The Control of Pyruvate Kinases of Escherichia coli

I. PHYSICOCHEMICAL AND REGULATORY PROPERTIES OF THE ENZYME ACTIVATED BY FRUCTOSE 1,6-DIPHOSPHATE*

E. Bruce Waygood‡ and B. D. Sanwal§
From the Department of Medical Genetics, Medical Sciences Building, University of Toronto, Toronto 181, Canada

SUMMARY

The pyruvate kinase of Escherichia coli activated by fructose 1,6-diphosphate has been purified to homogeneity. It is a tetramer of molecular weight about 240,000. By electrophoresis on sodium dodecyl sulfate polyacrylamide gels, Sephadex gel filtration in denaturing solvents, and ultracentrifugal experiments, the monomer molecular weight is found to be 60,000. Tryptophan is absent from the enzyme and no free NH₂ terminus can be found.

Of several nucleoside diphosphates which act as phosphate acceptors in the reaction, GDP, judging by its $K_m$ value of only 0.05 mM, is the best substrate for the enzyme. Magnesium or manganese is required as a divalent cation, but the kinetics of activation by fructose 1,6-diphosphate is different, depending upon which cation is used in assay mixtures. The enzyme is inhibited by GTP at concentrations much lower than any other nucleoside triphosphate, especially when GDP is used as the substrate. Succinyl-CoA by itself inhibits the enzyme to a small extent, but in the presence of ATP it exerts a cooperative inhibition.

Two allosteric pyruvate kinases (EC 2.7.1.40) are known to exist in Escherichia coli (1, 2). One of these is activated by fructose-1,6-P₂ and the other by AMP (2). In addition, the enzyme activated by fructose-1,6-P₂ is susceptible to a cooperative feedback inhibition by succinyl-CoA and ATP (3). The fructose-1,6-P₂-activated pyruvate kinases occur in diverse other organisms (review in Reference 4). Tanaka and his co-workers (5) have purified one from rat liver and have shown that it is regulated both at the activity level, i.e. by allosteric effectors, and at the level of its formation by dietary and hormonal influences. This enzyme has not been extensively studied, however, probably because of its instability. Much of our current knowledge regarding the structure and physicochemical properties of allosteric pyruvate kinase has been derived from studies of the enzyme from yeast (6–9), which is quite stable and obtainable in reasonable quantities.

The investigators in our laboratory have been interested for some time in the nature of regulation of amphibolic pathways in bacteria (review in Reference 10), and we have studied the structure and regulatory parameters of several enzymes of these pathways isolated from E. coli. Pyruvate kinase catalyzes one of the most important steps in the amphibolic Embden-Meyerhof pathway. The presence of two pyruvate kinases catalyzing the same reaction and the multiplicity of controls operative on these enzymes raise certain questions regarding the physiological roles and the mechanism of regulation of the two pyruvate kinases. In order to understand better certain aspects of these problems, we have undertaken to purify the pyruvate kinases to homogeneity, and to study the structure and mode of regulation and to elucidate the mechanism of the allosteric activation or inhibition of the enzymes by the various effectors.

In this report we present a method for the isolation of the fructose-1,6-P₂-activated pyruvate kinase from E. coli and a study of some of its physicochemical and regulatory properties.

EXPERIMENTAL PROCEDURE

Materials—P-enolpyruvate (cyclohexylammonium salt), carboxymethylcellulose, dansyl chloride, dithiothreitol, and Hesperidin were obtained from Calbiochem. Lactate dehydrogenase (type III, beef heart), NADH, sodium dodecyl sulfate, cytochrome c (horse), 2-mercaptoethanol, iodoacetamide, and Dowex resins were obtained from Sigma. All nucleotides and succinyl-CoA were obtained from P-L Biochemicals. Guanidine HCl and urea (ultrapure grade) and enzyme grade ammonium sulfate were obtained from Schwarz-Mann. Fructose-1,6-P₂ was obtained from Boehringer-Mannheim Corp. DEAE-cellulose used was DE52 from Reeve-Angel and the Sephadex, blue dextran, and Sepharose 4B were obtained from Pharmacia. Polyacrylamide sheets were obtained from the Ching Cheng Trading Co., Taiwan. DTNB was obtained from Aldrich. All other chemicals were reagent grade.

Organism and Growth Conditions—Wild type E. coli K-12, strain 3000, was used. Large quantities of cells for enzyme purification were obtained by growth with vigorous agitation under aerobic conditions in minimal medium at 37°C for 6 hours. 

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† Recipient of a Medical Research Council Studentship.
‡ Present address, Department of Biochemistry, University of Western Ontario, London, Ontario, Canada.
§ Present address, Department of Biochemistry, University of Toronto, Toronto 181, Canada.
umes were less than 1 ml were layered on top of the Sephadex samples containing approximately 0.2, 0.4, and 0.6 mg per ml of the enzyme were used. Centrifugations were performed under two different conditions. In one case the enzyme was suspended in 0.05 M Tris-HCl, pH 7.5, and was spun at 12,121 rpm at 9.8°. In the latter case the concentrations of the enzyme and the buffers were the same as before but also included 0.1 M KCl and were spun at 15,000 rpm at 17.25° and 32,000 rpm at 3.65°, respectively. The density of the solutions was determined using a Cahn RG electrobalance with a density cell attachment. The partial specific volume of the enzyme was obtained from the amino acid composition by the method of Cohn and Edsall (17).

**Amino Acid Analysis**—Amino acid hydrolysis in 6 M HCl of approximately 1 mg of enzyme per hydrolysis sample was carried out at 110° for 24, 48, and 72-hour periods in vacuo. Amino acids were separated and analyzed on a Beckman automatic amino acid analyzer. The methods used were based upon the original procedures of Spackman et al. (18). Cysteine and cystine were determined as cysteic acid by hydrolysis in the presence of dimethylsulfoxide as described by Spencer and Wohl (19). Tryptophan and tyrosine were determined independently by the spectrophotometric method of Elvehjem (20).

**Protein Determination** The protein concentration in crude extracts was determined by the method of Lowry et al. (21). Protein in pure enzyme preparations was estimated by using the value for specific extinction coefficient (E$_{1%}$) at 280 nm, of 1.8 which was obtained by dry weight determination and from the amino acid composition.

**NH$_3$ Terminus Analysis**—Pyruvate kinase (2.5 mg  40 nmol per subunit) was reacted for 30 min at room temperature with 12.5 mg of dansyl chloride in 0.02 M sodium phosphate, pH 8.2, which was 4 M in D$_2$O, 25% by volume dimethylformamide, and 10% by volume acetonitrile. The protein was then precipitated with 10% trichloroacetic acid, washed with acetone, dried in vacuo, and hydrolyzed in 6 M HCl for 4 hours at 110°. The dried hydrolysate was triturated with a few drops of ethyl acetate saturated with water, and this solution was spotted in a corner of a polyamide sheet (22). The chromatographs were developed with 1.5% aqueous formic acid in one dimension, followed after drying by benzene-acetic acid (9:1) in the second.

**Carbohydrate Analysis**—Glucosamine was routinely determined during amino acid analysis and was considered to represent 50% of the original content before the 24-hour hydrolysis (23). Separate determinations were made by hydrolyzing the enzyme in 3 M HCl for 4 hours at 110°. Neutral sugars were determined by gas liquid chromatography after the hydrolysate had been washed free of HCl and passed through Dowex 50-X4 (200 to 400 mesh) in the formate ion form to remove the amino acids present, 0.1 M 2-merecaptoethanol in 0.05 M Tris-HCl, pH 7.5, were spun at 29,750 rpm at 13.6°. In the latter case the concentrations of the enzyme and the buffers were the same as before but also included 0.1 M KCl and were spun at 15,000 rpm at 17.25° and 32,000 rpm at 3.65°, respectively. The density of the solutions was determined using a Cahn RG electrobalance with a density cell attachment. The partial specific volume of the enzyme was obtained from the amino acid composition by the method of Cohn and Edsall (17).

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**RESULTS**

**Purification of Enzyme**

**Crude Extracts**—Cells in suspension were disrupted by passing through the orifice of an Amino French pressure cell at 10,000 p.s.i. The extract was centrifuged for 10 min and the supernatant was used for further purification. All steps reported below were carried out at 4°. Centrifugations were performed at 48,000  $\times$  g. Unless mentioned otherwise the buffer was 0.05 M Tris-HCl, pH 7.5, containing 1 mm EDTA. The procedure is designed to give consistent results using about 500 g wet weight of cells.
Precipitate was suspended in as small a volume as possible. The suspension was centrifuged for 20 min and the precipitate was discarded.

Ammonium Sulfate Fractionation—Solid ammonium sulfate was added with stirring to the supernatant until 250 g per liter had been added. After standing for 15 min, the mixture was centrifuged, and the precipitate was discarded. To the supernatant 180 g of ammonium sulfate were added per liter with stirring. The mixture was allowed to stand for 1 hour or more and then centrifuged for 15 min. The supernatant was discarded and the precipitate was resuspended in 0.01 M Tris-HCl, pH 8.0, containing 0.2 M EDTA (approximately one-tenth of the original volume of the crude extract).

pH Dialysis—The suspension was dialyzed against 0.01 M sodium cacodylate buffer, pH 5.5, overnight, centrifuged for 10 min, and the precipitate discarded. The supernatant was adjusted to pH 7.0 by the addition of 1 M Tris and then to pH 8.0 by the addition of 1 M NaOH.

DEAE-cellulose Chromatography—The protein solution from the last step was applied to a DEAE-cellulose column (2.5 x 100 cm) equilibrated with 0.01 M Tris-HCl buffer, pH 8.0, containing 0.2 mM EDTA. The column was eluted by a 5-liter gradient from 0 to 0.3 M KCl in the same buffer, and 20-ml fractions were collected. Fractions showing at least a 2-fold greater specific activity were pooled. The protein in the pooled fractions was precipitated by the addition of 600 g of ammonium sulfate per liter. The pyruvate kinase eluted at a position equivalent to 0.15 M KCl, before the majority of the protein on the column.

Reverse Ammonium Sulfate Extraction—The precipitate from the last step was suspended in 60 ml of 50% (w/v) ammonium sulfate in the usual buffer and then centrifuged for 20 min, and the supernatant was discarded. The alternate suspension and centrifugation was repeated until the absorbance at 280 nm of the supernatant was below 0.5. The above procedure was repeated with 50% (w/v) and 40% (w/v) ammonium sulfate, until the supernatants showed significant pyruvate kinase activity. Pyruvate kinase was solubilized by repeated washing with 37.5% (w/v) ammonium sulfate. The pooled washes were brought to 90% (w/v) ammonium sulfate to precipitate the enzyme. The suspension was centrifuged for 20 min and the precipitate was suspended in as small a volume as possible.

Sephadex G-200 Chromatography—The enzyme from the last step was chromatographed on a Sephadex G-200 column (5.0 x 100 cm). Ten-milliliter fractions were collected, and those fractions showing a specific activity greater than 20 were pooled. The enzyme in the pooled fractions was precipitated by the addition of 600 g of ammonium sulfate per liter. The pyruvate kinase eluted at a position equivalent to 0.15 M KCl, before the majority of the protein on the column.

CM-cellulose Chromatography—The enzyme from the last step was chromatographed on a CM-cellulose column (0.9 x 25 cm) equilibrated with the same buffer as used for dialysis. The enzyme was eluted using a 0 to 1 M KCl gradient. Five milliliter fractions were collected, and those fractions showing a specific activity greater than 40 were pooled. The pH of the pooled fractions was adjusted to pH 7.5 by the addition of 1 M Tris, and pyruvate kinase was precipitated by placing the pooled fractions in a dialysis bag which was subsequently covered by solid ammonium sulfate.

Purity and Stability—The precipitate from the last step, when stored at 4°C in saturated ammonium sulfate, was stable for at least 3 months. Before use the precipitate dissolved in a small volume of buffer was dialyzed overnight. The purification procedure is summarized in Table I. The enzyme preparation obtained by this procedure gave a single band on polyacrylamide gels (Fig. 6).

Variation in Enzyme Activity under Different Growth Conditions

In order to understand what the physiological roles of the two pyruvate kinases may be, the organism was grown in minimal medium with supplements of various carbon sources, and the activity of both the AMP-activated and fructose-1,6-P₂-activated enzymes was measured. The results presented in Table II show that the fructose-1,6-P₂-activated enzyme is induced with glucose as the carbon source. With succinate or acetate as carbon sources, the enzyme level is reduced to about one-third of that present in glucose-grown cells. The AMP-activated enzyme, on the other hand, does not show much variation under different growth conditions.

Chemical Properties of Enzyme

General—The specific extinction coefficient (E<sub>1%cm</sub>) at 280 nm of the enzyme measured from dry weight analysis and calcula-
ments.

Amino Acid Composition—Table III gives the amino acid composition of the enzyme. Tyrosine and tryptophan were determined independently by the method of Edelhoch (20). The value obtained by this method for tyrosine was in agreement with the 24-hour hydrolysis value. The tryptophan content was found to be negligible after applying the correction for total cysteic acid. The partial specific volume of the enzyme, determined from the amino acid composition, was found to be 0.737.

NH2 Terminus Analysis—Using the dansylation procedure (22) we could not detect any NH2-terminal amino acid. The method used had a potential sensitivity to detect 2 nmoles of dansylated amino acid. The amount of protein used in this experiment was 2.4 mg, which, assuming identity of the subunits, should yield 40 nmoles of the NH2-terminal amino acid.

This preliminary evidence suggests that the NH2-terminal amino acid in the case of E. coli enzyme is either blocked or buried as in the case for the rabbit muscle enzyme (26) and the human erythrocyte enzyme (27).

Sulfhydryl Groups—As indicated in Table III there are about 14 cysteic acid residues per subunit of molecular weight 60,000. All of these sulfhydryl groups are not exposed on the surface of the enzyme. When an enzyme solution containing 20 nmoles of the monomer was treated with 1 nmoles DTNB at pH 8.0, the number of SH groups titrated approached four per monomer both in the absence (after 24 hours incubation) and presence of 2% sodium dodecyl sulfate. At the point where two SH groups per subunit had been titrated, the enzyme had lost about 96% of its activity (Fig. 1) but the remaining 4% became insensitive to activation by fructose-1,6-P2. In other words, blockage of two of the 14 or so possible sulfhydryl groups (estimated on the basis of amino acid analysis) of the protein led to a desensitization of the enzyme to the activator.

Carbohydrate Analysis—In three separate preparations glucosamine, recovered after 24-hour hydrolysis of the protein in 6 M HCl at 110°, averaged 2.6 moles per subunit (mol wt 60,000; see below). Assuming that this is a 50% recovery (23), the presence of 5 moles of glucosamine per subunit is indicated. Indeed, when milder conditions for hydrolysis were employed (3 M HCl for 4 hours at 110°), 5 moles of glucosamine per subunit were found.

Attempts were made to show that glucosamine (or, one of its easily hydrolyzable derivatives, such as N-acetylglucosamine) was covalently bound to pyruvate kinase, rather than merely being a contaminant. Two approaches were made. In one approach, heavily loaded (100 to 300 μg of protein) polyacrylamide gels were run and stained for glycoprotein using a periodate-Schiff method (28). In none of the trials was any stain found associated either with pyruvate kinase or with any other segment of the gel. If the enzyme is indeed a glycoprotein, this may mean that the carbohydrate content of pyruvate kinase is not high enough to give a color reaction with the periodate method. In the second approach, the enzyme was dialyzed overnight in 5 M guanidine HCl, carboxymethylated, and applied to a column (100 × 1.5 cm) of Sepharose 4B calibrated with light (mol wt 53,000) and heavy (mol wt 70,000) chains of

| Amino Acid Analysis of Enzyme | Hydrolysis time | Average | | |
|-----------------------------|----------------|---------|---------|---------|---------|---------|
|               | 24-hour | 48-hour | 72-hour | | |
|                | nmole | mg protein | | | | |
| Lysine         | 0.670 | 0.632 | 0.648 | 39 |
| Histidine      | 0.147 | 0.146 | 0.147 | 0 |
| Ammonia        | 0.989 | 1.04 | 0.985 | 60 |
| Arginine       | 0.380 | 0.355 | 0.375 | 22 |
| Aspartate      | 1.05 | 0.978 | 1.07 | 60 |
| Threonine      | 0.618 | 0.577 | 0.599 | 39 |
| Serine         | 0.470 | 0.409 | 0.386 | 30 |
| Glutamate      | 0.867 | 0.961 | 0.901 | 54 |
| Proline        | 0.256 | 0.242 | 0.274 | 15 |
| Glycine        | 0.766 | 0.845 | 0.747 | 47 |
| Alanine        | 0.806 | 0.813 | 0.827 | 49 |
| Valine         | 0.778 | 0.824 | 0.811 | 50 |
| Methionine     | 0.315 | 0.294 | 0.296 | 18 |
| Isoleucine     | 0.614 | 0.528 | 0.561 | 36 |
| Leucine        | 0.658 | 0.596 | 0.710 | 42 |
| Tyrosine       | 0.101 | 0.068 | 0.073 | 7 |
| Phenylalanine  | 0.223 | 0.226 | 0.240 | 14 |
| Cysteic acid   | 0.232 |            |        | 0 |
| Subunit molecular weight |             | 60,244 |

* Extrapolated to zero time for average.
† Highest values taken for average.
‡ Determined by the method of Spencer and Wold (19).
§ See text.
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Table IV

<table>
<thead>
<tr>
<th>Residue</th>
<th>24-hour hydrolysis after isolation from guanidine HCl column</th>
<th>24-hour hydrolysis before applying to guanidine HCl column</th>
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<tr>
<td>Aspartic</td>
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<tr>
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<td>Glutamine</td>
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<tr>
<td>Proline</td>
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<td>Glycine</td>
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</table>

* Residues per 60,000 molecular weight subunit.
* Value not determined.

human immunoglobulin G and equilibrated with 5.5 M guanidine HCl (29). The elution pattern is shown in Fig. 2. A single protein peak was eluted at a position corresponding to a molecular weight of 60,000, which as we show later, is the monomer molecular weight of pyruvate kinase. The fractions containing the protein were pooled and dialyzed extensively against distilled water. The protein which precipitated on dialysis was recovered and analyzed for glucosamine and neutral and acidic amino acids after hydrolysis in acid. The results are presented in Table IV. It will be noted that the amino acid composition of the native and guanidine HCl-treated pyruvate kinase is almost exactly the same, but instead of 5 moles of glucosamine per subunit found in the native protein, only 1 mole of glucosamine is recovered per mole of the subunit isolated by treatment with guanidine HCl. It is hard to determine from these results whether glucosamine is a contaminant in pyruvate kinase preparations, as the loss of the sugar during unfolding of the protein suggests that glucosamine, or some of its derivatives (such as

![Fig. 2. Isolation of pyruvate kinase subunit. Six milligrams of pyruvate kinase were dialyzed against 5.5 M guanidine HCl in 0.2 M Tris-HCl buffer, pH 8.0, overnight. After dialysis the enzyme was reduced by the addition of solid sodium dithiothreitol to the mixture and allowed to stand for 3 hours. Alkylation was achieved by the addition of solid iodoacetate to a concentration of 22 mM. This preparation was placed on a Sepharose 4B column (1.5 X 100 cm), equilibrated with 5.5 M guanidine HCl in 0.2 M Tris-HCl buffer, pH 8.0, and eluted by the same buffer. Fractions 61 to 70 were collected and used as described in the text. The peak at Fraction 48 is <2% of the major peak and corresponds to an approximate molecular weight of 180,000.

![Fig. 3. Molecular weight determination by Sephadex G-200. A Sephadex G-200 column (2 X 100 cm) equilibrated with 0.05 M Tris-HCl buffer, pH 7.5, containing 1 mM EDTA, was calibrated using the following proteins: cytochrome c (horse heart), catalase, ribonuclease A, ovalbumin, bovine serum albumin, alcohol dehydrogenase (yeast), and pyruvate kinase (rabbit muscle). V<sub>e</sub> is the elution volume and V<sub>0</sub> is the void volume as determined by blue dextran. The Escherichia coli fructose-1,6-P<sub>2</sub>-activated pyruvate kinase eluted ahead of the rabbit muscle pyruvate kinase and corresponded to a molecular weight of 247,000 ± 15,000.

![Fig. 4. Molecular weight determinations by sodium dodecyl sulfate polyacrylamide electrophoresis. The dodecyl sulfate polyacrylamide gels were calibrated using the proteins as shown. N-acetylglucosamine), is not bound covalently to the enzyme. We have ascertained that free glucosamine or N-acetylglu-

cosesamin does not affect the activity of the native enzyme. This problem is being investigated further.

Molecular Weight Studies

Sephadex G-200 Chromatography—Using a column calibrated by the method of Andrews (14), the estimated molecular weight of E. coli pyruvate kinase was found to be 247,000 ± 15,000 (Fig. 3). The average effective pore radius (15) for our Sepha-
dex G-200 column was 18.62 ± 0.3, which gave a Stokes radius of 6.78 ± 0.08 for pyruvate kinase.

Polyacrylamide Gel Electrophoresis—Fig. 4 shows results of a typical subunit molecular weight determination for pyruvate kinase. The molecular weight of the subunit found from four experiments was 60,000 ± 3,500.

Sedimentation Equilibrium Studies—Using the high speed equilibrium technique of Roark and Yphantis (16), the protein sedimented as a homogeneous species when either the native or dissociated (6.3 M guanidine HCl and 1% 2-mercaptoethanol) protein was used (Fig. 5). The molecular weights obtained from the gradient of these plots did not agree with previously described results. The value for the native protein at all three concentrations (0.2, 0.4, and 0.6 mg per ml), with or without
Fig. 5. Sedimentation equilibrium molecular weight determinations. Calcomp plotter plots of results obtained from sedimentation equilibrium studies of pyruvate kinase showing log (fringe displacement) against (radius)$^2/2$. A, pyruvate kinase (0.2 mg per ml) in 0.05 M Tris-HCl buffer, pH 7.5, containing 1 mM EDTA and 0.1 M KCl, was spun at 15,000 rpm at 17.25°. The molecular weight obtained from the gradient is 205,000. B, pyruvate kinase (0.2 mg per ml) in the above buffer, but also containing 6.3 M guanidine HCl and 1% 2-mercaptoethanol, was spun at 32,000 rpm at 3.65°. The molecular weight obtained from the gradient is 47,000 ± 15,000 in two separate determinations at the three protein concentrations noted above. The $\bar{M}_n$ and $\bar{M}_w$ estimates obtained from calculations according to Roark and Yphantis (16) did not give linear plots. Using the two-species analysis of Roark and Yphantis, no further clarification was obtainable. However, the Y1 and Y8 plots described by Roark and Yphantis (16), which correct for and eliminate Donnan charge effects, respectively, produced linear results with nearly no gradient. The values obtained from these were 234,000 ± 20,000 for the native protein and 57,000 ± 6,000 for the guanidine HCl-dissociated protein. Considering the evidence presented elsewhere (Figs. 3 and 4), we feel that the Y1 and Y8 values are the best estimates that could be obtained from these studies.

Quaternary Structure—Results described before suggest that pyruvate kinase in a native form is a tetramer of a basic subunit whose molecular weight is around 60,000. To confirm conclusions drawn from previous studies, we utilized the cross-linking procedure of Davies and Stark (13) to determine the subunit structure. In 5% sodium dodecyl sulfate polyacrylamide gels, samples of pyruvate kinase (0.3 mg per ml) previously cross-linked with 0.4 and 0.04 mg per ml of dimethylsuberimidate showed after dissociation and electrophoresis, bands corresponding to monomer, dimer, trimer, and tetramer (Fig. 6). Judging simply by the staining intensity of the different bands, it would seem that the 4 subunits of the enzyme are arranged in an isologous association as suggested by Carpenter and Harrington (30).

Kinetic Properties

General—The pyruvate kinase activated by fructose-1,6-P$_2$ described here requires either Mg$^{2+}$ or Mn$^{2+}$ (see below), but not K$^+$ or NH$_4^+$ for catalytic activity. The enzyme has a bell-shaped pH curve, with an optimum of 7.0. There is greater than 90% of the optimum activity at pH 6.0 or pH 8.0. The amount of activation caused by fructose-1,6-P$_2$ (see below) is about the same both at the acidic as well as alkaline pH values.

Substrate Kinetics—Using a constant level of Mg$^{2+}$ and P-enolpyruvate as the variable substrate, the rate-concentration curves show homotropic cooperativity (Fig. 7) with either ADP or GDP as the nucleotide substrate. In the presence of 1 mM fructose-1,6-P$_2$ the rate-concentration curve becomes hyperbolic. The Michaelis constants for P-enolpyruvate in the absence of the activator can be evaluated as 4 mM and 2.7 mM in the presence of ADP and GDP, respectively. The fully activated enzyme, i.e. in the saturating presence of fructose-1,6-P$_2$, yields a $K_m$ value of about 0.03 mM when either ADP or GDP is used.

The kinetic constants for the enzyme are quite different when Mn$^{2+}$ is used as the cation. In the presence of a saturating amount of Mn$^{2+}$ the rate-concentration curves show homotropic cooperativity with sigmoidal, much like that obtained in the presence of Mg$^{2+}$, but the $K_m$ for P-enolpyruvate is now only about 0.12 mM (Fig. 8). Fructose-1,6-P$_2$ also activates the enzyme in the presence of Mn$^{2+}$ and changes the $K_m$ of P-enolpyruvate to about 0.008 mM (Fig. 8) without significantly changing the value for $V_{max}$, which is considerably lower than that obtained using Mg$^{2+}$. 

Fig. 6. A, polyacrylamide gel electrophoresis of pyruvate kinase. The photograph shows the single band obtained after electrophoresis on polyacrylamide gels at pH 8.3. Protein concentration was 100 µg. The small band is the running dye. B, sodium dodecyl sulfate polyacrylamide gel electrophoresis of dimethylsuberimidate cross-linked pyruvate kinase. Pyruvate kinase was treated as described in the text. The four bands from top to bottom represent the tetramer, trimer, dimer, and monomer of the cross-linked pyruvate kinase.
FIG. 7 (left). P-enolpyruvate dependence of pyruvate kinase. Standard assays were carried out using 1.33 mM ADP and 10 mM MgCl₂ with or without fructose-1,6-P₃ (FDP). P-enolpyruvate shows homotropic cooperativity (Hill number = 3) in the absence of fructose-1,6-P₃ and has an approximate Kₘ of 4 mM. In the presence of 1 mM fructose-1,6-P₃, the kinetic response is Michaelis-Menten, and the inset shows a Lineweaver-Burk plot which gave a Kₘ for P-enolpyruvate of 30 μM. 1/V is the reciprocal of the initial velocity.

FIG. 8 (center). P-enolpyruvate dependence of pyruvate kinase. Standard assays were carried out in the presence of 2 mM MgCl₂ and 1.33 mM ADP with or without 1 mM fructose-1,6-P₃ (FDP). The Kₘ for P-enolpyruvate in the presence of fructose-1,6-P₃ is 8 μM as determined by the Lineweaver-Burk plot. 1/V is the reciprocal of the initial velocity. In the absence of fructose-1,6-P₃, P-enolpyruvate shows homotropic cooperativity, and has an approximate Kₘ of 0.12 mM.

FIG. 9 (right). GDP dependence of pyruvate kinase. A Lineweaver-Burk plot of the reciprocal of GDP concentration versus the reciprocal of initial velocity (V = micromoles of P-enolpyruvate utilized per mg of pyruvate kinase per min) obtained from standard assays in the presence of 1 mM P-enolpyruvate, 1 mM fructose-1,6-P₃, and 10 mM MgCl₂. The substrate GDP shows inhibition at higher concentrations and the Kᵢ for this inhibition is obtained from a Dixon plot (inset). The extrapolated Kₘ for GDP is 0.05 mM.

TABLE V

Specificity of enzyme for nucleoside diphosphates

The assays were carried out as described in the text in a mixture containing 10 mM MgCl₂, 1 mM fructose-1,6-P₃, and 1 mM P-enolpyruvate. The nucleoside diphosphate was used as the variable substrate. The Kₘ values reported are obtained by plotting the initial velocity data in a double-reciprocal form as described in the text. V_max refers to micromoles of P-enolpyruvate utilized per mg of pyruvate kinase per min.

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Kₘ (mM)</th>
<th>V_max</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP</td>
<td>0.24</td>
<td>55</td>
</tr>
<tr>
<td>GDP</td>
<td>0.05</td>
<td>110</td>
</tr>
<tr>
<td>UDP</td>
<td>0.42</td>
<td>88</td>
</tr>
<tr>
<td>IDP</td>
<td>0.17</td>
<td>94</td>
</tr>
<tr>
<td>CDP</td>
<td>0.67</td>
<td>47</td>
</tr>
</tbody>
</table>

Nucleotide Specificity—Using saturating concentrations of P-enolpyruvate, Mg²⁺, and fructose-1,6-P₃, the Michaelis constants for various nucleoside diphosphates were determined. Since several of these compounds produced substrate inhibition, only the initial part of the rate-concentration curve was used to evaluate the Michaelis constant (Fig. 9). These values are given in Table V. It will be noted that GDP is by far the best phosphate acceptor (Kₘ = 0.05 mM) even though it begins to show substrate inhibition at concentrations higher than 0.04 to 0.05 mM. Among the various nucleoside diphosphates tested, IDP and CDP also showed substrate inhibition, but this inhibition became apparent only at concentrations higher than 2 mM and was much less marked than that obtained with GDP. The remaining nucleotides, ADP and UDP, gave no inhibition up to concentrations of 10 mM. In the absence of fructose-1,6-P₃, the rate-concentration plots for the nucleotides, at least in the lower concentration ranges, were hyperbolic, but the Michaelis constants were higher than those reported in Table V and dependent upon P-enolpyruvate concentration.

Activation of Enzyme—Fructose-1,6-P₃ is an effective activator of the enzyme (Fig. 10). The 0.5 values (i.e. concentration at half-maximal velocity) for the activator under our experimental conditions are independent of the nature of nucleotide substrate (ADP or GDP). At low concentrations of P-enolpyruvate, the rate-concentration curves for fructose-1,6-P₃ are sigmoidal but change to a hyperbolic shape at high concentration of the substrate (Fig. 10). These heterotropic interactions are similar to those seen when P-enolpyruvate is the variable substrate (see Fig. 7). Various compounds related to fructose-1,6-P₃ such as glucose-1-P, glucose-6-P, fructose-1-P, fructose-6-P, glucosamine-6-P, and glucose-1,6-P₂ tested at a concentration of 1 mM neither activate nor inhibit the stimulation of activity caused by fructose-1,6-P₂. AMP does not activate this enzyme.

Inhibition of Enzyme—In an earlier communication (3) we demonstrated that succinyl-CoA was an inhibitor of pyruvate kinase. ATP, a product of the enzymatic reaction, did not cause any inhibition by itself at low (2.5 mM) concentration but when used together with succinyl-CoA caused cooperative inhibition, a phenomenon first described by Caskey et al. (31) with the enzyme glutamine:phosphoribosyl amidotransferase. With the finding that GDP is a better substrate for the E. coli pyruvate...
kinase reported above, it became of interest to determine whether GTP behaves in a manner analogous to ATP.

Using an assay mixture containing 0.2 mM P-enolpyruvate, 0.5 mM fructose-1,6-P₂ and either 1 mM GDP or 1.33 mM ADP, we tested the effect of GTP and various nucleoside triphosphates on the initial velocity of the enzyme. The particular concentrations of the substrates and the effector were chosen such that they were approximately those reported by Lowry et al. (32) and Nazar and Wong (33) to exist in vivo in actively growing cells and that they would give a half-activated enzyme. With ADP or GDP as the phosphate acceptor, the \( K_i \) values for GTP, ITP, UTP, and ATP were all higher than 10 mM, while GTP had a \( K_i \) of 1.8 mM in the presence of GDP and 2.3 mM in the presence of ADP. The exact \( K_i \) values for nucleotides other than GTP were not determined because at the high concentrations that these nucleoside triphosphates show inhibitory effect, complexation of the divalent cation (\( \text{Mg}^{2+} \)) used in the experiments becomes a serious problem (34) and it becomes hard to decide whether it is the nucleoside triphosphate effect or the nonavailability of free \( \text{Mg}^{2+} \) that leads to the inhibitory effect. It is apparent, however, that GTP inhibition is not due to the complexation of the cation. It is known that all of the ribonucleoside triphosphates have the same affinity for magnesium (34). Thus, the fact that GTP is an inhibitor while other triphosphates show little effect cannot be mainly the result of chelation of magnesium by this nucleotide.

While it does seem that GTP is a physiologically important inhibitor, its inhibitory potency nevertheless depends upon the concentration of the activator and the nature of the phosphate acceptor used in the assay mixtures. The effect of GTP on the inhibition of the enzyme with GDP as the phosphate acceptor in the presence of various levels of P enolpyruvate and fructose-1,6-P₂ is shown in Fig. 11. At low levels of substrate and activator, 2 mM GTP almost causes a complete inhibition of the enzyme. At high levels of the activator the inhibition is reduced to a plateau level (Fig. 11). In the presence of ADP as the phosphate acceptor, however, less inhibition is caused by 2 mM GTP (Figs. 11 and 12). These results suggest that GTP in a physiological range always inhibits the enzyme, especially when GDP is the substrate and the activator and P-enolpyruvate levels are low. In other words, fructose-1,6-P₂ seems to modulate the behavior of GTP as an inhibitory effector.

As was mentioned before and shown by us earlier (3) ATP is a very poor inhibitor of the enzyme, but together with succinyl-CoA, which gives some inhibition by itself, they produce a large cooperative inhibition. These studies (3) were carried out with ADP as the nucleotide substrate. Since finding that GDP is a better substrate than ADP and GTP a better inhibitor than ATP, it became of interest to repeat the experiment substituting GDP and GTP for ADP and ATP, respectively. The results are presented in Fig. 12. In the presence of 1 mM fructose-1,6-P₂, 2 mM ATP gives no inhibition when either ADP or GDP is the substrate. GTP, however, gives significant inhibition, which is stronger when GDP is the substrate. Succinyl-CoA causes inhibition at approximately the same level with either ADP or GDP as the substrate. As reported earlier, ATP and succinyl-CoA gives a cooperative inhibition, which is stronger when ADP is the substrate. GTP and succinyl-CoA do not show the same increased cooperative inhibition. GTP and ATP do not cooperate (results not shown).

**Metal Ion Requirement**—Both \( \text{Mg}^{2+} \) and \( \text{Mn}^{2+} \) ions show homotropic cooperativity in binding to the enzyme (Fig. 13). In both cases (shown only for \( \text{Mn}^{2+} \) in Fig. 13) sigmoid plots become hyperbolic in the presence of 1 mM fructose-1,6-P₂. In some other systems, such as the pyruvate kinase from human erythrocytes (85), \( \text{Mn}^{2+} \) has been reported to be required not only for catalytic activity but also acts as an allosteric activator. It is clear that such is not the case with the E. coli enzyme (Fig. 13).

The \( V_{\text{max}} \) for the enzyme in the presence or absence of the activator is the same when \( \text{Mn}^{2+} \) is used, and the value is approximately half of the value obtained when \( \text{Mg}^{2+} \) is used. Under fully activated conditions the \( K_m \) for \( \text{Mg}^{2+} \) is 0.4 mM and for \( \text{Mn}^{2+} \) is 0.04 mM. In the presence of a saturating concentration of \( \text{Mn}^{2+} \) (2.0 mM) the \( K_m \) values for activation by fructose-1,6-P₂ at 0.1 mM and 0.3 mM P-enolpyruvate are 13 \( \mu \)M and 5.5 \( \mu \)M, respectively.
with GDP as the nucleotide substrate show a profound substrate inhibition caused by this compound at higher concentrations. It seems likely that the so-called substrate inhibition is caused by GDP by binding at higher concentrations to a site other than the catalytic one. This inhibitory site could very well be the site at which GTP binds. The pyruvate kinase discussed here is unique from all of the other pyruvate kinases described from various sources in its nucleotide requirement and its inhibition by GTP.

In an earlier communication (3) we showed that succinyl-CoA is a feedback inhibitor of the \textit{E. coli} enzyme and causes a cooperative inhibition of enzyme activity with ATP. From the results presented here, GTP appears not to cooperate with succinyl-CoA to cause cooperative inhibition. It is hard to judge whether the inhibition by GTP and ATP plus succinyl-CoA is of an allosteric nature. To prove allosteric inhibition, it must be demonstrated that the enzyme has an inhibitor-binding site distinct from substrate-binding sites (37). Since kinetic studies are not very helpful in this regard, we have refrained from presenting kinetic competitive patterns with inhibitors in the presence of the various substrates. This aspect of the problem is being investigated by the use of equilibrium binding techniques and will be the subject of a separate communication.

Perhaps it is pertinent here to make a few comments on the physiological reasons for the presence of two pyruvate kinases in \textit{E. coli}. From molecular weight studies and allosteric properties of the two pyruvate kinases it seems that they are two different, rather than interchangeable, enzymes. The observation that may be interesting in this regard is that the activity of the fructose-1,6-P\textsubscript{2}-activated pyruvate kinase is repressed in succinate- and acetate-grown cells but increased in glucose-grown cells. This suggests that the fructose-1,6-P\textsubscript{2}-activated enzyme may be physiologically important only when glycolysis is occurring but not under conditions where gluconeogenesis is the predominant metabolic pathway being utilized by the cells.

As a final point, we should like to draw attention to the fact that P-enolpyruvate is converted in enteric bacteria to oxalacetate on one hand by means of P-enolpyruvate carboxylase and to pyruvate on the other by means of pyruvate kinase. Physiologically, it is interesting to find the mechanisms that determine the distribution of a key metabolite like P-enolpyruvate into the two channels. It may be significant that both of the enzymes at the branch point, P-enolpyruvate carboxylase and pyruvate kinase, show precursor activation by fructose-1,6-P\textsubscript{2} (10, 38, 39), but while the former is activated by GTP, the latter is inhibited by it. This modulation by GTP of the two enzymes may be a mechanism which controls the distribution of P-enolpyruvate into different channels according to the energy state of the cell.

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