The Control of Pyruvate Kinases of *Escherichia coli*

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

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**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 means, 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 Hepes⁴ 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 (ultrapore 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. Polyamide 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

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*The abbreviations used are: Hepes, N-2-hydroxyethylpiperazine-N'2-ethanesulfonic acid; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); dansyl, 5-dimethylaminonaphthalene-1-sulfonoyl.

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and aeration in a 200-liter capacity New Brunswick fermentor. The growth medium was made up of 10.5 g of K$_2$HPO$_4$, 4.5 g of KH$_2$PO$_4$, 1.5 g of (NH$_4$)$_2$SO$_4$, 0.05 g of MgSO$_4$, 4.0 g of glucose, and 10 mg of thiamine per liter. A 2-liter inoculum of cells grown on the same medium was used, and after 18 hours at 37°C, the cells were cooled and then harvested using a Sharples centrifuge. The cells were washed once in 0.05 M Tris-HCl, pH 7.5, containing 1 mM EDTA. After centrifugation the cells were suspended in 3 volumes of Tris buffer and were either frozen (−30°C) or used immediately.

Enzyme Assay—In crude extracts, where both the fructose-1,6-P$_2$- and AMP-activated pyruvate kinases are present, the fructose-1,6-P$_2$-activated pyruvate kinase was measured as an initial velocity difference between reaction mixtures with and without 1 μmole of fructose-1,6-P$_2$. The assay mixture contained in 1 ml, 1.33 μmoles of ADP, 0.2 μmole of P-enolpyruvate, 10 μmoles of MgCl$_2$, 0.15 μmole of NADH, 50 μg of lactate dehydrogenase, and 0.03 M Hepes buffer, pH 7.0. The fructose-1,6-P$_2$-activated pyruvate kinase was measured as the increase in activity upon the addition of 1 μmole of fructose-1,6-P$_2$. In purified extracts in which the AMP activated pyruvate kinase was not present, the same assay system was used with fructose-1,6-P$_2$ and with no correction for the AMP activated enzyme. The activity was measured as the change in absorbance at 340 nm using a Gilford 2400 recording spectrophotometer. All of the activators and inhibitors of pyruvate kinase discussed in this communication were tested for their effect on lactate dehydrogenase. None was found to inhibit or activate this enzyme.

The specific activity of the enzymes is defined as micromoles of P-enolpyruvate utilized per min per mg of protein, assuming that the ratio of NADH oxidized to P-enolpyruvate utilized is unity.

Polyacrylamide Gel Electrophoresis—Polyacrylamide gels were prepared and run according to the procedure of Davies (11), except that no stacking gel was used. The sample in 20% sucrose was layered on top of the gel. Sodium dodecyl sulfate polyacrylamide gels were prepared and used as described by Weber and Osborn (12), except that horse heart cytochrome c was used as the internal marker. Dimethylsuberimidate was used as described by Davies and Stark (13) to cross-link the subunits of the enzyme. The cross-linked enzyme after disaggregation was run in sodium dodecyl sulfate polyacrylamide gels.

**Sephadex G-200 Chromatography**—A column (2.5 × 100 cm) was calibrated according to Andrews (14) for molecular weight estimation and according to Ackers (15) for Stokes radius estimation. Sephadex G-25 was layered on top of the Sephadex G-200 to a depth of 1 cm. Samples in 20% sucrose whose volumes were less than 1 ml were layered on top of the Sephadex G-25 under the buffer. The column was equilibrated with 0.05 M Tris-HCl, pH 7.5, containing 1 mM EDTA. The void volume was measured by blue dextran and the final elution volume by ditriphenylalanine.

**Sedimentation Equilibrium Studies**—Sedimentation equilibrium studies were carried out with a Beckman model E analytical centrifuge. High speed equilibrium data were obtained from photographs of Rayleigh interference fringes and were processed using the computer program of Roark and Yphantis (16). 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°C. Similar samples which had been dialyzed against 6.3 M guanidine HCl and 1% 2-mercaptoethanol in 0.05 M Tris-HCl, pH 7.5, were spun at 29,750 rpm at 13.6°C. 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°C and 32,000 rpm at 3.65°C, 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**—Acid hydrolysis in 6 N HCl of approximately 1 mg of enzyme per hydrolysis sample was carried out at 110°C 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 dimethylsulfide 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 ($E_{280}$) at 280 nm, of 1.8 which was obtained by dry weight determination and from the amino acid composition.

**NH$_2$ Terminus Analysis**—Pyruvate kinase (2.5 mg = 40 nmole 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 1,10-phenanthroline, 25% by volume dimethyformamide, and 10% by volume acetonitrile. The protein was then precipitated with 10% trichloroacetic acid, washed with acetone, dried in vacuo, and hydrolyzed in 6 N HCl for 4 hours at 110°C. The dried hydrolysate was trituated 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 by drying with 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 N HCl for 4 hours at 110°C. Neutral sugars were determined by gas liquid chromatography after the hydrolysate had been washed free of HCl and passed through Dowex 50X4 (200 to 400 mesh) in the hydrogen ion form and Dowex 1-X8 (200 to 400 mesh) in the formate ion form to remove the amino acids and the amino sugars. Gas-liquid chromatography was performed according to standard procedures (24). Neutral sugar analysis was also carried out using paper chromatography and 1-butanol-pyridine-water (6:4:3) solvent system.

**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°C. Centrifugations were performed at 48,000 × 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. 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 mM 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 N 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 from the last step was applied to a Sephades G-200 column (5.0 x 100 cm) equilibrated with 0.01 M Tris-HCl, pH 8.0, containing 0.2 mM EDTA. The column was eluted by a 5-liter gradient at 280 nm of the enzyme measured from dry weight analysis and calculated.

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 protein solution from the last step was chromatographed on a Sephades G-200 column (5.0 x 100 cm). Ten-milliliter fractions were collected, and those 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.

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-P_2-activated enzymes was measured. The results presented in Table II show that the fructose-1,6-P_2-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\textsubscript{27}g\textsubscript{3} cm\textsuperscript{-1} mg\textsuperscript{-1}) at 280 nm of the enzyme measured from dry weight analysis and calculated.

### Table I

| Purification of pyruvate kinase from Escherichia coli K-12 |
|----------------|----------------|----------------|----------------|
| Purification step | Total protein | Specific activity | Total units | Recovery | Purification |
| mg | units/mg | |
| Crude | 50,400 | 0.096 | 5250 | 100 | 1 |
| 2% protamine sulfate | 30,200 | 0.18 | 6000 | 125 | 1.8 |
| Ammonium sulfate 0.45 to 0.6 saturated fraction | 27,900 | 0.22 | 6000 | 115 | 2.1 |
| pH 5.5 dialysis | 15,500 | 0.37 | 5400 | 103 | 3.6 |
| DEAE-cellulose chromatography | 1,615 | 2.8 | 4500 | 85 | 25.6 |
| Reverse ammonium sulfate precipitation | 560 | 5.6 | 3150 | 60 | 54 |
| Sephadex G-200 chromatography | 76 | 28 | 2100 | 40 | 270 |
| CM-cellulose chromatography | 22 | 48 | 1050 | 22 | 460 |

### Table II

<p>| Variation in activity of the two pyruvate kinases of Escherichia coli |
|----------------|----------------|----------------|
| Cells of E. coli K-12 strain were grown in 500-ml capacity Erlenmeyer flasks in minimal medium with constant agitation at 37°C. Cells were harvested in mid-log phase. The activities of the AMP-activated and fructose-1,6-P_2-activated enzymes were measured in crude extracts as described in the text. The values are given in terms of micromoles of P-enolpyruvate utilized per min per mg of extract protein. |</p>
<table>
<thead>
<tr>
<th>Carbon source</th>
<th>AMP-activated enzyme</th>
<th>Fructose-1,6-P_2-activated enzyme</th>
<th>AMP-activated/ fructose-1,6-P_2-activated</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.4% glucose</td>
<td>0.103</td>
<td>0.108</td>
<td>0.95</td>
</tr>
<tr>
<td>0.4% glycerol</td>
<td>0.106</td>
<td>0.044</td>
<td>2.48</td>
</tr>
<tr>
<td>1% succinate</td>
<td>0.077</td>
<td>0.032</td>
<td>2.40</td>
</tr>
<tr>
<td>1% acetate</td>
<td>0.075</td>
<td>0.031</td>
<td>2.45</td>
</tr>
</tbody>
</table>
Table III

Amino acid analysis of enzyme

<table>
<thead>
<tr>
<th>Residue</th>
<th>Hydrolysis time</th>
<th>Average ( \text{nmol/mg protein} )</th>
<th>Subunit molecular weight</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24-hour</td>
<td>48-hour</td>
<td>72-hour</td>
</tr>
<tr>
<td>Lysine</td>
<td>0.670</td>
<td>0.633</td>
<td>0.648</td>
</tr>
<tr>
<td>Histidine</td>
<td>0.147</td>
<td>0.144</td>
<td>0.147</td>
</tr>
<tr>
<td>Ammonia</td>
<td>0.989</td>
<td>1.04</td>
<td>0.985</td>
</tr>
<tr>
<td>Arginine</td>
<td>0.380</td>
<td>0.355</td>
<td>0.375</td>
</tr>
<tr>
<td>Aspartate</td>
<td>1.05</td>
<td>0.978</td>
<td>1.07</td>
</tr>
<tr>
<td>Threonine*</td>
<td>0.618</td>
<td>0.577</td>
<td>0.559</td>
</tr>
<tr>
<td>Serine*</td>
<td>0.470</td>
<td>0.409</td>
<td>0.386</td>
</tr>
<tr>
<td>Glutamate</td>
<td>0.867</td>
<td>0.961</td>
<td>0.901</td>
</tr>
<tr>
<td>Proline</td>
<td>0.256</td>
<td>0.242</td>
<td>0.274</td>
</tr>
<tr>
<td>Glycerine</td>
<td>0.766</td>
<td>0.845</td>
<td>0.744</td>
</tr>
<tr>
<td>Alanine</td>
<td>0.806</td>
<td>0.813</td>
<td>0.827</td>
</tr>
<tr>
<td>Valine*</td>
<td>0.778</td>
<td>0.824</td>
<td>0.814</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.315</td>
<td>0.294</td>
<td>0.296</td>
</tr>
<tr>
<td>Isoleucine*</td>
<td>0.614</td>
<td>0.528</td>
<td>0.561</td>
</tr>
<tr>
<td>Leucine</td>
<td>0.698</td>
<td>0.726</td>
<td>0.710</td>
</tr>
<tr>
<td>Tyrosine*</td>
<td>0.101</td>
<td>0.068</td>
<td>0.073</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.235</td>
<td>0.226</td>
<td>0.240</td>
</tr>
<tr>
<td>Cysteic acid*</td>
<td>0.232</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tryptophan*</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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.

Fig. 1. Reaction of DTNB with pyruvate kinase. The reaction mixture contained 20 nmole of pyruvate kinase in 0.05 M Tris-HCl buffer, pH 8.0, to which DTNB was added at time zero to a concentration of 1 mM in a volume of 0.4 ml. The reaction of DTNB with the sulphydryl groups of pyruvate kinase was measured by the change in absorbance at 412 nm. The activity of pyruvate kinase was measured in the standard assay containing 1.33 mM ADP, 2 mM P-enolpyruvate, 10 mM MgCl₂, with (●—●) and without (▲—▲) 1 mM fructose-1,6-P₂. The data given for the —SH groups reacted are a combination of two separate experiments.

Lutated from the amino acid composition data presented in Table III was 1.8. This low coefficient reflects the low tyrosine content and the complete absence of tryptophan from the enzyme. The fluorescence spectrum of the enzyme was typical of proteins that do not contain tryptophan (22). When the exciting wave length was 275 nm or 287 nm, only one emission peak at 314 nm was visible without any shoulder at 340 to 350 nm.

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 nmole 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 nmole 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).

Sulphydryl Groups—As indicated in Table III there are about 14 cysteic acid residues per subunit of molecular weight 60,000. All of these sulphydryl groups are not exposed on the surface of the enzyme. When an enzyme solution containing 20 nmole of the monomer was treated with 1 nmo DTNB at pH 8.0, the number of —SH groups titrated approached eight per monomer 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 sulphydryl 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 mole 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 5.5 M guanidine HCl, carboxymethylated, and applied to a column (100 x 1.5 cm) of Sepharose 6B calibrated with light (mol wt 53,000) and heavy (mol wt 70,000) chains of...
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. 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-Cl 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.

**TABLE IV**

**Amino acid analyses of oligomer and isolated subunit**

<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>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>Threonine</td>
<td>38</td>
<td>38</td>
</tr>
<tr>
<td>Serine</td>
<td>30</td>
<td>29</td>
</tr>
<tr>
<td>Glutamine</td>
<td>60</td>
<td>50</td>
</tr>
<tr>
<td>Proline</td>
<td>N.D.b</td>
<td>14</td>
</tr>
<tr>
<td>Glycine</td>
<td>43</td>
<td>50</td>
</tr>
<tr>
<td>Alanine</td>
<td>47</td>
<td>50</td>
</tr>
<tr>
<td>Valine</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>Methionine</td>
<td>17</td>
<td>21</td>
</tr>
<tr>
<td>Isoleucine</td>
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</tr>
<tr>
<td>Leucine</td>
<td>42</td>
<td>48</td>
</tr>
<tr>
<td>Tyrosine</td>
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<td>7</td>
</tr>
<tr>
<td>Phenylalanine</td>
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<td>14</td>
</tr>
<tr>
<td>Glucosamine</td>
<td>1</td>
<td>5</td>
</tr>
</tbody>
</table>

* Residues per 60,000 molecular weight subunit.

**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-Cl buffer, pH 8.0, overnight. After dialysis the enzyme was reduced by the addition of 10 mM dithiothreitol to the mixture and allowed to stand for 3 hours. Alklylation was achieved by the addition of solid iodoacetate to a concentration of 22 mm. This preparation was placed on a Sephadez G-200 column (2.5 X 100 cm) equilibrated with 5.5 M guanidine HCl and 1% 2-mercaptoethanol, alcohol dehydrogenase (yeast), and pyruvate kinase (rabbit muscle). V,) is the elution volume and V, is the void volume as determined by blue dextran. The E. coli fructose-1,6-P₂-activated pyruvate kinase eluted ahead of the rabbit muscle pyruvate kinase and corresponded to a molecular weight of 247,000 ± 15,000.

**FIG. 3. Molecular weight determination by Sephadez G-200.** A Sephadez G-200 column (2.5 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, is the elution volume and V, is the void volume as determined by blue dextran. The E. coli fructose-1,6-P₂-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-acetylglucosamine does not affect the activity of the native enzyme. This problem is being investigated further.

**Molecular Weight Studies**

**Sephadez 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 Sephadez 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. Calemp 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°C. 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°C. The molecular weight obtained from the gradient is 47,000.

General—The pyruvate kinase activated by fructose-1,6-P₂ described here requires either Mg²⁺ or Mn²⁺ (see below), but not K⁺ or NH₄⁺ 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-P₂ (see below) is about the same both at the acidic as well as alkaline pH values.

Substrate Kinetics—Using a constant level of Mg²⁺ 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-P₂ 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-P₂ 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 Mn²⁺ is used as the cation. In the presence of a saturating amount of Mn²⁺ the rate-concentration curve for P-enolpyruvate as the variable substrate is sigmoidal, much like that obtained with Mg²⁺, but the $K_m$ for P-enolpyruvate is now only about 0.12 mM (Fig. 8). Fructose-1,6-P₂ also activates the enzyme in the presence of Mn²⁺ 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 Mg²⁺.

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 analogous association as suggested by Carpenter and Harrington (30).

Kinetic Properties

General—The pyruvate kinase activated by fructose-1,6-P₂ described here requires either Mg²⁺ or Mn²⁺ (see below), but not K⁺ or NH₄⁺ 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-P₂ (see below) is about the same both at the acidic as well as alkaline pH values.

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The activities evaluated were those caused by fructose-1,6-P₂. AMP does not activate the stimulation 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). 

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
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 (Mg²⁺) used in the experiments becomes a serious problem (34) and it becomes hard to decide whether it is the nucleoside triphosphate effect per se or the nonavailability of free Mg²⁺ 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 Mg²⁺ and Mn²⁺ ions show homotropic cooperativity in binding to the enzyme (Fig. 13). In both cases (shown only for Mn²⁺ 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 (35), Mn²⁺ 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 $V_{max}$ for the enzyme in the presence or absence of the activator is the same when Mn²⁺ is used, and the value is approximately half of the value obtained when Mg²⁺ is used. Under fully activated conditions the $K_m$ for Mn²⁺ is 0.4 mM and for Mn²⁺ is 0.04 mM. In the presence of a saturating concentration of Mn²⁺ (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 μM and 5.5 μM, 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 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 E. coli. From molecular weight studies and allosteric properties of the two pyruvate kinases it seems that they are two different, rather than interconvertible, enzymes. The observation that may be interesting in this regard is that the activity of the fructose-1,6-P₂-activated pyruvate kinase is repressed in succinate- and acetate-grown cells but increased in glucose-grown cells. This suggests that the fructose-1,6-P₂-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 oxaloacetate 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-P₂ (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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AND REGULATORY PROPERTIES OF THE ENZYME ACTIVATED BY
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