Purification and Properties of Glycerol Kinase from *Escherichia coli*

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**SUMMARY**

Glycerol kinase of *Escherichia coli* has been purified and crystallized. It has a molecular weight of \( \sim 3 \times 10^6 \). The enzyme phosphorylates glycerol exclusively to \( L-\alpha\)-glycerophosphate. It also catalyzes the phosphorylation of dihydroxyacetone and L-glyceraldehyde but with values of \( k_m \) much higher than that for glycerol. \( \alpha\)-Glyceraldehyde has a catalytic effect in promoting the conversion of adenosine triphosphate to adenosine diphosphate and \( P_i \) in the presence of the enzyme. Among the nucleoside triphosphates tested, only adenosine triphosphate was active as the phosphoryl group donor. Mn\(^{2+}\) substitutes for Mg\(^{2+}\), although with less activity at equal molar concentration.

A specific inducible kinase is obligatory for the utilization of glycerol by *Escherichia coli* K12 (1). Glycerol, entering the cell presumably by free diffusion (2), is converted by this enzyme to \( L-\alpha\)-glycerophosphate, which is then dehydrogenated to triose phosphate (1, 3).

Recently we have purified the *E. coli* glycerol kinase to a crystalline form in order to prepare specific antisera for the immunochromatographic measurement of the enzyme. With the aid of a mutant capable of producing a cross-reacting material (4), we were able to induce the kinase in the absence of free glycerol (4), as in the case of the \( L-\alpha\)-glycerophosphate transport system and the \( L-\alpha\)-glycerophosphate dehydrogenase (3, 5).

In this paper the purification and properties of the kinase are presented. Comparisons are made with the enzymes from rat liver (6), pigeon liver (7), and Candida mycoderma (8), which have been purified and studied in detail in other laboratories.

**EXPERIMENTAL PROCEDURE**

**Chemicals**—\(^{14}C\)-Glycerol (4C per mole) was purchased from New England Nuclear. Glycerol and glucose were obtained from Merck; dihydroxyacetone was obtained from Mann; dicyclohexylammonium \( L-\alpha\)-glycerophosphate, \( L-\alpha\)-glyceraldehyde, DL-3-chloro-1,2-propanediol, and DL-glyceraldehyde 3-phosphate were from Calbiochem; \( \alpha\)-glyceraldehyde, phosphoenolpyruvate, fructose 1,6-diphosphate, and ATP were obtained from Sigma; DL-1,2-propanediol, 1,3-propanediol, ethylene glycol, and D-mannitol came from Eastman; 2,3-butanediol were products of L. Light and Company; \( \alpha\)-thioglycerol was a K and K product; \( \epsilon\)-erythritol, ribitol, \( \beta\)-arabitol, and L-arabitol were from Pfannstiel Laboratories, Inc., Waukegan, Illinois; NAD, NADH, CTP, UTP, TTP, GTP, and ITP were Pabst products; and crystalline \( L-\alpha\)-glycerophosphate dehydrogenase, \( \alpha\)-glycerophosphate dehydrogenase, lactate dehydrogenase, pyruvate kinase, and aldolase were from C. F. Boehringer and Sons.

**Bacterial Strains**—Cells of *E. coli* K12, strain 7, were used as the source of the kinase because they produce this enzyme constitutively (3) up to 5% of the extractable protein.

**Growth of Cells**—A 50-liter culture was grown at 37° in a Biogen fermenter with vigorous aeration. The medium contained 2% casein acid hydrolysate, 0.7% \( K_2HPO_4 \), 0.2% \( KH_2PO_4 \), \( H_2O \), 0.1% (\( NH_4 \))_2SO_4, and 0.01% \( MgSO_4 \cdot 7H_2O \) at a pH of 7.0. Cells were harvested by a Sharples centrifuge after the culture had reached stationary phase.

**Enzyme Assays**—Four methods of assay for glycerol kinase activity were used, each for a different experimental purpose. All were carried out at 25°.

**Assay I** employed routinely to follow the purification of the enzyme, was essentially that of Wieland and Suyter (7). The phosphorylation of glycerol was coupled to the reduction of NAD by the action of \( L-\alpha\)-glycerophosphate dehydrogenase at pH 9.5. The reaction mixture contained the following: 30 \( \mu \)moles of glycerol, 60 \( \mu \)moles of ATP, 60 \( \mu \)moles of MgCl\(_2\), 4 \( \mu \)moles of NAD, 900 \( \mu \)moles of hydratase, 500 \( \mu \)moles of sodium carbonate, 0.3 mg of \( L-\alpha\)-glycerophosphate dehydrogenase from rabbit muscle, and glycerol kinase in a final volume of 3.0 ml. The \( L-\alpha\)-glycerophosphate dehydrogenase was omitted from the blank. NADH formation was followed at 340 

**Assay II** used to determine the pH optimum of the kinase, was carried out in two stages in the manner described by Dubilit and Kennedy (6). The phosphorylation of glycerol was allowed to proceed in a system consisting of the following: 10 \( \mu \)moles of glycerol, 20 \( \mu \)moles of ATP, 20 \( \mu \)moles of MgCl\(_2\), 50 \( \mu \)moles each of phosphate, Tris-HCl, and bicarbonate at a given pH, 2 mg of serum albumin, and 1 \( \mu \)g of crystalline glycerol kinase in a final volume of 1.0 ml. After an incubation period of 10 min, 1 ml of 10% trichloroacetic acid was added, and the mixture was
centrifuged. An aliquot of the supernatant fluid was neutralized with NaOH and incubated with 1 μmole of NAD, 0.06 mg of crystalline L-α-glycero-P dehydrogenase, and 600 μmoles of hydrazine at pH 9.5 in a final volume of 1.0 ml. The maximal formation of NADH was determined. The yield of NADH with a known amount of L-α-glycero-P was about 95% of the stoichiometric value.

Assay III, adapted for the study of substrate specificity of the kinase, was based on the stimulation of ADP release from ATP by the compound tested for acceptor activity. ADP appearance in turn was measured by enzymatic coupling to NADH oxidation. The complete reaction mixture included the following: 3 μmoles of the test compound, 3 μmoles of ATP, 30 μmoles of MgCl₂, 3 μmoles of phosphoenolpyruvate, 0.5 μmole of NADH, 0.2 mg of crystalline pyruvate kinase, 0.1 mg of crystalline lactate dehydrogenase, 100 μmoles of potassium phosphate at pH 7.5, and 0.1 to 5 μg of crystalline glyceraldehyde kinase in a final volume of 3.0 ml. Glycerol kinase was omitted from the blank. The reaction was monitored by absorbance at 340 nm. The relatively low concentration of ATP was employed in order to minimize the blank reaction due to the trace amount of ADP in the ATP preparation.

Assay IV was devised as a sensitive method for the measurement of the activation of the kinase for glyceraldehyde. The procedure, the details of which have already been published (9), required the use of 14C-labeled glyceraldehyde and the precipitation of the labeled product, L-α-glycero-P, as the lead salt subsequent to dilution details of which have already been published (2), required the use of 14C-labeled glyceraldehyde and the precipitation of the labeled product, L-α-glycero-P, as the lead salt subsequent to dilution.

**Determination of ADP**—ADP in a sample was measured enzymatically by Assay III with appropriate modification. Under the conditions employed, the reaction went to completion within 1 min, and known amounts of ADP caused the oxidation of the stoichiometric quantities of NADH.

**Determination of P<sub>i</sub>**—Orthophosphate was measured by the method of Chen, Toribata, and Warner (6). Inubation was carried out at 37° for 1 hour.

**Polyacrylamide Disc Gel Electrophoresis**—Electrophoresis in 7.5% polyacrylamide gel was performed by a modification of the method of Ornstein and Davis (10). All of the manipulations were conducted at room temperature unless otherwise specified. Glass columns (0.5 × 7 cm) were loaded with 1.6 ml of “lower gel” which was allowed to polymerize in the presence of ammonium persulfate for 15 min. Afterward, 0.25 ml of “upper gel” was introduced and allowed to polymerize for a similar period of time under the influence of fluorescent light. A mixture of 0.1 ml of enzyme sample and 0.1 ml of 50% sucrose was applied on the top of the gel column. After carefully overlaying the enzyme with 0.05 M Tris-glycine buffer at pH 8.8, the columns were installed in the electrophoresis apparatus with the tops connected to the upper compartment holding the same buffer with bromphenol blue as the tracking dye, and the bottoms were submerged in a solution of 0.05 M Tris-glycine at pH 8.3 contained in the lower compartment. Electrophoresis was performed at 4 °C with a current of 3 ma per tube for 2 hours at about which time bands of the tracking dye approached the bottom of the columns. The gels were then removed for analyses. To locate the glyceraldehyde kinase, the gel was put in a small test tube with 1 ml of a solution containing 10 μmoles of glyceraldehyde, 10 μmoles of ATP, 10 μmoles of MgCl₂, 1 μmole of NAD, 40 μmoles of Tris-ICl at pH 8.8, 150 μg of L-α-glycero-P dehydrogenase, 20 μg of phenazine methosulfate, and 800 μg of p-iodonitrotetrazolium violet. Protein was stained by immersing the gel in a solution of 0.25% Amido schwarz reagent in 7% acetic acid for 5 min followed by repeated washings with 7% acetic acid.

**Molecular Weight Determination**—Light scattering measurements were carried out with a RAO photometer at a wave length of 436 μm. Protein concentration ranged from 1.3 to 7.6 mg per ml. The molecular weight was obtained by extrapolating to zero protein concentration.

Equilibrium sedimentation was carried out in a Spinco model E analytical ultracentrifuge equipped with Rayleigh interference optics. The enzyme solution, 0.1 mg of protein in 0.2 ml of 0.01 M potassium phosphate at pH 7.0, was centrifuged in a double sector cell at 12,500 rpm at 20° for 40 hours. Interspin patterns were photographed with Kodak spectroscopic plates, type II-G. Measurements of the fringe patterns were made with a Nikon two-dimensional microcomparator. A specific volume of 0.74 was assumed in the calculation of the molecular weight (11).

**RESULTS**

**Enzyme Purification**

**Protection of Enzyme**—Throughout the process of purification, the enzyme was kept in the presence of 0.01 M glyceralde, 0.001 M EDTA, and 0.001 M β-mercaptoethanol to prevent inactivation (6). Except at the heating step, all the procedures were carried out at 2-4°.

**Preparation of Crude Extract**—About 0.4 kg of freshly harvested cells was suspended in 1 liter of 1% KCl and sonically treated, 50 ml at a time, in a Raytheon 10-kc sonic oscillator for 10 min. The preparation was centrifuged at 40,000 × g for 20 min, and the precipitate was discarded.

**Streptomycin Treatment**—The crude extract was adjusted to pH 6.0 with 0.1 N acetic acid, and 0.10 volume of 20% streptomycin sulfate solution was added slowly with efficient mixing. The turbid solution was kept overnight before the precipitate was removed by centrifugation.

**Heat Treatment**—The supernatant fraction from the above treatment was heated rapidly to 52° batchwise, kept at the temperature for 5 min, and quickly chilled in an ice bath. The precipitate was removed by centrifugation, and the floating material was eliminated by straining through glass wool.

**First Ammonium Sulfate Fractionation**—To the clarified solution, solid ammonium sulfate was added gradually to 60% saturation. After 1 hour of stirring, the precipitate, containing most of the enzyme, was spun down and dissolved in 50 ml of 0.01 M sodium acetate at pH 5.5.

**Dialysis**—The dissolved enzyme was dialyzed against 0.01 M sodium acetate, pH 5.5, with several changes of the buffer. The precipitate formed during the first 10 hours of dialysis was collected and then discarded after it was found to contain relatively little activity. The supernatant fraction was dialyzed again for another 20 hours, and the precipitate formed during this period was collected and dissolved in 0.1 M potassium phosphate at pH 6.0. The insoluble residue was removed by centrifugation.

**Second Ammonium Sulfate Fractionation**—Saturated ammonium sulfate solution was added dropwise to the enzyme solution until 40% saturation was reached. The precipitated protein was removed after 1 hour of equilibration. The ammonium sulfate saturation of the supernatant fraction was gradually raised to 40%. The precipitate was collected and dissolved in 0.02 M potassium phosphate at pH 7.5 and dialyzed against the same buffer for 18 hours.
**FIG. 1.** DEAE-cellulose chromatography of glycerol kinase. For conditions of elutions see the text. Fractions of 5 ml were collected. •—•, absorbance at 280 nm; ○—○, enzyme activity.

**Fig. 2.** Crystals of E. coli glycerol kinase. A, × 840; B, × 840; C, × 320.

**DEAE-cellulose Column Chromatography**—The dialyzed enzyme was applied on a DEAE-cellulose column (2 x 30 cm) which had been equilibrated with 0.02 M potassium phosphate at pH 7.5. Elution was carried out by increasing the concentration of KCl and decreasing the pH. A linear gradient apparatus was used: the mixing chamber initially was filled with 500 ml of 0.02 M potassium phosphate at pH 7.5, and the reservoir chamber with 500 ml of 0.5 M KCl in 0.02 M potassium phosphate at pH 6.0. As shown in Fig. 1, the bulk of the eluted protein was distributed under a single peak closely reflecting the distribution of glycerol kinase activity, which indicates a high degree of purity of the enzyme.

To concentrate the recovered enzyme, the active fractions were combined, diluted 5-fold with 0.02 M potassium phosphate at pH 7.5, and passed through a DEAE-cellulose column (2 x 15 cm) pre-equilibrated with the same buffer. The enzyme was eluted with a small volume of 0.4 M KCl in 0.02 M potassium phosphate at pH 6.0, precipitated by the addition of solid ammonium sulfate to 70% saturation, and redissolved in a small volume of 0.1 M potassium phosphate at pH 6.0.

**First Crystallization**—A saturated solution of ammonium sulfate was added very slowly to the enzyme solution over a period of several hours with constant stirring. When the mixture became slightly turbid, at about 40% saturation, the precipitate was removed by centrifugation. The supernatant fraction was kept close to 0°C for 5 days, during which period small amounts of saturated ammonium sulfate solution were added to promote crystallization. The precipitate, consisting of semicrystalline crystals and amorphous material, was collected and redissolved in 0.1 M potassium phosphate at pH 7.0.

**Second Crystallization**—The enzyme solution was adjusted to pH 7.0, and a saturated solution of ammonium sulfate was slowly added with constant mixing until turbidity began to develop. Semicrystalline crystals appeared at first (Fig. 2A). Two days later, however, rod crystals with rectangular cross-sections emerged and after 1 week became the predominant crystal type (Fig. 2B). Very little amorphous material could be detected.

In another run of purification, the crystals formed were chrysanthemum-like (Fig. 2C) instead of rectangular. The factors which determine the crystal morphology are still obscure, although less amorphous material seems to collect at pH 7 than at pH 6. However, it is pertinent to note that the specific enzyme activity did not vary with the form of the crystal.

The yields and the specific activities of the enzyme at various stages of purification are summarized in Table I.

**Homogeneity of Final Preparation**—The recrystallized glycerol kinase migrated as a single symmetrical peak during ultracentrifugation (Fig. 3) with a sedimentation constant of 8.9 S.

**Disc gel electrophoresis** revealed, however, five faint protein bands in addition to a major band. Zones containing the glycerol kinase activity coincided with the major band and also with one of the minor bands.

An immunochemical examination showed a single sharp band of precipitate in the agar of an Ouchterlony plate between a well charged with pure enzyme and a well loaded with a specific antiserum. There was, moreover, complete immunochemical identity between the crystallized enzyme and the enzyme in a crude extract (4).

**TABLE I**

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume</th>
<th>Protein</th>
<th>Enzyme</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>1220</td>
<td>44.3</td>
<td>141</td>
<td>3.2</td>
</tr>
<tr>
<td>Streptomycin supernatant</td>
<td>1350</td>
<td>32.0</td>
<td>133</td>
<td>4.2</td>
</tr>
<tr>
<td>Heat treatment</td>
<td>1600</td>
<td>19.4</td>
<td>112</td>
<td>5.8</td>
</tr>
<tr>
<td>Ammonium sulfate I</td>
<td>97</td>
<td>8.9</td>
<td>99</td>
<td>11.8</td>
</tr>
<tr>
<td>Precipitation by dialysis</td>
<td>68</td>
<td>2.5</td>
<td>69</td>
<td>28.4</td>
</tr>
<tr>
<td>Ammonium sulfate II</td>
<td>47</td>
<td>1.2</td>
<td>55</td>
<td>47</td>
</tr>
<tr>
<td>DEAE eluate</td>
<td>105</td>
<td>0.56</td>
<td>44</td>
<td>79</td>
</tr>
<tr>
<td>First crystallization</td>
<td>26</td>
<td>0.46</td>
<td>44</td>
<td>94</td>
</tr>
<tr>
<td>Second crystallization</td>
<td>26</td>
<td>0.35</td>
<td>35</td>
<td>100</td>
</tr>
</tbody>
</table>
ATP. Yet n-glyceraldehyde 3-phosphate was not detectable as a product by the use of a coupled system consisting of n-glyceraldehyde dehydrogenase, NAD, and arsenate. If to a system aldolase and fructose 1,6-diphosphate were supplemented, instant reduction of NAD occurred; thus there was no interference in the detection system. Together these observations compel the conclusion that n-glyceraldehyde participates in a catalytic manner in the enzymatic cleavage of ATP to ADP and Pi. The possibility that this n-glyceraldehyde-dependent adenosine triphosphatase activity was due to a contaminating enzyme was excluded by the suppression of the reaction in the presence of glycerol.

The reaction of L-glyceraldehyde was not investigated further because there was nothing unusual about the stoichiometry and the lack of convenient method for the demonstration of L-glyceraldehyde 3-phosphate. The phosphorylation of dihydroxyacetone by the kinase was apparently straightforward since the expected amount of dihydroxyacetone phosphate was revealed by

![Image of ultracentrifugal analysis of glycerol kinase.](image)

**Fig. 3.** Ultracentrifugal analysis of glycerol kinase. The sample contained 10 mg of protein per ml of 0.01 m potassium phosphate at pH 6.0 with glycerol, EDTA, and d-mercaptoethanol as protectors. The centrifugation was carried out at 59,780 rpm at 20° in a Spinco model E ultracentrifuge. Pictures were taken at 16, 32, 48, and 64 min. The boundary moved from left to right.

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### Properties of Enzyme

**Stoichiometry**—When crystallized glycerol kinase (0.2 unit per ml), $3.3 \times 10^{-4} \text{M}$ glycerol, $6.6 \times 10^{-3} \text{M}$ ATP, and $10^{-2} \text{M}$ MgCl$_2$ were incubated in 0.05 m potassium phosphate at pH 7.5 until the reaction approached completion, 93% of the input glycerol was accounted for as L-α-glycero-P (modification of Assay II). The amount of ADP released was equivalent to 94% of the glycerol input. Thus the E. coli enzyme catalyzes the phosphorylation of glycerol also in a highly stereospecific manner.

**Michaelis Constants**—The $K_m$ of the enzyme for glycerol was $1.3 \times 10^{-4} \text{M}$ as determined by the lead precipitate method (2). The apparent $K_m$ for ATP was $4 \times 10^{-4} \text{M}$ when measured in the presence of $10^{-2} \text{M}$ MgCl$_2$. In Table II, these $K_m$ values are compared with those of the glycerol kinases from pigeon liver (7) and C. mycoderma (8). The E. coli enzyme has the lowest $K_m$ for glycerol and the highest for ATP.

**Substrate Specificity**—The ability of the E. coli kinase to catalyze the phosphorylation of compounds structurally similar to glycerol was assayed by the release of ADP from ATP (Assay III). Among the compounds tested only dihydroxyacetone, L-glyceraldehyde, and n-glyceraldehyde were active, but their Michaelis constants were much higher than that for glycerol (Table III).

In the course of these studies it was noticed that n-glyceraldehyde, unlike the other substrates, caused a continuous release of ADP beyond the extent explicable on a stoichiometric basis. To check this finding, another experiment was carried out in which the final yields of both ADP and Pi were compared in reaction mixtures with the test substrate in limiting concentration and ATP in excess. The results presented in Table IV show that, in contrast to glycerol and L-glyceraldehyde which caused the formation of an equivalent amount of ADP with little liberation of Pi, n-glyceraldehyde caused the appearance of more than 9 eq of ADP with little liberation of Pi, i.e., virtually complete hydrolysis of the input ATP. Yet n-glyceraldehyde 3-phosphate was not detectable as a product by the use of a coupled system consisting of n-glyceraldehyde 3-phosphate dehydrogenase, NAD, and arsenate. If to such a system aldolase and fructose 1,6-diphosphate were supplemented, instant reduction of NAD occurred; thus there was no interference in the detection system. Together these observations compel the conclusion that n-glyceraldehyde participates in a catalytic manner in the enzymatic cleavage of ATP to ADP and Pi. The possibility that this n-glyceraldehyde-dependent adenosine triphosphatase activity was due to a contaminating enzyme was excluded by the suppression of the reaction in the presence of glycerol.

The reaction of L-glyceraldehyde was not investigated further because there was nothing unusual about the stoichiometry and the lack of convenient method for the demonstration of L-glyceraldehyde 3-phosphate. The phosphorylation of dihydroxyacetone by the kinase was apparently straightforward since the expected amount of dihydroxyacetone phosphate was revealed by

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**TABLE II**

Comparison of $K_m$ values of glycerol kinases from three different biological origins

<table>
<thead>
<tr>
<th>Parameter</th>
<th>E. coli</th>
<th>C. mycoderma</th>
<th>Pigeon liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_m$ for glycerol</td>
<td>$1.3 \times 10^{-4}$</td>
<td>$6 \times 10^{-5}$</td>
<td>$4 \times 10^{-4}$</td>
</tr>
<tr>
<td>$K_m$ for ATP</td>
<td>$4 \times 10^{-3}$</td>
<td>$9 \times 10^{-3}$</td>
<td>$2.8 \times 10^{-3}$</td>
</tr>
</tbody>
</table>

* These values were taken from Bergmeyer et al. (8).
* These values were taken from Wieland and Suyter (7).

**TABLE III**

Values of $V_{max}$ and $K_m$ for various substrates

All the measurements were made with Assay III, except for the determination of $K_m$ for glycerol in which case Assay IV was used.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$V_{max}$</th>
<th>$K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol</td>
<td>100</td>
<td>$1.3 \times 10^{-4}$</td>
</tr>
<tr>
<td>Dihydroxyacetone</td>
<td>187</td>
<td>$5 \times 10^{-4}$</td>
</tr>
<tr>
<td>L-Glyceraldehyde</td>
<td>81</td>
<td>$3 \times 10^{-3}$</td>
</tr>
<tr>
<td>n-Glyceraldehyde</td>
<td>35</td>
<td>$5 \times 10^{-4}$</td>
</tr>
</tbody>
</table>

**TABLE IV**

A stoichiometric study of relationship between amount of substrate added and amounts of ADP and Pi released

Each reaction mixture initially contained the following: 100 pmol of the test substrate, 1 μmol of ATP, 5 μmol of MgCl$_2$, 10 μmol of Tris-HCl at pH 8.0, and 250 μg of glycerol kinase in a final volume of 0.5 ml. After 30 min of incubation at 37°, the reaction was stopped by the addition of 0.5 ml of 10% trichloroacetic acid, and the precipitate was removed by centrifugation. The supernatant fraction was neutralized with NaOH, adjusted to a volume of 2.0 ml, and analyzed for ADP and Pi as described under "Experimental Procedure." Corrections were made for a blank reaction in which no test substrate was added.

<table>
<thead>
<tr>
<th>Substrate tested</th>
<th>ADP released</th>
<th>Pi released</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol</td>
<td>1.2</td>
<td>0.2</td>
</tr>
<tr>
<td>L-Glyceraldehyde</td>
<td>0.9</td>
<td>0.3</td>
</tr>
<tr>
<td>n-Glyceraldehyde</td>
<td>9.1</td>
<td>9.7</td>
</tr>
</tbody>
</table>
indicated. Enzyme activities were assayed after 1 day (O-O) and 4 days (O- - - O).

The following compounds were inactive when tested at $10^{-4}$ M as substrate: \( \text{D-1,2-propanediol, 1,3-propanediol, D-3-chloro-1,2-propanediol, 2,3-butanediol, } \alpha\text{-thioglycerol, ethylene glycol, f-erythritol, n-arabitol, l-arabitol, ribitol, mannitol, and glucose.} \)

**Glycerol Kinase of E. coli**

The following compounds were inactive when tested at $10^{-4}$ M as substrate: \( \text{d-1,2-propanediol, 1,3-propanediol, D-3-chloro-1,2-propanediol, 2,3-butanediol, } \alpha\text{-thioglycerol, ethylene glycol, f-erythritol, n-arabitol, l-arabitol, ribitol, mannitol, and glucose.} \)

**Nucleoside Triphosphate and Divalent Cation Specificity**—No formation of \( \text{n-glycer-1-P} \) was detected by Assay I when ATP was replaced by any of the other nucleoside triphosphates, such as GTP, CTP, UTP, ITP, and TTP, at $10^{-3}$ M.

No glycerol-phosphorylating activity could be shown in the absence of any added divalent cation. The apparent \( K_m \) for Mg\(^{++} \) was $3 \times 10^{-3}$ M when determined in the presence of $2 \times 10^{-2}$ M ATP. At $10^{-2}$ M, Mn\(^{++} \) gave a rate of reaction which was only 30% as high as that observed with Mg\(^{++} \). Higher concentration of Mn\(^{++} \) could not be tested because it caused precipitation of ATP.

**Specific Inhibition**—Since it is well known that hexokinases from animal tissues can be noncompetitively inhibited by glucose 6-phosphate (12), the glycerol kinase was also tested for inhibition by the immediate phosphorylated product with the rate of release of ADP as an assay. In the presence of $10^{-4}$ M glycerol, no effect by L-\( \alpha \)-glycero-P was found even when it was added up to $3 \times 10^{-3}$ M. This lack of inhibition by the immediate product could have been deduced from the finding that cells possessing glycerol kinase but lacking L-\( \alpha \)-glycero-P dehydrogenase were subject to toxic accumulation of L-\( \alpha \)-glycero-P when exposed to glycerol (13).

A recent study on the control of glycerol utilization by glucose, however, revealed remote product inhibition of glycerol kinase. The effect was identified as fructose 1,6-diphosphate. A description of the nature of this inhibition and its physiological implications has been published (14).

**pH Optimum**—The enzyme reproducibly exhibited a peculiar pH activity relationship as depicted in Fig. 4. The activity rises sharply with pH in the region of neutrality, levels off in the region of 7.3 to 9.3, and rises again with a peak at 9.8 (Assay II).

**Stability**—When kept in 0.1 M potassium phosphate at very low protein concentration, the enzyme was found to lose activity rapidly. Even in the range of pH 6 to 7, within which the decay was slowest, the half-life of the active enzyme was only about 24 hours at 0°C (Fig. 5). Like the kinases from the other sources (6-8), the E. coli enzyme could be stabilized markedly by glycerol. Further protection could be provided by the presence of 0.001 M EDTA and 0.001 M B-mercaptoethanol. When kept at close to 0°C in crystalline form under a half-saturated ammonium sulfate solution containing the protectors, no appreciable loss of activity occurred during a period of 2 years.

**Molecular Weight**—By the light scattering method the molecular weight of the enzyme was found to be $2.8 \times 10^5$. The value calculated on the basis of sedimentation equilibrium was $8.5 \times 10^5$.

**DISCUSSION**

The significance of the low \( K_m \) of the E. coli enzyme for glycerol has been examined in connection with the role of this enzyme in capturing the substrate from the environment (2). Such a high apparent affinity is expected to require considerable complementarity between the active surface of the enzyme and the substrate. In view of this requirement and the fact that the E. coli enzyme, similar to its counterparts in other species (6,15), acts on glycerol stereospecifically to yield \( \alpha \)-glycero-P, it is surprising to find \( \alpha \)-glyceraldehyde not only reactive, but actually displaying a \( K_m \) lower than that for \( \alpha \)-glyceraldehyde.

This stereochemical paradox and the continuous hydrolysis of ATP catalyzed by the kinase in the presence of limited \( \alpha \)-glyceraldehyde might be explained if the latter attaches to the enzyme with the carbonyl end instead of the primary hydroxyl end directed toward the reactive center. Since aldehyde groups undergo ready hydration (16), the nucleophilic attack on the terminal phosphorol group of ATP may be exerted by one of the gem diol groups (see Scheme I). Such a phosphorylation is likely to be abortive, leading to the liberation of P\(_i\) with the regeneration of the free triose.
In a previous study, Bublitz and Kennedy observed that glycerol kinase from rat liver catalyzed the liberation of hydrogen ions in the presence of \( \beta \)-glyceraldehyde and ATP (6). Since \( \alpha \)-glyceraldehyde 3-phosphate was not detected as a product, it was suggested that only \( \alpha \)-glyceraldehyde served as a substrate. However, if \( \alpha \)-glyceraldehyde served catalytically for the hydrolysis of ATP in this system, it would have escaped notice.

With respect to the phosphoryl group donor, the \( E. \ coli \) enzyme exhibited a high degree of specificity. The stringent requirement for ATP is in marked contrast to the rat liver enzyme which is active with UTP as well as ATP (6) and to the \( C. \ mycoderma \) enzyme which is active with ATP, UTP, GTP, and ITP (8). Whether there is a functional basis for the fastidious characteristic of the \( E. \ coli \) enzyme has yet to be solved.

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