Catalytic and Allosteric Properties of Glycerol Kinase from Escherichia coli*

(Received for publication, October 30, 1972)

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SUMMARY

Catalytic and allosteric properties of the normal and of a genetically altered glycerol kinase from Escherichia coli were examined. Normal glycerol kinase exhibited Michaelis-Menten kinetics for glycerol with an apparent $K_m$ of 10 $\mu$M. Gel filtration studies indicated that, at saturation, 3.7 moles of [14C]glycerol bound per mole of enzyme, and a dissociation constant of 10 $\mu$M was determined by ultrafiltration. The response of reaction velocity to MgATP concentration was not hyperbolic, and double reciprocal plots displayed downward curvature, indicating two apparent $K_m$ values of 80 to 100 $\mu$M and 400 to 500 $\mu$M. The unusual MgATP kinetic data were obtained by two different assay procedures, were observed throughout the course of purification, were the same for different enzyme preparations, and were unchanged by aging of the enzyme. Product inhibition studies suggested an ordered mechanism for the enzyme, with glycerol as the first substrate to bind.

Fructose 1,6-bisphosphate was a noncompetitive inhibitor with respect to both substrates. The response of reaction velocity to fructose 1,6-bisphosphate concentration was slightly sigmoid, maximum inhibition was about 80%, and double reciprocal plots of fractional inhibition against inhibitor concentration indicated an apparent $K_i$ of 0.5 $\mu$M. Gel filtration studies showed that, at saturation, 3.5 moles of [14C]fructose 1,6-bisphosphate bound per mole of enzyme, with a dissociation constant of 0.06 $\mu$M determined by ultrafiltration. Inhibition by fructose 1,6-bisphosphate was greatly reduced by high pH, high ionic strength, or 0.2 M guanidine hydrochloride, some of which also stimulated enzyme activity. Under such conditions, [14C]fructose 1,6-bisphosphate still bound to the enzyme.

A genetically desensitized glycerol kinase was purified to apparent homogeneity. It had a specific activity twice that of the normal enzyme, but was identical in size, charge, and subunit composition. Although the mutant enzyme still bound [14C]fructose 1,6-bisphosphate, it was totally insensitive to inhibition. The mutant enzyme also displayed a non-hyperbolic response to MgATP.

Both the normal and mutant glycerol kinases were markedly stabilized against heat inactivation by glycerol, but not by fructose 1,6-bisphosphate. In the absence of specific ligands, about 12 sulfhydryl groups per molecule of enzyme reacted with 5,5'-dithiobis(nitro-2-benzoic acid). In the presence of glycerol, only two sulfhydryl groups per molecule reacted with the reagent, while fructose 1,6-bisphosphate had no effect.

These results are discussed in terms of the subunit structure of the enzyme and in terms of the mechanism of fructose 1,6-bisphosphate inhibition of glycerol kinase.

We recently obtained crystalline glycerol kinase (ATP:glycerol phosphotransferase, EC 2.7.1.30) from Escherichia coli by a new purification procedure and demonstrated that the enzyme is an oligomeric protein composed of four identical subunits (1). Glycerol kinase is subject to inhibition by fructose bisphosphate that is reflected in an altered rate of catalysis rather than in an altered substrate affinity (2). Thus, glycerol kinase belongs to the little studied group of allosteric enzymes that have been termed "V" systems (3), and its catalytic and allosteric properties are therefore of considerable interest. In this paper, we present kinetic and chemical studies of the interaction of glycerol kinase with its substrates and products and with the inhibitor fructose bisphosphate. We also report the purification and properties of a genetically desensitized glycerol kinase, and describe the factors which reduce or eliminate fructose bisphosphate inhibition of the normal enzyme.

EXPERIMENTAL PROCEDURE

Materials

Radiochemicals were obtained from the following sources: [1,3-14C]glycerol, [2-14C]glycerol, and D-[1-14H]glucose from New England Nuclear; L-[6-3H] isoleucine from Schwarz-Mann; and [U-14C]fructose bisphosphate from Amersham Searle. [1,3-14C]Glycerol was treated before use with microgranular DEAE-cellulose (Whatman DE-52) to remove anionic impurities.
Reagent chemicals were obtained from the following sources: ATP from Schwarz-Mann; ADP from Pabst Laboratories; VIII. ECTEOLA-cellulose eluant. Other reagents were obtained from sources already described; p-hydroxymercuriphenyl sulfonic acid from Sigma; Omnitol (phenylmethanesulfonic acid) from New England Nuclear; and ECTEOLA-cellulose (epichlorohydrin triethanolamine cellulose, Cellex E) from Bio-Rad. Other reagents were obtained from sources already described (1).

Bacterial Strains and Growth Conditions

The following strains of *E. coli* K-12, all of which produce glycerol kinase constitutively, were obtained from Dr. Edmund C. C. Lin: strain 72, which produces the normal enzyme (4); and strains 43, 48, and 244, which produce glycerol kinase insensitive to fructose bisphosphate inhibition (2, 5, 6). All strains were grown as described previously (1) in a medium containing, in grams per liter: Difco casein acid hydrolysate, 20; K2HPO4, 7; KH2PO4, 1.8; (NH4)2SO4, 1; and MgSO4, 0.5; at about pH 7.0.1

### Enzyme Preparations

Crystalline glycerol kinase, purified from strain 72 as described previously (1), was used for all experiments unless otherwise stated.

A genetically desensitized glycerol kinase was purified from strain 244 by the procedure summarized in Table I. This was similar to that used for the purification of the normal enzyme (1), except for the following modifications. For precipitation of nucleic acids, only 0.1 g of Ethodin per g of protein in the crude extract was used. After removal of nucleic acids, the material which precipitated between 30 and 35 g of solid ammonium sulfate per 100 ml of the supernatant solution was used in the next purification step. After elution from Sephadex G-200 (Fraction VI), the preparation was only about 90% pure, still exhibiting two additional minor bands upon electrophoresis in standard polyacrylamide gels. To remove these contaminants, the material was further purified by chromatography on ECTEOLA-cellulose. Fraction VII in 700 ml of standard buffer (10 mM glycerol, 50 mM triethanolamine hydrochloride, 1 mM EDTA, 1 mM 2-mercaptoethanol, pH 7.0) containing 0.01 M KCl was applied over the course of 24 hours to a column (340 ml) of ECTEOLA-cellulose (0.38 meq per g). The column was washed with 100 ml of standard buffer containing 0.01 M KCl, and the protein eluted with a linear gradient (2.5 liters) from 0.01 to 0.15 M KCl in standard buffer. Enzyme activity emerged concomitant with the major protein peak at about 0.09 M KCl. The peak fractions were pooled and concentrated to a volume of about 40 ml by ultrafiltration through a PM-10 Diaflo membrane (Amicon Corp.). The concentrated solution was clarified by centrifugation for 20 min at 12,000 × g and stored at 0º. The total purification achieved was over 30-fold with a yield of 11%.

### Enzyme Assays

**Assay I**—In this procedure, glycerol kinase activity was measured as glycerol-dependent conversion of [γ-32P]ATP to an acid-stable phosphate ester, as already described (1). Under conditions where less than 20% of the initial ATP was consumed, the product formed was a linear function of the amount of enzyme added and of time.

**Assay II**—Enzymic activity was measured by adsorption of the [3H]glycerol-3-P formed from radioactive glycerol and ATP to disks of DEAE-cellulose filter paper (7). An appropriate amount of glycerol kinase in standard buffer lacking glycerol but with 0.1 mg per ml of gelatin was added to a mixture containing [1,3-3H]glycerol (20 nmoles, ~12,000 cpm per nmole), MgCl2 (1 μmol), gelatin (0.5 mg), and triethanolamine hydrochloride, pH 7.0 (25 μmoles). The reaction was initiated by the addition to this mixture of a solution containing ATP (0.5 μmol), which gave a final volume of 0.5 ml. The tubes were incubated for 10 min at 25º in a water bath. The reaction was terminated by chilling and immediate addition of ice-cold 2 M glycerol (0.5 ml). Samples (0.05 ml) from each tube were spotted on disks (2.3 cm diameter) of DEAE-cellulose filter paper (Whatman DE-81). After 1 min, the papers were immersed in about 500 ml of 0.1 M glycerol and washed for about 30 min with two more changes of 0.1 M glycerol. Essentially all the [3H]glycerol-3-P was retained by the filters, whereas the excess radioactive glycerol was removed. The disks were placed in vials containing 0.1 N HCl (1 ml), and Bray's scintillation fluid (10 ml) (8) was added for scintillation counting in a Packard Tri-Carb scintillation counter. The experimental values were corrected by subtracting blank values, less than 5 pmoles, obtained from incubations without enzyme. Retention of [3H]glycerol-3-P was unaffected by concentrations of ATP from 1 to 10 mM at either 1 or 10 mM Mg2+. Under conditions where less than 20% of the initial glycerol was consumed, the assay was linear with respect to the amount of enzyme added and with respect to time.

### Protein Determinations

Protein was estimated by the biuret method (9) or by a micromodification of this method (10). In some experiments, enzyme concentration was determined spectrophotometrically. At a path length of 1 cm, glycerol kinase (1 mg per ml) had an absorbance at 280 nm of 1.4. This value was obtained by comparing the absorbances of a solution of glycerol kinase in a buffer containing 0.1 mM diethiothreitol, 0.1 mM EDTA, and 10 mM potassium phosphate, pH 7.5, with the dry weight of samples of the enzyme solution and of the buffer alone.

### Electrophoresis in Polyacrylamide Gels

Electrophoresis in standard and SDS-polyacrylamide gels was performed as described previously (1).

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1 The composition of the medium was given incorrectly in Ref. 1.

2 The abbreviations used are: SDS, sodium dodecyl sulfate; DTNB (Ellman reagent), 5,5'-dithiobis(2-nitrobenzoic acid).
**Sulphydryl Determinations**

Glycerol kinase was prepared for sulphydryl group titration as previously described (1). Samples (0.05 ml, 0.211 mg of protein) were added to cuvettes containing either 20 μl of buffer (1 mM EDTA, 50 mM triethanolamine hydrochloride, pH 7.0), or 20 μl of 50 mM glycerol, or 20 μl of 100 mM fructose bisphosphate. The sulphydryl groups were titrated immediately by the addition of 20 μl of 10 mM DTT in 50 mM potassium phosphate, pH 7.5, and the reaction was monitored with a Unicam SP.800A spectrophotometer over the course of 2 hours. Then 10 μl of aqueous 10% SDS were added and the reaction followed for another 40 min. The number of sulphydryl groups that had reacted was calculated using a molar absorptivity of 13,600 M⁻¹ cm⁻¹ for the p-thionitrobenzoic acid produced in the reaction (11).

**Ligand-binding Measurements**

**Ultrafiltration Method**—Small disks (7.5 mm diameter) of PM-10 Diaflo membrane were exhaustively washed with water shortly before use to remove glycerol. For glycerol-binding studies, a freshly prepared solution of crystalline enzyme in 0.1 mM glycerol, 1 mM MgCl₂, 1 mM 2-mercaptoethanol, and 50 mM triethanolamine hydrochloride, pH 7.0, was dialyzed overnight against several changes of the same buffer lacking glycerol. Samples (10 μl) of the enzyme solution (∼10 mg per ml) were added to mixtures (0.49 ml) containing [1,3-¹³C]glycerol (2.5 nmole; ∼10,000 cpm per nmole), MgCl₂ (0.3 μmole), 2-mercaptoethanol (0.3 μmole), triethanolamine hydrochloride (pH 7.0); 15 μmole), and varying amounts of unlabeled glycerol. The tubes were incubated for 10 min at room temperature (23°), and the solutions were then subjected to filtration on the ultrafiltration apparatus of Paulus (12). After filtration, the membranes were transferred to vials containing 1 ml of H₂O, and Bray’s scintillation fluid (10 ml) was added for scintillation counting. Blank values were determined by filtering samples without protein, to correct for the small volume of solution (3 to 5 μl) retained by the Diaflo membranes. In the absence of the enzyme, no adsorption of [¹³C]glycerol to the PM-10 membranes was observed over the range of glycerol concentrations used (5 to 49 μM). To reveal other possible artifacts in the use of the ultrafiltration technique for measuring glycerol binding to the enzyme, additional control experiments were performed. The concentration of glycerol kinase in solution was found to remain essentially constant during the course of filtration, suggesting that the protein was effectively deposited on the filter. Furthermore, the amount of [¹³C]glycerol bound was a linear function of the enzyme concentration.

For fructose bisphosphate-binding experiments, crystalline glycerol kinase was redissolved in standard buffer containing 40% ethylene glycol and stored at −15°. A sample of this stock solution (11.8 mg per ml) was diluted into the same buffer and samples (40 μl) of the enzyme solution (0.74 mg per ml) were added to mixtures (0.56 ml) containing [U-¹⁴C]fructose bisphosphate (12 nmoles; ∼1000 cpm per nmole), glycerol (0.6 μmole), 2-mercaptoethanol (0.6 μmole), potassium phosphate (pH 7.2; 30 μmole), n-[¹-³H]glucose (12 nmoles; ∼1000 cpm per nmole), and varying amounts of unlabeled fructose bisphosphate. A portion (0.5 ml) of each mixture was transferred to the channels of the ultrafiltration apparatus, and filtration was conducted as described above. For scintillation counting, the membranes were placed in vials containing 1 ml of dimethylformamide to dissolve the membranes; then 5 ml of a toluene scintillation fluid (4 g of Omnifluor per liter) were added. Blank values were determined by filtering samples without protein. The [³H]-glucose present permitted accurate correction for the small volume of solution retained by the disks. In the absence of the enzyme, no adsorption of [¹⁴C]fructose bisphosphate to the PM-10 membranes was observed. Furthermore, at low protein concentrations (5 to 20 μg), the amount of [¹⁴C]fructose bisphosphate bound was a linear function of enzyme concentration.

**Gel Filtration Method**—To measure fructose bisphosphate binding by the method of Hummel and Dreyer (13), a Plexiglas column (0.3 × 45 cm) of Sephadex G-25 (20 to 80 μl) was equilibrated at room temperature with 0.1 mM [U-¹⁴C]fructose bisphosphate (∼100 cpm per nmole), 1 mM L-[¹-³H]isoleucine (∼400 cpm per nmole), 1 mM glycerol, 1 mM 2-mercaptoethanol, and 50 mM potassium phosphate, pH 7.2. A portion (50 μl) of the stock solution of glycerol kinase (11.8 mg per ml) was applied to the column, which was then eluted with the equilibration buffer. Fractions of 0.086 ml were collected. A sample (50 μl) of each fraction was transferred to a scintillation vial containing 1 ml of H₂O, and Bray’s scintillation fluid (10 ml) was added for scintillation counting. The [³H]isoleucine present allowed accurate determination of fraction size. Glycerol kinase activity in the fractions was measured by Assay I.

Glycerol binding was measured by a similar procedure, except that the column used was slightly longer (0.3 × 60 cm) and the equilibration and elution buffer contained 50 μM [2-¹⁴C]glycerol (∼100 cpm per nmole), 1 mM L-[¹-³H]isoleucine (∼400 cpm per nmole), 1 mM ADP, 2 mM MgCl₂, 1 mM 2-mercaptoethanol, and 50 mM potassium phosphate, pH 7.2.

**RESULTS**

**Catalytic Properties**

**Substrate Kinetics**—Glycerol saturation curves were hyperbolic, yielding linear double reciprocal plots (Fig. 1). The apparent Kₘ for glycerol of 10 μM was independent of the MgATP concentration over a 5-fold range. At pH 9.5 and at 0°, the apparent Kₘ for glycerol increased to 50 and 25 μM, respectively (not shown). Ultrafiltration studies indicated that glycerol bound to glycerol kinase with a dissociation constant of about 10 μM, the same as the value of its Kₘ, and that the enzyme was almost saturated at 40 μM glycerol (Fig. 2, inset). The maximum amount of [¹⁴C]glycerol bound varied from 1 to 2 moles of glycerol per mole of enzyme. These low and variable values were probably due to inactivation of glycerol kinase during dialysis prior to ultrafiltration, since the enzyme is labile in the absence of glycerol. To avoid this problem, the gel filtration method of Hummel and Dreyer (13) was used which did not require the prior removal of glycerol from the enzyme. It was found by this method that at 50 μM glycerol, 3.7 moles of [¹⁴C]glycerol were bound per mole of enzyme (Fig. 2).

The response of reaction velocity to ATP concentration at different Mg²⁺ concentrations, and the response of reaction velocity to Mg²⁺ concentration at different ATP concentrations, indicated MgATP to be the substrate of the enzyme (not shown). High concentrations of free ATP or of free Mg²⁺ were only slightly inhibitory. To ensure that all ATP was present as the MgATP complex and that the concentration of free Mg²⁺ was essentially constant, all subsequent assays employed a concentration of Mg²⁺ that was 1 mM greater than the total nucleotide concentration (19). Under these conditions, the relationship of reaction velocity to MgATP concentration, even at saturating glycerol, was unusual in that double reciprocal plots were hyper-
Fig. 1. Relationship between reaction velocity and glycerol concentration. Assay II was performed at 0.2 mM (X), 0.8 mM (■), and 1.0 mM (○) MgATP with 8, 2, and 1.2 ng of glycerol kinase, respectively.

Fig. 2. Binding of [14C]glycerol to glycerol kinase. Binding of [14C]glycerol to glycerol kinase (0.59 mg) was measured by gel filtration as described under "Experimental Procedure." Fractions of 0.078 ml were collected. (—) [14C]glycerol bound, (— - - -) enzyme activity. Inset, binding of [14C]glycerol to 61 μg (■) and to 100 μg (○) of glycerol kinase was measured by ultrafiltration, as described under "Experimental Procedure."

Fig. 3. Relationship between reaction velocity and MgATP concentration. The activity of glycerol kinase at varying concentrations of MgATP was measured by Assay II at 30 μM [14C]-glycerol and 2 μg of enzyme (○), or by Assay I at 1 mM glycerol and 400 ng of enzyme (○).

Fig. 4. Effect of ADP on the relationship between reaction velocity and MgATP concentration. A, Assay I was performed with 0.2 μg of glycerol kinase at varying concentrations of MgATP in the absence (○) and in the presence (○) of 0.5 mM ADP. B, the activity of glycerol kinase (0.2 μg) was measured by Assay I at 0.2 mM (○), 0.4 mM (▲), 0.6 mM (■), and 0.8 mM (○) MgATP at different concentrations of ADP. The amount of Mg2+ added exceeded the total nucleotide concentration by 1 mM.

Glycerol kinase, with limiting slopes that suggested two apparent $K_m$ values for MgATP, one in the range 80 to 100 μM and the other between 400 to 500 μM (Fig. 3). As shown, the unusual kinetic data did not depend on the assay procedure or enzyme concentration. The "biphasic" behavior was observed throughout the course of purification, was the same for two different crystalline preparations, and was not changed by aging of the enzyme. In fact, a commercial preparation of E. coli glycerol kinase (Calbiochem), that was purified by a different procedure and still contained several contaminants, displayed the same type of MgATP kinetics as our homogeneous preparations. Glycerol kinase that was 50% inactivated with p-hydroxymercuriphenyl sulfonate still exhibited hyperbolic double reciprocal plots. At pH 9.5, in 0.4 M KCl, or in 0.2 M guanidine hydrochloride, this behavior was less pronounced but not eliminated (not shown).

Product Inhibition—The effect of ADP on the response of reaction velocity to MgATP concentration was rather complex, especially at low concentrations of MgATP (Fig. 4).
higher concentrations of MgATP, the inhibition appeared to be mainly competitive with a $K_i$ of 0.5 mM (Fig. 4B). ADP was clearly a noncompetitive inhibitor with respect to glycerol at limiting MgATP concentration (Fig. 5).

Glycerol-3-P was not inhibitory when glycerol was saturating. At limiting glycerol concentrations, glycerol-3-P was a competitive inhibitor with respect to glycerol with a $K_i$ of about 2 mM (Fig. 6).

**Allosteric Properties**

**Inhibition by Fructose Bisphosphate**—Fructose bisphosphate was a noncompetitive inhibitor with respect to both substrates (Fig. 7). The response of reaction velocity to fructose bisphos-

![Fig. 5. Effect of ADP on the relationship between reaction velocity and glycerol concentration. The activity of glycerol kinase (0.1 µg) was measured at 0.2 mM MgATP by Assay I in the presence of varying concentrations of ADP at 50 µM (△) and 1 mM (●) glycerol. The amount of Mg$^{2+}$ added exceeded the total nucleotide concentration by 1 mM.](http://www.jbc.org/)

![Fig. 6. Effect of glycerol-3-P on the relationship between reaction velocity and glycerol concentration. The activity of glycerol kinase (1.2 ng) was measured by Assay II in the absence (●) and in the presence (△) of 1 mM glycerol-3-P or in the presence of 1 mM glycerol-2-P (○).](http://www.jbc.org/)

![Fig. 7. Effect of fructose bisphosphate on the activity of glycerol kinase. A, the activity of glycerol kinase (1.2 ng) was measured by Assay II in the absence (○) and in the presence (●) of 1 mM fructose bisphosphate. B, the data from A were replotted, with $v_o$ and $v_i$ representing the enzyme activities measured in the absence and the presence of fructose bisphosphate, respectively.](http://www.jbc.org/)

![Fig. 8. Relationship between fructose bisphosphate (FDP) concentration and reaction velocity. A, the activity of glycerol kinase (0.3 µg) was measured at varying concentrations of fructose bisphosphate by Assay I as described under "Experimental Procedure" except that Mg$^{2+}$ was present in 1 mM excess over the combined concentrations of ATP and fructose bisphosphate. B, the data from A were replotted, with $v_o$ and $v_i$ representing the enzyme activities measured in the absence and the presence of fructose bisphosphate, respectively.](http://www.jbc.org/)
for fructose bisphosphate of about 0.5 mM (Fig. 8B). Electrophoresis in polyacrylamide gels, containing 1 mM fructose bisphosphate, of glycerol kinase that had been incubated with 1 mM fructose bisphosphate for 1 hour at 4° showed that no aggregation or dissociation had occurred. Of numerous intermediary metabolites tested for possible effects on glycerol kinase, only fructose bisphosphate was inhibitory (14). Moreover, no multivalent effects were found when adenosine ribonucleotides, pyridine nucleotides, or other compounds were present together with fructose bisphosphate (14).

Desensitization—It was found that inhibition of glycerol kinase by fructose bisphosphate could be greatly reduced by manipulation of the ionic conditions. As shown in Fig. 9, the activity of glycerol kinase was about half-maximal at pH 7.0, where inhibition by fructose bisphosphate was greatest. As the pH was increased, an inflection occurred in the activity profile, accompanied by a decline in sensitivity to inhibition, which was reduced to one-third at the optimum, pH 9.5. If the enzyme was exposed to pH 9.5 and reassayed at pH 7.0, inhibition by 1 mM fructose bisphosphate was reduced from 55 to 30% and remained low for at least 30 min after the enzyme was returned to neutral pH. In contrast, if fructose bisphosphate was present during the initial exposure to high pH, desensitization was completely prevented, even when the inhibition was measured at high pH. Gel filtration on Sephadex G-200 indicated that the enzyme did not dissociate at pH 9.5.

At high concentrations of KCl, glycerol kinase was stimulated and sensitivity to fructose bisphosphate was reduced (Fig. 10A). Other salts also desensitized the enzyme but did not enhance activity (Table II). Low concentrations (50 mM) of guanidine hydrochloride stimulated enzyme activity, while higher concentrations (above 200 mM) were inhibitory (Fig. 10B). At 200 mM, guanidine hydrochloride produced no net stimulation of enzyme activity but almost completely eliminated inhibition by 1 mM fructose bisphosphate. Gel filtration on Sephadex G-200 showed that glycerol kinase was not dissociated in 160 mM guanidine hydrochloride.

Other treatments, some of them considerably harsher, did not desensitize the enzyme. Glycerol kinase that was heated in standard buffer at 70° for 10 min lost 25% of its initial activity but remained unchanged in its sensitivity to fructose bisphosphate inhibition. Inhibition by 1 mM fructose bisphosphate of heated and unheated samples was 55 and 50%, respectively. Enzyme that was 50% inactivated by p-hydroxymercuriphenyl sulfonate was still sensitive to fructose bisphosphate inhibition. Treated and untreated glycerol kinase was inhibited 50 and 62%, respectively, by 1 mM fructose bisphosphate.

On the other hand, mutant forms of glycerol kinase could be

<table>
<thead>
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<th>Salt</th>
<th>[P]Glycerol-3-P formed</th>
<th>Inhibition by 1 mM FDP</th>
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<tbody>
<tr>
<td>No FDP</td>
<td></td>
<td>%</td>
</tr>
<tr>
<td>+ 0.4 M KCl</td>
<td>73.7</td>
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<td>70.6</td>
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<tr>
<td>+ 0.4 M NaCl</td>
<td>73.5</td>
<td>65.1</td>
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* FDP, fructose bisphosphate.
readily obtained that were insensitive to inhibition by fructose bisphosphate. Strains of E. coli producing genetically desensitized glycerol kinases have been selected by several different procedures (2, 5, 6), and we purified the enzyme from one such mutant (strain 244). A single band was observed when the purified mutant enzyme (Fraction IX, Table I) was subjected to electrophoresis in standard polyacrylamide gels at two different pH values as well as in the presence of SDS, indicating that the preparation was homogeneous. The specific activity of the mutant enzyme (Table I) was almost twice that obtained for crystalline preparations of the normal glycerol kinase (618 units per mg) (1). The electrophoretic mobility of the mutant enzyme in both standard and SDS-polyacrylamide gels was indistinguishable from that of the normal glycerol kinase, suggesting that the two enzymes were identical in charge, size, and subunit composition. Upon gel filtration on Sephadex G-200, the enzyme from strain 244, as well as another enzyme insensitive to fructose bisphosphate inhibition produced by strain 43, emerged at exactly the same position as the normal enzyme from strain 72. The enzyme from another desensitized mutant, strain 48, was very unstable. Although freshly prepared cell-free extracts had a low level of glycerol kinase activity, no activity could be recovered after gel filtration.

As shown in Fig. 11, the mutant glycerol kinase purified from strain 244 was totally insensitive to concentrations of fructose bisphosphate that inhibited the normal enzyme to an extent of 65 to 70%. Several of the other properties of the mutant enzyme were different from those of the normal glycerol kinase. Double reciprocal plots of the response of reaction velocity to MgATP were hyperbolic, but the value of the lower $K_m$ (55 $\mu M$) was one-half of that observed with the normal enzyme, whereas the higher $K_m$ value was the same. The mutant enzyme displayed a broad pH optimum, with essentially no change in activity between pH 7.0 and 9.5.

**Effect of Desensitization on Fructose Bisphosphate Binding—** Under normal conditions, fructose bisphosphate bound to glycerol kinase with a dissociation constant of about 0.06 mM (Fig. 12, inset). Gel filtration of glycerol kinase in the presence of a nearly saturating concentration (0.1 mM) of fructose bisphosphate showed the binding of 3.5 moles of $[^{14}C]$fructose bisphosphate per mole of enzyme (Fig. 12). Binding $[^{14}C]$fructose bisphosphate of fructose bisphosphate, measured at a nonsaturating concentration (0.06 mM), was also observed under the conditions that promoted desensitization (Table III). In fact, fructose bisphosphate binding at high pH and with the mutant enzyme was almost as great as with the normal enzyme at pH 7.0.

**Effects of Ligands on Protein Structure**

**Heat Stability—** Glycerol kinase was very labile in the absence of glycerol. Even at 0°C, the purified enzyme lost over 50% of its activity after 18 hours of dialysis against standard buffer lacking glycerol. In the presence of glycerol, however, enzyme (0.19 mg per ml in standard buffer) that was left for 2 days at room temperature showed no loss of either activity or sensitivity to fructose bisphosphate inhibition and relatively high temperatures were required to inactivate both the normal and mutant glycerol kinases, the mutant protein being less stable. When the normal enzyme was heated for 10 min at 70°C, half-maximal protection occurred at about 1 mM glycerol (Fig. 13). The enzyme was also stabilized by ADP and Mg$^{2+}$, and the presence of ADP and Mg$^{2+}$ afforded additional protection in the presence

![Fig. 11. Effect of fructose bisphosphate (FDP) on the activity of normal and mutant glycerol kinases. The activities of normal enzyme (0.4 $\mu$g) (•) and of mutant enzyme (0.2 $\mu$g) (○) were measured at varying concentrations of fructose bisphosphate by Assay I.](http://www.jbc.org/)

**Fig. 12. Binding of $[^{14}C]$fructose bisphosphate ($[^{14}C]$FDP) to glycerol kinase. Binding of $[^{14}C]$fructose bisphosphate to glycerol kinase (0.59 mg) was measured by gel filtration, as described under "Experimental Procedure." (---) $[^{14}C]$fructose bisphosphate bound; (---), enzyme activity. Inset, binding of $[^{14}C]$fructose bisphosphate to glycerol kinase (16 $\mu$g) was measured by ultrafiltration, as described under "Experimental Procedure." FDP, fructose bisphosphate.**

**TABLE III**

<table>
<thead>
<tr>
<th>Conditions</th>
<th>$[^{14}C]$fructose bisphosphate bound (moles/mole enzyme)</th>
<th>Relative sensitivity</th>
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<td>Normal enzyme</td>
<td>At pH 7.0: 1.7</td>
<td>[100]</td>
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<tr>
<td></td>
<td>At pH 9.2: 1.5</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>At 0.4 M KCl: 0.53</td>
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<td>At 0.18 M guanidine hydrochloride: 0.74</td>
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<tr>
<td>Mutant enzyme</td>
<td>At pH 7.0: 1.1</td>
<td>0</td>
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of glycerol, lowering the concentration of glycerol required for half-maximal protection to about 0.3 mM (Fig. 13). Fructose bisphosphate had no effect on the stability of either the normal or mutant glycerol kinases in the absence or the presence of glycerol.

**Sulphydryl Group Reactivity**—It had been shown previously (1) that about 12 of the 20 sulphydryl groups of glycerol kinase reacted with DTNB under nondenaturing conditions. However, in the presence of a saturating concentration of glycerol, only two sulphydryl groups per molecule reacted at a measurable rate with the Ellman reagent (Fig. 14). In contrast, fructose bisphosphate had no effect on the reactivity of the sulphydryl groups in the native enzyme.

**DISCUSSION**

An examination of both the substrate kinetics and the product inhibition patterns of a bisubstrate enzymic reaction can often distinguish between possible catalytic mechanisms (15). Since double reciprocal plots of the response of reaction velocity to the concentration of each substrate at varying concentrations of the other substrate yielded families of lines that intersected on the abscissa, a ping-pong mechanism for glycerol kinase was ruled out. It was found that ADP was a noncompetitive inhibitor with respect to glycerol, while ATP inhibition with respect to MgATP was complex, being mainly competitive at higher MgATP concentrations. In contrast, glycerol-3-P was a competitive inhibitor with respect to glycerol. Insofar as the complexity of the MgATP kinetics permits an interpretation, this product inhibition pattern is more consistent with an ordered than with a random mechanism for glycerol kinase, with glycerol as the first substrate to bind to the enzyme.

It is perhaps unexpected that a small molecule such as glycerol rather than the nucleotide must bind first to the enzyme; however, several lines of evidence indicate that glycerol promotes a major conformational change in glycerol kinase. First, the presence of glycerol, at low concentrations, markedly stabilized the enzyme against inactivation. Second, in the presence of glycerol only two sulphydryl groups per molecule of enzyme reacted with DTNB, while in its absence 12 sulphydryl groups reacted with the Ellman reagent. The removal of 10 of the 12 exposed cysteine residues from contact with the solvent could hardly be due to simple steric protection by glycerol; it more likely results from a major conformational change. Finally, binding experiments showed that the dissociation constant of the enzyme-glycerol complex, in the absence of MgATP, was $10^{-5}$ M, indicative of a free energy of glycerol binding of $-6.8$ Cal per mole ($1,600$ J per mole). Since the $K_m$ for glycerol was found to increase with decreasing temperature, the enthalpy of glycerol binding must be positive (about $+6$ Cal per mole or $1.4$ J per mole as calculated from Van't Hoff plots). Consequently, the entropy of glycerol binding must also have a positive value (about $+45$ cal mole$^{-1}$ deg$^{-1}$ or $11$ J mole$^{-1}$ deg$^{-1}$). Since the binding reaction per se will involve an entropy loss, glycerol binding must be accompanied by some reaction in which entropy is gained, presumably a conformational change in the protein.

Another interesting aspect of the interaction of glycerol kinase with its substrates was the unusual response of reaction velocity to MgATP concentration that resulted in hyperbolic double reciprocal plots. This type of behavior could be caused by any of the following factors: (1) an artifact of the assay procedure; (2) heterogeneity of the enzyme preparation; (3) direct interaction between ATP molecules; (4) a nonordered bisubstrate mechanism; or (5) the existence of several types of active site on a single enzyme molecule. Let us consider each of these possibilities in turn.

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**Fig. 13.** Protection against heat inactivation by glycerol in the absence and presence of MgADP. Solutions of glycerol kinase (20 μg per ml) in standard buffer containing gelatin (0.1 mg per ml) and various concentrations of glycerol, without (●) and with (■) 1 mM ADP and 2 mM MgCl$_2$, were heated at 70° for 10 min. After rapid cooling to 0°, the activity of samples (0.1 ml) was measured by Assay I. *Inset,* the data were replotted in terms of the difference between the rate constants of heat inactivation with and without glycerol. $A_R$ and $A_E$ are the activities remaining after 10 min at 70° in the absence and presence of glycerol, respectively.

**Fig. 14.** Sulphydryl group titrations of glycerol kinase in the absence and presence of various ligands. A freshly prepared solution of crystalline glycerol kinase was dialyzed overnight against several changes of standard buffer and then separated from excess 2-mercaptoethanol by gel filtration on Sephadex G-200 equilibrated with 1 mM EDTA and 50 mM triethanolamine hydrochloride, pH 7.0. After gel filtration, samples were immediately titrated with DTNB as described under "Experimental Procedure," either in absence of specific ligands (▪), in the presence of $2$ mM fructose bisphosphate (■), or in the presence of $1$ mM glycerol (■).
The same kinetic behavior with respect to MgATP was observed using two different assay methods that were conducted at protein concentrations that differed by two orders of magnitude (Fig. 3) and under conditions that assured measurement of initial reaction velocities. Moreover, by carrying out the assays at a concentration of Mg\(^2+\) that exceeded the total ATP concentration by 1 mM, we were assured of the virtual absence of free ATP and of the presence of constant levels of free Mg\(^2+\). It is therefore unlikely that the nonlinear double reciprocal plots are an artifact of the assay procedure, and, indeed, other workers have observed similar ATP kinetics with glycerol kinases from different sources (16, 17).

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2. Enzyme heterogeneity could be due to the presence of glycerol kinase isozymes, to modification of the enzyme during the purification procedure by limited proteolysis or oxidation of sulfhydryl groups, or to the presence of conformational isomers or different states of aggregation. The following observations make these possibilities unlikely. The unusual MgATP kinetic data were observed with our homogeneous preparations of glycerol kinase in which all 20 sulfhydryl groups were intact (I), immediately after crystallization and also after prolonged aging, with fractions obtained at all stages of purification, with the enzyme that had been obtained from the genetically desensitized mutant strain 244, with a commercial preparation of glycerol kinase purified by a different procedure, and with enzyme that had been 50% inactivated by p-hydroxymercuriphenyl sulfonate. The kinetic response to MgATP was not affected by glycerol concentration (Fig. 3), by the presence of the allosteric inhibitor fructose bisphosphate (Fig. 7), nor by a 200-fold variation in enzyme concentration (Fig. 3). The glycerol kinetic data were strictly hyperbolic and the apparent \(K_m\) for glycerol was the same at high and low concentrations of MgATP (Fig. 1). Finally, all known mutations in glycerol kinase map at a single locus on the bacterial chromosome (19) and simultaneously alter the properties of the whole population of enzyme molecules (2, 5, 6, 20), making the existence of isozymes very unlikely.

3. Direct interaction between ATP molecules on different sites on the same protein molecule is difficult to eliminate until x-ray crystallographic data on the enzyme-substrate complex are available. However, interactions of this type would most likely be electrostatic ones and would be more pronounced at a high pH where ATP is more fully ionized. Since, at pH 9.5, the double reciprocal plots are less hyperbolic than at neutral pH, it is unlikely that the observed behavior is due to this kind of interaction.

4. Nonlinear double reciprocal plots often result when a substrate can combine with more than one form of an enzyme, as in nonordered biubstrate reactions (18). However, product inhibition patterns and substrate kinetics implicate an ordered mechanism for glycerol kinase and are not in accord with the patterns predicted for any one of the possible nonordered two-substrate two-product reactions (18).

5. The remaining possibility is the existence of two types of active site on a single enzyme molecule which would give rise to hyperbolic double reciprocal plots if the sites differed in their affinity for MgATP. This situation could arise in two ways: by a conformational change which occurs upon binding of MgATP to some of the subunits, decreasing the affinity of the remaining subunits for MgATP, so-called "negative cooperativity" (21); or by the existence of some intrinsic asymmetry in the arrangement of subunits, resulting in nonequivalence of the active sites. Although "negative cooperativity" has been invoked as an explanation for the complex substrate kinetics of several oligomeric enzymes (21–25), it is very difficult to prove that the kinetic behavior is indeed caused by a ligand-induced conformational change. On the other hand, nonequivalent protomer orientation within oligomers composed of identical subunits has recently been shown by x-ray crystallography to exist in yeast hexokinase (26), and rapid kinetic measurements by the stopped flow method have shown two types of active sites in E. coli alkaline phosphatase (27) and in alcohol dehydrogenase (28). Also, alkylation and acylation studies on rabbit muscle glyceraldehyde-3-P dehydrogenase have suggested that the four active sites of the enzyme are not equivalent (29).

Glycerol kinase from E. coli is an oligomer composed of four chemically identical subunits (I), and the fact that 4 moles of glycerol and 4 moles of fructose bisphosphate were bound per mole of enzyme at saturating concentrations of these ligands suggests that there are one catalytic and one allosteric site per subunit. Yet, nonequivalence of subunit orientation with E. coli glycerol kinase is indicated by the observation that, while in the absence of specific ligands, 12 sulfhydryl groups per tetramer reacted rapidly with DTNB, in the presence of glycerol, only two sulfhydryl groups per tetramer were reactive. This type of subunit nonequivalence in the presence of glycerol would be sufficient to account for the nonhyperbolic MgATP saturation curves and it is therefore not necessary to invoke MgATP-induced conformational changes.

It had previously been shown that glycerol kinase from E. coli was specifically inhibited by fructose bisphosphate (2) while other common metabolites had no significant effect on the activity of the enzyme (14). Since fructose bisphosphate was a noncompetitive inhibitor with respect to both substrates and since inhibition could be dissociated from catalytic activity by changes in ionic conditions and by mutation (2, 5, 6), it appears unlikely that fructose bisphosphate inhibits by binding directly to the active site. In fact, inhibition by fructose bisphosphate cannot be due to inhibitor binding per se, for treatments which reduced or abolished sensitivity of the enzyme to inhibition did not prevent fructose bisphosphate binding and a genetically desensitized enzyme was still able to bind the inhibitor. Although effectors have been shown to influence the state of aggregation of some allosteric enzymes (30–33), the presence of fructose bisphosphate caused neither aggregation nor dissociation of glycerol kinase. Inhibition of the enzyme by fructose bisphosphate must therefore be due to a conformational change that accompanies the binding of the ligand. This conformational response may, however, be quite subtle, since heat stability and sulfhydryl group reactivity of the enzyme were not affected by the presence of saturating concentrations of the inhibitor.

The kinetics of interaction of fructose bisphosphate with glycerol kinase had the following characteristics. First, the response of reaction velocity to fructose bisphosphate concentration was only slightly sigmoid. Second, even at saturating concentrations of fructose bisphosphate, inhibition was incomplete, the maximum extent of inhibition being about 80%. Finally, the \(K_i\) for fructose bisphosphate was higher by almost an order of magnitude than the dissociation constant of the enzyme-fructose bisphosphate complex, determined by binding studies. Especially the last observation seems rather puzzling; nevertheless, these properties can be readily explained in terms
model of Monod et al. (3). It is assumed that the two states of the enzyme, \( R \) and \( T \), have equal affinity for substrate but that only State \( R \) is enzymically active. \( A \), calculated relationship between inhibitor concentration and per cent inhibition (\% inhibition = 100 \([1 + (d \beta)^n - (1 + \beta)^n]/[1 + (d \beta)^n + (1 + \beta)^n] \) ); \( B \), calculated relationship between inhibitor concentration and per cent saturation (% saturation = 100 \([\alpha(1 + \beta)^{n-1} + Ld\alpha(1 + d \beta)^{n-1}/(1 + \beta)^{n-1} + L(1 + \beta)^n] \) ); where \( n \) is the number of protomers; \( L \) is the equilibrium constant for the allostERIC transition in the absence of ligands (\( K_a \)); \( d \) is the ratio of the microscopic dissociation constants of the inhibitor from States \( R \) and \( T \) (\( K_a : K_T \)); and \( \beta \) is the inhibitor concentration relative to its dissociation constant from State \( R \) (\( 1/K_D \)). The curves were drawn with a Hewlett-Packard model 9125A plotter in conjunction with the model 9100B calculator, with the following set of parameters: \( L = 0.003, n = 4, \) and \( d = 5.88 \) (---) or \( d = 2.0 \) (--.--). \( K_T \) and \( K_R \) are the calculated inhibitor concentrations that produce half-maximal inhibition or half-saturation, respectively. The inhibitor concentration is expressed in terms of arbitrary units which were chosen to correspond approximately to the millimolar concentrations of fructose bisphosphate used in the experiments with glycerol kinase.

Fig. 15. Relationship between inhibitor concentration and inhibition and saturation of an enzyme according to the allosteric model of Monod et al. (3). It is assumed that the two states of the enzyme, \( R \) and \( T \), have equal affinity for substrate but that only State \( R \) is enzymically active. \( A \), calculated relationship between inhibitor concentration and per cent inhibition (\% inhibition = 100 \([1 + (d \beta)^n - (1 + \beta)^n]/[1 + (d \beta)^n + (1 + \beta)^n] \) ); \( B \), calculated relationship between inhibitor concentration and per cent saturation (% saturation = 100 \([\alpha(1 + \beta)^{n-1} + Ld\alpha(1 + d \beta)^{n-1}/(1 + \beta)^{n-1} + L(1 + \beta)^n] \) ); where \( n \) is the number of protomers; \( L \) is the equilibrium constant for the allostERIC transition in the absence of ligands (\( K_a \)); \( d \) is the ratio of the microscopic dissociation constants of the inhibitor from States \( R \) and \( T \) (\( K_a : K_T \)); and \( \beta \) is the inhibitor concentration relative to its dissociation constant from State \( R \) (\( 1/K_D \)). The curves were drawn with a Hewlett-Packard model 9125A plotter in conjunction with the model 9100B calculator, with the following set of parameters: \( L = 0.003, n = 4, \) and \( d = 5.88 \) (---) or \( d = 2.0 \) (--.--). \( K_T \) and \( K_R \) are the calculated inhibitor concentrations that produce half-maximal inhibition or half-saturation, respectively. The inhibitor concentration is expressed in terms of arbitrary units which were chosen to correspond approximately to the millimolar concentrations of fructose bisphosphate used in the experiments with glycerol kinase.

maximal inhibition (\( K_D \)) is about 10 times greater than the concentration of fructose bisphosphate that half-saturates the binding sites (\( K_p \)), in good agreement with the experimental results. In the context of this model, it is also of interest to examine the effect of changes in the relative affinities of fructose bisphosphate to the two states of the allosteric enzyme on inhibition and ligand binding. As indicated by the broken lines in Fig. 15, merely a 3 fold change in this parameter, so that the ratio of the relative affinities to states \( T \) and \( R \) becomes 2, causes an almost complete loss of sensitivity to inhibition while the binding of fructose bisphosphate to the enzyme is only slightly affected. This could account for our finding that glycerol kinase is so readily desensitized to fructose bisphosphate inhibition by manipulation of ionic conditions (Figs. 9 and 10) and by mutation (2, 5, 6) and the fact that the desensitized enzyme still bound fructose bisphosphate (Table III). 5

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5 A change in the allosteric constant, \( L \), would produce a similar effect, but a relatively large decrease in this parameter (about 100-fold) would be required to afford the same degree of desensitization as a 3-fold change in the relative affinities to the two states.
Catalytic and Allosteric Properties of Glycerol Kinase from Escherichia coli
Jeremy W. Thorner and Henry Paulus


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