP_i \quad (3)$$

$$\text{sum: pyruvate} + \text{ATP} + CO_2 + 2P_i \rightleftharpoons \text{oxalacetate} + \text{AMP} + 2PP_i \quad (5)$$

Cooper and Kornberg (1965) had observed that the synthesis of P-enolpyruvate from pyruvate occurs in *E. coli* as shown in reaction 6 and is catalyzed by an enzyme named P-enolpyruvate synthase.

$$\text{pyruvate} + \text{ATP} \rightleftharpoons \text{P-enolpyruvate} + \text{AMP} + P_i \quad (6)$$

It appeared (Wood, 1968) that the same reaction occurred in propionibacteria but it then was found that P_i was required for reaction 1 as well as for reaction 3. The synthase apparently involves a hydrolytic cleavage in reaction 1b, whereas the dikinase involves a phosphorolytic cleavage, thus yielding PP_i instead of P_i.

Reeves *et al.* (1968) determined the equilibrium of the dikinase reaction using enzyme from *B. symbiosus* and obtained a value of 1140 for K_{eq} at pH 7.0 in the direction of pyruvate synthesis. Unlike *B. symbiosus* and *E. histolytica*, which lack pyruvate kinase, propionibacteria contain a large amount of pyruvate kinase (unpublished results) and it seems unlikely that the dikinase functions physiologically in propionibacteria to form pyruvate from P-enolpyruvate. If a pyrophosphatase were linked with the dikinase in propionibacteria it would overcome the unfavorable equilibrium for P-enolpyruvate synthesis. A preliminary investigation has shown that this enzyme is present in *P. shermanii*. The equilibrium of the carboxytransphosphorylase reaction is in favor of oxalacetate formation (Wood *et al.*, 1966). Therefore the formation of oxalacetate *via* reaction 5 would be favorable when linked with pyrophosphatase, or when linked with other reactions of metabolism which remove oxalacetate. That pyruvate phosphate dikinase functions physiologically for formation of P-enolpyruvate is indicated by the 6- to 10-fold increase of the enzyme when the bacteria are grown with lactate instead of glycerol as a substrate. Growth on lactate requires formation of P-enolpyruvate from pyruvate as opposed to growth on glycerol where P-enolpyruvate is formed *via* phosphoglycerate.

The purification of the dikinase from *P. shermanii* is difficult because of the instability of the enzyme. The enzyme is not only cold labile, but also loses activity even if the purification is performed at room temperature. Addition of EDTA, dithiothreitol, pyruvate, and $MgCl_2$ alone and in various combinations does not prevent the loss of activity during purification. Another source of difficulty is the small quantity of the enzyme present in the extracts of the bacteria, thus requiring the processing of a large amount of bacteria to obtain significant yields.

Pyruvate phosphate dikinase from *P. shermanii* has both similarities to and differences from the dikinases purified from *Zea mays* (Hatch and Slack, 1968) and *E. histolytica* and *B. symbiosis* (Reeves *et al.*, 1968). The cold lability of the dikinase from propionibacteria is similar to that observed with the plant enzyme and this loss of activity is not dependent on freezing. In contrast the enzyme from *B. symbiosus* was reported to lose only 10% of its activity over a 2-week period at 4°. Another similarity between the enzyme from propionibacteria and the plant enzyme is the requirement for free Mg^{2+} in the reaction toward P-enolpyruvate. However the dikinase from propionibacteria has a requirement for a monovalent cation just as does the enzyme from *B. symbiosis*, but this requirement was not observed with the enzyme from plants or the amoeba. Another major difference from the plant enzyme is the optimum pH. It is 8.3 for plants whereas it is 6.5–7.0 for propionibacteria. It is noted that aspartate transaminase and P-enolpyruvate carboxylase were used in the assay of the enzyme from plants. The transaminase has an optimal pH of 8.0–8.5 and P-enolpyruvate carboxylase from peanut cotyledons also has been shown to have an optimal pH of 8.0–8.2 (Maruyama *et al.*, 1966). Perhaps these coupling enzymes become limiting at lower pH values. Andrews and Hatch (1969) observed that the velocity of the reaction in the direction of P-enolpyruvate synthesis was about six times the velocity of the reverse reaction at pH 8.3. The enzyme from *P. shermanii* also shows a higher rate of synthesis of P-enolpyruvate than of pyruvate at pH values between 8 and 8.5, but at its optimum pH (6.5–7.0) the rate in the direction of formation of pyruvate is the most rapid.

The K_m values of the enzyme from different sources do not differ greatly.

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Purification and Properties of Malate Dehydrogenase from *Chlorella pyrenoidosa*. Catalytic Mechanism of the Particulate Form*

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ABSTRACT: By means of ammonium sulfate fractionation and column chromatography, particulate and supernatant forms of malate dehydrogenase (MDH) from *Chlorella pyrenoidosa* have been purified to specific activities of 33 and 84, respectively. The two forms have been shown to be chromatographically, electrophoretically, and immunologically different, each showing a characteristic MDH activity when chromatographed on DEAE-cellulose, when electrophoresed on cellulose-acetate, and when developed by Ouchterlony double diffusion. Both forms have a molecular weight of approximately 61,000 as determined by Sephadex gel filtration. The kinetic properties of the particulate MDH were studied by: (1) analysis of initial velocity patterns in the direction of DPNH oxidation at pH 7.4 in 0.1 M potassium phosphate buffer; (2) determination of product inhibition patterns with DPN^+ and malic acid as product inhibitors; and (3) analysis of inhibition patterns of monoethyl oxaloacetic acid with respect to DPNH and oxaloacetic acid. All three studies yielded results supportive of a compulsory ordered addition mechanism for the particulate MDH. The K_m of DPNH of 4×10^{-5} M and K_m of oxaloacetic acid of 5×10^{-5} M as well as the substrate inhibition by oxaloacetic acid at levels above 8.48×10^{-4} M for the particulate MDH are data similar to those obtained with animal mitochondrial malate dehydrogenases.

The malate dehydrogenase system (L-malate:DPN oxidoreductase, EC 1.1.1.37) of animal, plant, and microbial origin has been shown to generate a complex electrophoretic pattern. Two MDH[1] proteins, one associated with the supernatant fraction of the cell and the other with the particulate fraction, can generally be demonstrated in animal and microbial species; several plant systems have been suggested to consist of three MDH proteins (Mukerji and Ting, 1969; Ting, 1968; Yamazaki and Tolbert, 1969).

Physical properties such as subunit composition (Wolfenstein *et al.*, 1969), fluorescence spectra (Cassman and Englard, 1966a), amino acid composition (Kitto and Kaplan, 1966), and immunological reactivities (Henderson, 1968) have been examined for a number of highly purified animal MDH enzymes. In a few animal species, kinetic studies have led to the elucidation of catalytic mechanisms: either a basic compulsory ordered addition ("ordered bi–bi") mechanism or some variation thereof has been suggested in each case. But in plant species, though multiple forms of MDH from several systems (Ting, 1968; Mukerji and Ting, 1969) have been studied in terms of physical and kinetic properties, including thermal inactivation and molecular weight determinations and electrophoretic, chromatographic, and pH profiles, no catalytic mechanism has been defined. Examining the MDH system of *Chlorella pyrenoidosa*, Cole and his associates (1968) have demonstrated the existence of multiple electrophoretic forms. This communication reports the partial purification of MDH activities from this algal species and includes molecular weight determinations as well as immunological and electrophoretic studies. A detailed kinetic analysis of that MDH activity associated with the particulate fraction of the cell is presented.

Experimental Procedures

Organism and Culture Conditions. Steady-state cultures of the high-temperature strain (7-11-05) (Sorokin and Myers, 1953) of *C. pyrenoidosa* were cultured as described previously (Cole *et al.*, 1968).

Purification. Cells were harvested by centrifugation (12,100-*g*) at 4°. The cell pellet, washed twice with 0.1 M Tris-HCl buffer, pH 8.6, and twice with distilled water, was recovered in 50 ml of 0.2 M potassium phosphate buffer with 0.3 M sucrose, pH 6.5. The sample was mixed with a one-third volume aliquot of glass powder in a 125-ml beaker and sonicated with the blunt tip probe of a Branson Sonic Power Sonifier, Model M52, operated on high power for a total of 3–5 min, in 15-sec periods. The beaker was immersed in an acetone–ice bath and the sample maintained at 5°. After sonic disruption, the sample was centrifuged at 480*g* for 5 min to sediment only

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[1] Abbreviation used is: MDH, malate dehydrogenase.