The glpR gene encoding the repressor for the sn-glycerol 3-phosphate regulon of Escherichia coli was cloned downstream from the strong pl promoter of bacteriophage λ. This allowed overproduction of the repressor in an expression system. The cI857 region from λ harboring the cI857 gene. The repressor was purified 40-fold to homogeneity from an induced strain. The purification scheme utilized polyethyleneimine and ammonium sulfate fractionation, followed by phosphocellulose and DEAE-Sephadex chromatography. Purification was monitored by measuring the binding of radiolabeled inducer (sn-glycerol 3-phosphate) to the repressor. The purified repressor migrated as a single band exhibiting a subunit molecular weight of 30,000 assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The molecular weight of the repressor under non-denaturing conditions was 100,000–130,000 suggesting the repressor is a tetramer under native conditions. Interaction of the repressor with sn-glycerol 3-phosphate was studied using flow dialysis. Scatchard analysis of the data indicated four binding sites/repressor tetramer and a dissociation constant of 31 μM. Interaction of the repressor with DNA was studied using band-shift electrophoresis. The repressor specifically bound DNA fragments containing the control regions for the gldD, gldK, and gldP-A genes. Binding of DNA by the repressor was diminished in the presence of sn-glycerol 3-phosphate.

Escherichia coli and other bacteria are able to utilize glycerol, sn-glycerol 3-phosphate, and glycerolphosphodiester as carbon/energy sources and as precursors for phospholipid biosynthesis (Lin, 1976; Raetz, 1986). The proteins which carry out the catabolic steps required for dissimilation of glycerol-P and its precursors are encoded by the genes of the glp regulon. These genes are located at three different positions on the linkage map of E. coli (Lin, 1976). The gldK and glpF genes, encoding cytoplasmic glycerol kinase and cytoplasmic membrane-associated glycerol diffusion facilitator, respectively, map near Minute 88. The glpTQ operon encoding cytoplasmic membrane-associated glycerol-P permease (Larson et al., 1982) and periplasmic glycerophosphodiesterase (Larson et al., 1983) maps near Minute 49. The glpACB operon encoding cytoplasmic membrane-associated anaerobic glycerol-P dehydrogenase is directly adjacent to and is transcribed divergently from the glpTQ operon (Kuritzkes et al., 1984; Ehrmann et al., 1987). The glpD gene, encoding cytoplasmic membrane-associated aerobic glycerol-P dehydrogenase, maps near Minute 75 (Schweizer and Larson, 1987). The glpR gene, encoding a specific repressor for the glp regulon, and two genes of unknown function, glpE and glpG, map adjacent to glpD (Schweizer et al., 1986).

The glp genes comprise a regulon because they are all negatively regulated by a specific repressor encoded by the glpR gene (Lin, 1976; Schweizer et al., 1985). Repression mediated by the glp repressor can be relieved to differing extents (depending on the operon involved) in the presence of glycerol-P, the inducer for the system. Binding of the inducer by the repressor presumably decreases the affinity of the repressor for its specific binding sites (operators) on the DNA. Results of experiments employing a strain with a thermolabile glp repressor indicated that the repressor binds differentially to its binding sites at each operon. Expression of the glpD gene was most sensitive to the glp repressor; expression of glpT was less sensitive, and expression of glpK was least sensitive to the repressor (Lin, 1976). Expression of the glp genes is also differentially sensitive to catabolite repression (Lin, 1976). The degree of sensitivity to catabolite repression is reversed when compared to specific repression exerted by the repressor.

The experiments described in the present paper were initiated so that insight concerning the molecular details of the differential regulation of glp gene expression will ultimately be obtained. The repressor has been overexpressed, facilitating its purification to homogeneity. The subunit and native molecular weights of the repressor have been determined, and the interaction of the repressor with glycerol-P and operator DNA has been characterized.

EXPERIMENTAL PROCEDURES

Materials

Sigma supplied the following chemicals: ampicillin, polyethyleneimine, casein, phenylmethylsulfonyl fluoride, phosphocellulose, DEAE-Sephadex (A-50–120), Sephadex G-200–120, glycerol kinase (Candida), glycerol-P dehydrogenase (rabbit muscle), α-amylase (sweet potato), alcohol dehydrogenase (yeast), albumin (bovine serum and chicken egg), carbonic anhydrase (bovine erythrocytes), and Trizma base. Bio-Rad supplied the reagents for electrophoresis. Yeast extract and Tryptone were from Difco. T4 DNA ligase, Klenow fragment of DNA polymerase I, and restriction endonucleases were from Boehringer Mannheim. Ecoscint scintillation mixture was...
obtained from National Diagnostics, Du Pont-New England Nuclear supplied [α-32P]dATP and [2-3H]glycerol. Radiolabeled glycerol was phosphorylated using glycerol kinase to produce [2-3H]glycerol-P (Schlossman and Bell, 1978). The concentration of glycerol-P was determined spectrophotometrically using glycerol-P dehydrogenase and NAD, assuming a molar absorbance coefficient of 6300 M⁻¹ cm⁻¹ for NADH at 340 nm (Beisenherz et al., 1985).

Construction of pSH40

A 3-kilobase pair EcoRI-SalI fragment containing the glpR gene (Schweizer et al., 1985) was ligated into the expression vector pPLc2833 (Remaut et al., 1983) which had been cleaved with EcoRI and SalI. The ligation mixture was used to transform strain NM303 (mgl deaΔ [5]) (Schweizer et al. and Gottesman, 1982) to overexpression of glpR.

Growth of Bacteria

Strain N4830(pSH40) was used as the source of the glp repressor. The cultures were grown in minimal medium containing 0.05 M MOPS (pH 6.5), 0.4 mg/ml casein, 55 μM [2-3H]glycerol-P (80 cpm/pmole), and repressor in 1 ml. After a 5-min incubation at room temperature, 0.35 ml of ice-cold saturated ammonium sulfate was added; the tubes were vortexed and then centrifuged for 5 min at 10,000 g for 15 min. The supernatant was aspirated, and the pellet was washed with 100 ml of 20 mM Tris-HCl (pH 7.5), 50 mM NaCl, 0.1 mM EDTA, and the cell paste stored at -80°C. The yield was 40.3 g of cells.

Assay of glp Repressor

The glp repressor was monitored using a modification of the ligand-binding assay of the catechol gene activator protein of E. coli (Blay and Ullmann, 1986). Assays (in 1.5-ml microcentrifuge tubes) contained 0.05 M MOPS (pH 6.5), 0.4 mg/ml casein, 55 μM [2-3H]glycerol-P (80 cpm/pmole), and repressor in 1 ml. After a 5-min incubation at room temperature, 0.55 ml of ice-cold saturated ammonium sulfate was added; the tubes were vortexed and then centrifuged for 5 min at 4°C. The supernatant was aspirated, and the pellet was washed with 0.5 ml of cold saturated ammonium sulfate. The final pellet was resuspended in 0.1 ml of water, and the radioactivity was determined directly in the microcentrifuge tubes after addition of 1 ml of scintillation fluid. The assay was linear from 0-2 μg of repressor. One unit of repressor activity is defined as the amount of repressor required to bind 1 pmol of glycerol-P.

Purification of glp Repressor

The following scheme was used for isolation of the repressor from 13.6 g of frozen cell paste. All steps were carried out at 0-4°C. Buffers were adjusted to the indicated pH values at room temperature. A summary of the purification is given in Table I. Protein concentrations were estimated using the method of Bradford (1976) with bovine serum albumin serving as the standard.

1. Preparation of Crude Extract—Cells were suspended in 120 ml of Buffer A (0.02 M Tris-HCl (pH 7.5), 0.4 M NaCl, 0.1 M EDTA, 0.2 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride) and then disrupted by a single passage through a French pressure cell. Cell debris was removed by centrifugation at 40,000 × g for 50 min. The supernatant was diluted with Buffer A to 7 mg of protein/ml (crude extract, Table I).

2. Polyelectrolyme Fractionation—The crude extract was stirred rapidly, and 7.6 ml of 2.5% polyethyleneimine were added over the course of 1 min. After 20 min, the suspension was clarified by centrifugation at 10,000 × g for 20 min. The pellet was suspended by homogenization in 100 ml of Buffer A, stirred for 30 min, and the centrifugation repeated. The pellet was then suspended in 100 ml of Buffer A containing 1 M NaCl and stirred for 30 min. The centrifugation was repeated. The supernatant is the polyelectrolyme fraction (Table I).

3. Ammonium Sulfate Fractionation—Ammonium sulfate (13 g) was added with stirring to the previous fraction. After 20 min, insoluble material was removed by centrifugation at 14,000 × g for 20 min. Additional ammonium sulfate (17 g) was added with stirring to the supernatant fraction, and the centrifugation was repeated for 30 min. The pellet was suspended in 50 ml of 10 mM sodium phosphate (pH 7.1), 1 mM EDTA, 0.2 mM dithiothreitol. Insoluble material was removed by centrifugation at 8,000 × g for 10 min. The supernatant is the ammonium sulfate fraction (Table I).

4. Polysaccharide Chromatography—The previous fraction was applied at 32 ml/h to a 2.5 × 34-cm column of polysaccharide equilibrated with Buffer B (20 mM sodium phosphate (pH 7.1), 1 mM EDTA, 0.1 mM dithiothreitol) containing 0.2 M NaCl. The column was washed with 30 ml of equilibration buffer and then eluted with a 500-ml linear gradient of 0.3-1 M NaCl in Buffer B. A final wash with 250 ml of Buffer B containing 1.2 M NaCl completed the elution. Repressor activity was eluted as a broad peak starting at 0.6 M NaCl. Fractions containing repressor activity were pooled, and 0.35 g of ammonium sulfate were added per ml of pool. Precipitated proteins were removed by centrifugation at 10,000 × g for 30 min and dissolved in 100 ml of Buffer C (0.02 M Tris-HCl (pH 7.5), 1 mM EDTA, 0.1 mM dithiothreitol). Insoluble material was removed by centrifugation at 18,000 × g for 15 min. The supernatant is the polysaccharide fraction (Table I). This material was divided into aliquots and stored at -80°C.

Polyacrylamide Gel Electrophoresis

SDS-polyacrylamide gel electrophoresis was carried out on 12% slab gels using the buffer system of Laemmli (1970). Nondenaturing gradient polyacrylamide gel electrophoresis was carried out using the above buffer system without the SDS (Rock and Cronan, 1981). The slab gels contained a 5-25% linear gradient of polyacrylamide stabilized with a 0-25% linear gradient of glycerol. The ratio of acrylamide to N,N'-methylenebisacrylamide was 37:5:1. Samples were dissolved in 0.125 M Tris-HCl (pH 6.8), 1 mM dithiothreitol, 10% glycerol, 0.005% bromphenol blue prior to electrophoresis. Electrophoresis was carried out overnight at 80 V until the tracking dye reached the bottom of the gel.

Gel Filtration Chromatography

A column (1.5 × 50 cm) of Sephadex G-200 was equilibrated with Buffer C containing 0.5 M KCl and 15% glycerol at 7 ml/h (4°C). Standard proteins and the glp repressor (0.5 mg) dissolved in 1 ml of Buffer C were applied individually to the column. The elution volumes (V,) were determined by counting the number of drops collected between the time of sample application and the peak of absorbance at 280 nm determined in a flow cell. The void (V0) and total (V,) volumes were determined using plasmid DNA and ATP, respectively. A standard curve of log molecular weight versus V, was used to estimate the size of the glp repressor, where K, = (V, - V0)/(V, - Vo). The subunit molecular weights of the standard proteins were determined using SDS-polyacrylamide gel electrophoresis and the standards shown in Fig. 2.

Flow Dialysis

Binding of glycerol-P by the glp repressor was measured at room temperature using flow dialysis (Coldwiek and Woomack, 1969). A Spectra/Por 2 dialysis membrane was used between the 1.5-ml lower chamber and the upper chamber which contained 1.0 ml of repressor at 1.2 mg/ml (approximately 40 μM monomer of M, 30,000). The buffer was the same as that used for storage of the repressor (Buffer C containing 0.5 M KCl and 15% glycerol) and was pumped through the lower chamber at 6 ml/min. The initial concentration of [3H]glycerol-P was 10 μM; 10-μl additions of 1 or 2 mM unlabeled glycerol-P were made to give final concentrations of about 20, 30, 40, 60, 80, and 100 μM (corrected for increase in volume in Fig. 5). The final addition was 10 μl of 0.8 M glycerol-P.
**Glycerol 3-Phosphate Repressor of E. coli**

**Fig. 1. Structure of pSH40.** The arrows indicate the directions of transcription of the glpR and blp genes (encoding the glp repressor and β-lactamase, respectively).

**Band-shift Electrophoresis**

Interaction of the glp repressor with radiolabeled DNA restriction fragments was assessed using the methods developed by Garner and Revzin (1981) and by Fried and Crothers (1981). For these experiments appropriate DNA fragments containing the glpD, glpK, and the glpT-glpa operator regions were cloned into the Bluescript-KS (+) phagemid vector (Stratagene Cloning System). The fragments were end labeled by filling recessed 3' ends (generated by restriction endonuclease or exonuclease III digestion) using the Klenow fragment of DNA polymerase I and [α-32P]dATP (Maniatis et al., 1982). Fragments labeled on one end were generated by subsequent digestion with a second enzyme. A 374-base pair EcoRI-Nael fragment derived from the vector served as control. The fragments were purified by phenol extraction and ethanol precipitation, with subsequent elution and β-lactamase, respectively.

To assess binding of the glp repressor to the DNA fragments, repressor and DNA (0.7–1.5 nM) were incubated together at 37 °C in 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 50 mM KCl, 5% glycerol, 0.025% Triton X-100 in a final volume of 7.5 μl. After 10 min. 18.7 μl of 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 5% glycerol, 0.025% Triton X-100, 100 μg/ml bovine serum albumin, and 0.025% bromophenol blue were added, and the samples were applied immediately to a 6% polyacrylamide gel containing 0.07% bisacrylamide. Electrophoresis was carried out at room temperature for 2–3 h at 1.5 mA/cm of gel using Tris borate-EDTA buffer. The mobilities of the DNA fragments were assessed by autoradiography of the dried gel. The conditions employed for analysis of DNA binding by the Gal repressor (Majumdar et al., 1987) and by the OmpR protein (Norioka et al., 1986).

**RESULTS**

**Overproduction of the glp Repressor**—Knowledge of the position and direction of transcription of the glpR gene facilitated construction of pSH40 (Fig. 1) where the repressor gene is positioned downstream from the λ P.l promoter. Thermal induction of the cryptic λ lysogen N4830 (cI857) harboring pSH40 resulted in rapid induction of glp repressor activity, assessed by measuring binding of radiolabeled glycerol-P (Table II). The activities were determined after partial purification, because contaminating glycerol-P binding activities gave high backgrounds in crude extracts. The specific activity reached a maximum after 1 h of induction at 42 °C. The activity was quite stable, because no substantial loss in specific activity was observed even after prolonged growth at 42 °C. Cells grown overnight at 42 °C were used as starting material for purification of the glp repressor.

**Purification of the glp Repressor**—Table I shows a summary of the protocol developed for the purification of the glp repressor. More than 6 mg of repressor were isolated from 12.5 g of cells obtained from a 4-liter culture. The repressor required a 40-fold purification to apparent homogeneity with a 19% yield. The purification fold and yield are minimum values, because proteins in the crude extract other than the glp repressor bound glycerol-P.

**Table I**

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Volume (ml)</th>
<th>Protein (mg)</th>
<th>Specific activity (units/mg)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Crude extract</td>
<td>180</td>
<td>1,260</td>
<td>470 (100)</td>
<td></td>
</tr>
<tr>
<td>2. Polyethyleneimine</td>
<td>98</td>
<td>265</td>
<td>1,356 (60)</td>
<td></td>
</tr>
<tr>
<td>3. Ammonium sulfate</td>
<td>52</td>
<td>114</td>
<td>2,540 (49)</td>
<td></td>
</tr>
<tr>
<td>4. Phosphocellulose</td>
<td>100</td>
<td>16</td>
<td>16,100 (43)</td>
<td></td>
</tr>
<tr>
<td>5. DEAE-Sephadex</td>
<td>4.4</td>
<td>6.2</td>
<td>18,000 (19)</td>
<td></td>
</tr>
</tbody>
</table>

**Fig. 2. SDS-polyacrylamide gel electrophoretic analysis of the glp repressor during purification. Lanes:** 1, crude extract (36 μg); 2, polyethyleneimine fraction (21 μg); 3, ammonium sulfate fraction (16 μg); 4, phosphocellulose fraction (6 μg); 5 and 6; DEAE-Sephadex fraction (6 and 12 μg, respectively). The molecular weights (×10^6) of protein standards (β-galactosidase, phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, and lysozyme) are indicated on the left.
of the repressor relative to the elution positions for standard proteins is shown in Fig. 3A. The molecular weight of the repressor was estimated to be 130,000. The apparent size of the repressor estimated by gel filtration was dependent upon the ionic strength of the buffer. Aggregation was apparent when a buffer of lower ionic strength (20 mM sodium phosphate, 0.2 M NaCl, 1 mM EDTA, and 0.1 mM dithiothreitol) was used, with the peak of absorbance eluting at a position corresponding to a molecular weight of 170,000.

The native size of the repressor was also determined by comparison of its migration position on gradient (5-25%) polyacrylamide slab gels with those of standard proteins. The results (Fig. 3B) suggested the repressor has a native molecular weight of 100,000. Taken together, the results indicate the repressor is a tetramer composed of four identical subunits.

**Interaction of the g lp Repressor with Glycerol-P**—Binding of the inducer was studied under a variety of conditions by using precipitation of repressor-inducer complexes with ammonium sulfate as described for the standard assay. Binding of glycerol-P was independent of ionic strength (0-0.2 M NaCl) and did not require divalent metal ions (5 mM MgCl₂ decreased binding). The pH optimum for binding was quite broad, occurring between pH 6.5 and 7.5 (data not shown).

The specificity of binding was investigated by testing the effects of addition of unlabeled compounds to binding assays containing 28 μM [³H]glycerol-P (Table III). Addition of glycerol-2-P, glucose-6-P, phosphoenolpyruvate or P, at an 18-fold molar excess had no effect on binding of glycerol-P by the repressor. Compounds resembling dihydroxyacetone-P and glycerophosphodiesters had a small but significant effect on binding. P, at higher concentrations also decreased binding of inducer. Fosfomycin, an antibiotic recognized by the g lpT-dependent transport system for glycerol-P, had very little affinity for the repressor. All of the results indicate that the repressor is very specific for binding of glycerol-P, because large excesses of competing compounds were required to elicit their effects.

Binding of glycerol by the repressor was also tested. Glycerol did not compete with binding of labeled glycerol-P. Furthermore, the repressor did not bind [³H]glycerol, as assessed by using the standard assay for binding of [³H]glycerol-P.

**The binding affinity of the repressor for glycerol-P** was estimated using two techniques. Using the standard assay (coprecipitation of glycerol-P with repressor in cold saturated ammonium sulfate), estimates of the Kd for binding of glycerol-P ranged from 20 to 50 μM (data not shown). Because the association may be affected by addition of the cold ammonium sulfate solution, flow dialysis was also used for estimation of the binding of glycerol-P. The Kd for glycerol-P was determined for partially purified preparations.

**Addition** | **Concentration** | **Binding** | **Specific activity**
---|---|---|---
None | 0.5 | 100 | 80
Glycerol-2-P | 0.5 | 100 | 91
Glucose-6-P | 0.5 | 99 | 92
Phosphoenolpyruvate | 0.5 | 59 | 90
Dihydroxyacetone-P | 2.5 | 56 | 84
Fosfomycin | 0.5 | 70 | 36
Glycerophosphoglycerol | 0.5 | 46 | 97
Glycerophosphocholine | 0.5 | 80 | 97
Glycerophosphocholine | 0.5 | 84 | 56
Sodium phosphate | 2.5 | 36 | 46
Sodium phosphate | 5 | 91 | 84
Sodium phosphate | 25 | 56 | 36

*FIG. 3. Native molecular weight of the g lp repressor.* A, chromatography on Sephadex G-200. The standard proteins employed and their molecular weights (× 10⁶) were: 1, β-amylase (202); 2, alcohol dehydrogenase (167); 3, lactoperoxidase (76); 4, bovine serum albumin (66); 5, ovalbumin (45); 6, carbonic anhydrase (29). The elution position of the g lp repressor is indicated by the open circle. B, gradient polyacrylamide gel electrophoresis. The numbers and symbols refer to the same proteins employed in part A, except that d₂ refers to the dimer of bovine serum albumin.

**TABLE II**

*Overproduction of the glp repressor*

Strain N4830(pSH40) was grown at 30 °C and then shifted to 42 °C as described under "Experimental Procedures." At the indicated time points, 100-ml aliquots were removed, and crude extracts were prepared in a final volume of 6 ml. The polyethyleneimine purification step was carried out on 1 ml of each of the crude extracts. The specific activity of the repressor was determined for the partially purified preparations.

<table>
<thead>
<tr>
<th>Time after shift to 42 °C</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>&lt;10</td>
</tr>
<tr>
<td>0.5</td>
<td>790</td>
</tr>
<tr>
<td>1</td>
<td>970</td>
</tr>
<tr>
<td>2</td>
<td>860</td>
</tr>
<tr>
<td>4</td>
<td>780</td>
</tr>
<tr>
<td>7</td>
<td>800</td>
</tr>
</tbody>
</table>

**TABLE III**

*Effects of phosphorylated compounds on binding of glycerol-P by the g lp repressor*

Binding was measured using standard assays containing 1.4 μg of purified repressor, 28 μM [³H]glycerol-P, and the indicated additions.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Concentration</th>
<th>Binding</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.5</td>
<td>100</td>
<td>80</td>
</tr>
<tr>
<td>Glycerol-2-P</td>
<td>0.5</td>
<td>100</td>
<td>91</td>
</tr>
<tr>
<td>Glucose-6-P</td>
<td>0.5</td>
<td>99</td>
<td>92</td>
</tr>
<tr>
<td>Phosphoenolpyruvate</td>
<td>0.5</td>
<td>59</td>
<td>90</td>
</tr>
<tr>
<td>Dihydroxyacetone-P</td>
<td>2.5</td>
<td>56</td>
<td>84</td>
</tr>
<tr>
<td>Fosfomycin</td>
<td>0.5</td>
<td>70</td>
<td>36</td>
</tr>
<tr>
<td>Glycerophosphoglycerol</td>
<td>0.5</td>
<td>46</td>
<td>97</td>
</tr>
<tr>
<td>Glycerophosphocholine</td>
<td>0.5</td>
<td>80</td>
<td>97</td>
</tr>
<tr>
<td>Glycerophosphocholine</td>
<td>0.5</td>
<td>84</td>
<td>56</td>
</tr>
<tr>
<td>Sodium phosphate</td>
<td>2.5</td>
<td>36</td>
<td>46</td>
</tr>
</tbody>
</table>
The results indicate that the repressor binds specifically to DNA containing its specific recognition sequence. The interaction between the repressor and the DNA fragments with the glpD, glpK, and glpT-glpa operators resulted in decreased mobility of the DNA. The presence of glycerol-P at concentrations tested had no apparent effect on the interaction between the repressor and the DNA fragments. It is apparent that the repressor binds with less affinity to the DNA fragment harboring the glpK operator, because higher concentrations of repressor are required to decrease the electrophoretic mobility of the DNA band. Also, the presence of the inducer did not cause dissociation of the repressor in the case of the glpD or glpT operators, which are known to bind repressor more avidly than the glpK operator (Lin, 1976). When the repressor was present at a lower concentration (5 nM), 5 mM glycerol-P decreased but did not eliminate binding of repressor to the glpD operator (data not shown).

**DISCUSSION**

In the present work, we have developed the methods required for the isolation of active homogeneous glp repressor in amounts sufficient for physical, chemical, and biochemical characterization. The purification protocol utilizes standard techniques and could be scaled up. The degree of overproduction of the repressor probably could be increased if the glpR gene were cloned in closer proximity to the pL promoter. In pSH40 (Fig. 1) glpR is located more than 1 kilobase pair of DNA away from pL. Knowledge of the nucleotide sequence of this region will facilitate construction of plasmids designed for greater overproduction of the repressor. Greater overproduction should be possible, because expression of the glpR gene is not subject to autoregulation.

Under nondenaturing conditions, the glp repressor exists as a tetramer of 30,000-dalton subunits. The glp repressor is, therefore, like other regulatory proteins which bind to DNA. Such proteins are generally oligomeric. For example, the λ repressors are dimeric, and the lac repressor is tetrameric (Riggs and Bourgeois, 1968).

The interaction of the inducer, glycerol-P, with the repressor was characterized using the standard assay and flow dialysis. The apparent Kd was estimated to be 31 μM. This is much higher than the Kd for the interaction between the lac repressor (the prototypical repressor for a catabolic operon) and allolactose, which is about 0.6 μM (Barkely and Bourgeois, 1980). One might expect a relatively high Kd in the case of the glp repressor if glycerol-P destined for degradation (and presumably free to bind to the glp repressor) is present in the same metabolic pool as that destined for biosynthesis of phospholipids. This is so because the cellular concentration of glycerol-P must be maintained at a level allowing optimal synthesis of phospholipids, has a high turnover rate, and is not subject to autoregulation.

We have not determined the affinity of the repressor-operator complex for glycerol-P. In the case of the lac operon, the affinity of the inducer (isopropyl-1-thio-β-D-galactoside) for the repressor-operator complex is much lower than the affinity of this inducer for free repressor (Barkely and Bourgeois, 1980). If the situation is somewhat analogous in the case of the glp regulon, particularly the glpD and glpT-dehydrogenases, the absence of exogenous sources of glycerol-P, continued expression of the dehydrogenases would create a futile cycle by degrading glycerol-P provided by glycerol-P synthase.

**Fig. 4.** Scatchard analysis of the binding of glycerol-P by the glp repressor. Binding of [3H]glycerol-P was determined in two separate experiments using different preparations of repressor. R designates the concentration of repressor tetramer, B designates bound glycerol-P, and F designates free glycerol-P in μM.

**Fig. 5.** Binding of operator DNA by the glp repressor assessed by band-shift electrophoresis. V, vector DNA; D, glpD DNA; K, glpK DNA; T-A, glpT-glpa DNA. Incubation mixtures contained either no repressor (lanes labeled 1), 30 nM repressor tetramer (lanes 2 and 5), 100 nM repressor (lanes 3 and 6), or 300 nM repressor (lanes 4 and 7). Incubations run in lanes 5–7 also contained 5 mM glycerol-P.

binding of glycerol-P was 28.7 μM in one case and 32.9 μM in the other.

Interaction of the glp Repressor with Operator DNA—The electrophoretic band-shift method for analysis of DNA-protein interactions (Garner and Revzin, 1981; Fried and Crothers, 1981) was used to assess binding of DNA by the purified repressor. As shown in Fig. 5, preincubation of the glp repressor with DNA fragments containing either the glpD, glpK, or glpT-glpa operators resulted in decreased mobility of the DNA (relative to the control with no repressor added) upon subsequent electrophoresis on a polyacrylamide gel. The repressor did not interact with a DNA fragment isolated from the vector, which should have no specific repressor binding sites. The results indicate that the repressor binds specifically to DNA containing its specific recognition sequence.

Addition of 5 mM glycerol-P to the incubation mixtures caused dissociation of the repressor from the DNA fragment containing the glpK operator. The presence of glycerol-P at the concentration tested had no apparent effect on the interaction between the repressor and the DNA fragments with either the glpD or glpT-glpa control regions. It is apparent that the repressor binds with less affinity to the DNA fragment harboring the glpK operator, because higher concentrations of repressor are required to decrease the electrophoretic mobility of the DNA band. Also, the presence of the inducer
pressor, assessed in vivo in a strain harboring a thermolabile repressor (Lin, 1976). Our results obtained using band-shift electrophoresis for assessing repressor-DNA interactions also indicated that the glpD operator has the highest affinity for the repressor. Levels of repressor lower (5 nM) than those employed for the experiment described in Fig. 5 were effective in shifting the mobility of the DNA fragment harboring the glpD operator (data not shown). The fact that 5 mM glycerol-P had no effect on the interaction of the repressor with the glpD operator also indicates that the repressor binds more tightly to this operator when compared to the glpK operator. The presence of glycerol-P caused dissociation of the repressor from the glpK operator. A relatively high concentration of P had no effect on the interaction of the repressor with the glpK operator. Levels of repressor lower (5 nM) than those required to demonstrate binding to the glpK operator (Lin, 1976). Our results obtained using band-shift electrophoresis for assessing repressor-DNA interactions also indicated that the repressor binds more tightly to this operator when compared to the glpK operator. The presence of glycerol-P caused dissociation of the repressor from the glpK operator. A relatively high concentration of repressor was required to demonstrate binding to the glpK operator, which also indicates that the glpK operator has the lowest affinity for the repressor. The results are in agreement with the reported differential binding affinities of the repressor for the different glp operators (Lin, 1976). It will be interesting to elucidate the basis for the differential binding affinity of the repressor for the control sites and to determine the actual binding constants for the interaction of the gkp repressor with each of its binding sites using the purified components. Such experiments await determination of the sequences of the control regions and localization of the repressor binding sites in each of the operons.

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REFERENCES
Green, P. R., Merrill, A. H., Jr., and Bell, R. M. (1981) J. Biol. Chem. 256, 11151–11159