Periplasmic Glycerophosphodiester Phosphodiesterase of Escherichia coli, a New Enzyme of the glp Regulon*

(Received for publication, December 9, 1982)

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The promoter-proximal gene (glpT) of the glpT-glpQ operon of Escherichia coli encodes a membrane permease responsible for active transport of sn-glycerol 3-phosphate. Promoter-distal glpQ encodes a periplasmic protein which is not required for active transport of sn-glycerol 3-phosphate (Larson, T. J., Schumacher, G., and Boos, W. (1982) J. Bacteriol. 152, 1008–1021). This periplasmic protein has now been identified as a phosphodiesterase which hydrolyzes glycerophosphodiesters into sn-glycerol 3-phosphate plus alcohol. The enzyme exhibited broad substrate specificity with respect to the alcohol moiety; sn-glycerol 3-phosphate was released from glycerophosphoethanolamine. Glycerophosphocholine, glycerophosphoglycerol, and bis(glycerophospho)glycerol. The enzyme was specific for phosphocholine, glycerophosphoglycerol, and bis(glycerophospho)glycerol. The enzyme was specific for glycerophosphodiesters; bis(p-nitrophenyl)phosphate, a substrate for other phosphodiesterases, was not hydrolyzed. In a coupled spectrophotometric assay utilizing sn-glycerol 3-phosphate dehydrogenase and NADH, apparent activity was optimal at pH 9 and was stimulated by Ca2+. The substrates of the phosphodiesterase had no affinity for the glpT-encoded active transport system. Thus, the glpQ gene product expands the catalytic capability of the glp regulon to include a variety of glycerophosphodiesters.

The catabolism of glycerol and glycerol-3-P in Escherichia coli is mediated by the components of the glp regulon (1) (Fig. 1). The regulon is composed of a membrane diffusion facilitator for glycerol (encoded by glpF), a cytoplasmic glycerol kinase (glpK), an active transport system for glycerol-3-P (glpT), and two glycerol-3-P dehydrogenases, one of which functions aerobically (glpD), the other anaerobically (glpA). The glp genes map in three different regions of the chromosome. All are negatively regulated by glpR; inducer for the system is glycerol-3-P (1).

The enzymatic steps of the dissimilatory pathway have been studied using the purified components (1, 4, 5). However, characterization of the proteins involved in membrane permeation of the substrates into the glp-encoded dissimilatory system, close correlation between active transport function and the presence of a M, ≈ 40,000 periplasmic protein was observed (6-9). This protein appeared to be part of the glpT system and was therefore named GLPT-protein (6). The role of this protein in the transport process remained obscure. Membrane vesicles devoid of the protein still accumulate glycerol-3-P (7, 9). Furthermore, the purified protein exhibits no binding affinity for glycerol-3-P, indicating it is not a periplasmic binding protein (9). These results, along with finding only one genetic complementation group among glpT mutations (10), suggest that a membrane permease alone should suffice for growth on glycerol-3-P. That this is indeed the case was shown by isolation of recombinant plasmids which carry the glpT gene, but are deleted for the gene encoding periplasmic GLPT-protein (11). Such plasmids still encode the active transport system for glycerol-3-P and indicate that glpT is promoter-proximal to the gene (renamed glpQ) encoding the periplasmic protein (11).

Since the glpQ gene product is not required for active transport of glycerol-3-P, we considered the possibility that it may have an enzymatic function. The experiments described in this paper show that the protein is an enzyme which catalyzes the hydrolysis of glycerophosphodiesters.

Glycerol-3-P + H2O → glycerol-3-P + ROH

ROH can be any of several alcohols such as ethanolamine, choline, glycerol, or glycerophosphoglycerol. This periplasmic phosphodiesterase thereby provides the cell with the capability of channeling a wide variety of glycerophosphodiesters into the glp-encoded dissipatory system.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions—The bacterial strains and plasmids utilized in this work are shown in Table I. Strains harboring recombinant plasmids were grown until late exponential phase in LB medium (15) containing 0.4% glycerol and the appropriate antibiotic (50 μg/ml of tetracycline HCl or 50 μg/ml of ampicillin) prior to preparation of cold osmotic shock fractions (16) or measurement of glycerol-3-P transport activity. When minimal media were used, the A and B salts of Clark and Maaløe (17) were supplemented with 2 μg/ml of thiamine HCl and the indicated carbon sources at 0.4%.

Preparation of Substrates—The phosphophosphodiesterases used as substrates were purchased from Sigma (glycerophosphoethanolamine and glycerophosphocholine (grade I, CdCl2 complex)) or else were prepared from phosphatidylglycerol and cardiolipin (Sigma) by mild alkaline deacylation (18) to form glycerophosphoglycerol and bis(glycerophospho)glycerol. The mild alkaline deacylation products of crude E. coli phospholipids (isolated from frozen cell paste by chloroform/methanol extraction followed by acetone precipitation (18)) were used as substrate in the initial experiments.

The chromatographic properties and purities of the deacylation products were assessed using ascending chromatography on Whatman No. 3MM paper in the solvent system 3-propanol/water/concentrated ammonia (2:2:1). The Rf values of phosphorus-containing spots were: 0.47 (glycerophosphoglycerol), 0.44 (glycerophosphoethanolamine), 0.36 (glycerophosphocholine), 0.34 (bis(glycerophospho)glycerol), and 0.3, 0.4, and 0.5 (decayed E. coli phospholipids). These Rf values are close to those expected (18). The substrates freshly obtained from

* This research was supported by Grant SFB138 from the Deutsche Forschungsgemeinschaft and by grants from the Fonds der Chemischen Industrie and NATO. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported by an Alexander von Humboldt Postdoctoral Research Fellowship. To whom reprint requests may be addressed at, the Department of Biochemistry, Duke University Medical Center, Durham, N. C. 27710.

1 The abbreviation used is: glycerol-3-P, sn-glycerol 3-phosphate.

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ity was determined of 4.86 ml of hydrazine hydrate plus 1.5 g of glycine to 100 ml with 20 units/ml of glycerol-3-P dehydrogenase (Sigma), 0.5 mM glycero-

...rounds of alkali hydrolysis of commercial phospholipids contained only one phosphorus-positive spot; glycerophosphocholine and glycerophospho-

**Glycerol-3-P metabolism in E. coli.** The known genes are glpF, glycerol diffusion facilitator; glpK, glycerol kinase; glpD, aerobic glycerol-3-P dehydrogenase; glpA, anaerobic glycerol-3-P dehydrogenase; glpT, glycerol-3-P active transport; glpQ, glycerophos-

**TABLE I**  
Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype</th>
<th>Reference</th>
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<tr>
<td>E. coli K12</td>
<td>araD139 Δ(argF-lac)U169</td>
<td>12</td>
</tr>
<tr>
<td>MC4100</td>
<td>5'</td>
<td>11</td>
</tr>
<tr>
<td>TS100</td>
<td>MC4100 glpR2</td>
<td>10</td>
</tr>
<tr>
<td>DL291</td>
<td>TS100 gvrA Δ(glpT</td>
<td>10</td>
</tr>
<tr>
<td>GL304</td>
<td>(gvrA)593 recA</td>
<td>13</td>
</tr>
<tr>
<td>BB2636</td>
<td>HfrC phoA8 relA glpD3 glpR2</td>
<td>P(DL291→BB2636)</td>
</tr>
<tr>
<td>GL305</td>
<td>gvrAΔ(glpT-</td>
<td>11</td>
</tr>
<tr>
<td>BL86</td>
<td>gvrA593</td>
<td>11</td>
</tr>
<tr>
<td>Plasmid*</td>
<td>tet bla</td>
<td>14</td>
</tr>
<tr>
<td>pBR222</td>
<td>tet glpT-glqP</td>
<td>11</td>
</tr>
<tr>
<td>pGT221</td>
<td>tet ΔglpT-glqP</td>
<td>11</td>
</tr>
<tr>
<td>pGS34</td>
<td>tet glpT500-glqP</td>
<td>11</td>
</tr>
<tr>
<td>pGS3100</td>
<td>tet gvrA-Δ(glpT-glqP)</td>
<td>11</td>
</tr>
<tr>
<td>pB13</td>
<td>blu gvrA-glqP</td>
<td>11</td>
</tr>
<tr>
<td>pB111</td>
<td>blu glpT-glqP</td>
<td>11</td>
</tr>
</tbody>
</table>

* Wild type genes are indicated unless a defect is specified.

...and 0.1–1 µg of cold osmotic shock protein. The rate of NAD reduction was followed by recording the increase in absorbance at 340 nm. Conditions were used where enzymatic activity was linear with respect to added shock proteins. One unit of phosphodiesterase activity is defined as the amount of enzyme required to hydrolyze 1 µmol of glycerophosphodiester/min under these condi-

**RESULTS**

**Glycerophosphodiesterases Are Hydrolyzed by a Phosphodiesterase Encoded by glpQ.** Recent experiments demonstrated that the periplasmic protein encoded by glpQ is not required for active transport of glycerol-3-P (11). Therefore, we considered a possible enzymatic function for the protein. It has been reported that *E. coli* contain low levels of a soluble enzyme which catalyzes the hydrolysis of glycerophospho-

**Coordinate Expression of Glycerophosphodiester Phos-
phodiesterase and the Glycerol-3-P Active Transport System. Because the glpT and glpQ genes are organized in a transcriptional unit (11), it can be predicted that they are coordinately expressed. Results showing this to be the case are shown in Table III. Here, strain MC4100 (wild type for the glp regulon) and the isogenic glpR (constitutive) strain TS100 were grown on minimal medium containing the indicated carbon source. After overnight growth, glycerol-3-P transport activity was measured in washed cells, and the glycerophosphoglycerol phosphodiesterase specific activity was determined in cold osmotic shock fractions. In both strains, transport and phosphodiesterase activity were induced by growth on glycerol or glycerol-3-P. Growth on glucose repressed both activities, although the glpR allele decreased the degree of catabolite repression. Both activities were expressed constitutively in the glpR strain TS100 after growth on succinate. Under these conditions, the activities were uninduced in the wild type strain MC4100.

By comparison of the induced level of the phosphodiesterase (Table III) with that found in the periplasm of strains harboring cloned glpT-glqQ (Table II), it is apparent that the multicyclic plasmids caused a 10- to 15-fold increase in phosphodiesterase specific activity. Glycerol-3-P transport rates in strains harboring multicopy glpT-glqQ3 (see Table VI) were not significantly greater than those found for the induced strains shown in Table III. This could be due to rate-limiting diffusion of glycerol-3-P through the outer envelope layers under the conditions employed (0.3 μM [14C]glycerol-3-P).

Characterization of the Glycerophosphoglycerol Phosphodiesterase Activity—To determine optimal conditions for assay of the phosphodiesterase, several parameters were investigated. Because the coupled spectrophotometric assay described above is carried out in 1 M hydrazine buffer at pH 9 (to trap dihydroxyacetone phosphate produced by formation of the hydrazone derivative (19)), the influence of hydrazine on phosphodiesterase activity was determined using a two-step assay. In the first step, glycerophosphoglycerol was incubated with phosphodiesterase in 0.1 M Tris-HCl (pH 8.4) containing increasing amounts of hydrazine buffer (pH 9). After stopping the reaction by heating (90 °C, 5 min), the total glycerol-3-P produced was measured spectrophotometrically using glycerol-3-P dehydrogenase in the usual manner. It was found that hydrazine (0.8 m) had no apparent inhibitory effect on phosphodiesterase activity. Thus, the coupled spectrophotometric assay should be valid for determination of enzyme activity.

The assay was optimized with respect to glycerol-3-P dehydrogenase and phosphodiesterase. Under standard conditions, activity was linear with respect to added shock proteins (from DL291/pGS31) in the 0.1- to 1-μg range.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Plasmid genotype</th>
<th>Glycerophosphate-glycerol phosphodiesterase units/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGS31</td>
<td>glpT-glqQ</td>
<td>23</td>
</tr>
<tr>
<td>pGS3100</td>
<td>glpT600-glqQ8</td>
<td>10.6</td>
</tr>
<tr>
<td>pBS3</td>
<td>glpT-glqQ</td>
<td>4.6</td>
</tr>
<tr>
<td>pGS4</td>
<td>ΔglpT-glqQ</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>pBR322</td>
<td>(Vector)</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

* glpT600 is a nonpolypeptide mutation.
* glpT603 is an amber nonsense mutation.

To determine the optimal pH for the assay, 1 M hydrazine/glycine buffer was adjusted to pH values ranging from 7.5 to 10.5 using HCl or NaOH. Very little activity was observed at these extreme values. Between these values, a symmetrical bell-shaped activity profile was observed, with optimal activity at pH 9. It should be pointed out that the pH of the reaction may influence not only phosphodiesterase activity, but also glycerol-3-P dehydrogenase and the chemical reaction of dihydroxyacetone phosphate with hydrazine.

The influence of various ions in the coupled assay was investigated. Addition of 10 mM Na2HPO4, 20 mM KNO3, or 70 mM NaCl had no influence on apparent enzyme activity. However, the presence of divalent ions did influence enzyme activity (Table IV). Whereas Mg2+ and Mn2+ at 4 mM decreased activity, Ca2+ had a stimulatory effect. Addition of EDTA caused marked inhibition of activity. EDTA or Ca2+ did not influence the excess glycerol-3-P dehydrogenase present in the assay, which suggests that Ca2+ specifically affects phosphodiesterase activity. ZnCl2 had no apparent influence on activity.

Based upon the above results, the standard assay was done in 1 M hydrazine/glycine buffer (pH 9.0) containing 10 mM CaCl2, 0.5 mM NAD, 20 units/ml of glycerol-3-P dehydrogenase and phosphodiesterase (0.1-1 μg of shock fluid proteins). Glycerophosphodiesterases employed as substrates were present at 0.5 mM except where otherwise indicated.

Substrate Specificity of the Glycerophosphodiester Phosphodiesterase—Many hydrolytic enzymes found in the periplasm exhibit broad substrate specificity (24). Active transport systems are generally not available for the substrates but are available for the product(s) of these enzymes (24). The phospho-
[Phosphodiesterase encoded by glpQ] apparently falls into this category of lyticolytic enzymes, since several glycerophosphodiesterases tested were hydrolyzed (Table V) but were not recognized by the glpT-encoded transport system (see below). Determination of the apparent $K_a$ and $V_{max}$ values for the substrates showed that glycerophosphoethanolamine, glycerophosphocholine, and glycerophosphoglycerol were essentially equivalent as substrates ($K_a$ values of 0.2 mM; $V_{max}$ values of 22-30 units/mg). The enzyme had less affinity for the deacylation product of cardiolipin, bis(glycerophospho)glycerol ($K_a = 0.6$ mM). $V_{max}$ using this substrate was about the same as for the above substrates.

Initial rate measurements for hydrolysis of bis(glycerophospho)glycerol were not corrected for the possibility of this substrate yielding 2 mol of glycerol-3-P/mol of substrate. The apparent $V_{max}$ reported in Table V should be valid unless the glycerophosphodiesterolytic system formed reacts much more quickly than the bulk bis(glycerophospho)glycerol present. Rates were determined as soon as possible after addition of phosphodiesterase, before the theoretical concentration of glycerophosphoglycerol had reached 0.01 mM.

Glycerol-3-P was also produced when the phosphodiesterase was incubated with cyclic sn-1,3(2)-2-phosphoglycerol, but at a lower rate when compared to the other substrates tested (Table V). The apparent $K_a$ with respect to cyclic sn-1,3(2)-phosphoglycerol was also higher. Cyclic phosphoglycerol can be released from membrane-derived oligosaccharides in vitro by action of a periplasmic phosphoglycerol transferase (25). But, because it is likely this enzyme functions as a transferase in vivo and because the stereochemistry of the cyclic phosphoglycerol formed is presumed to be sn-1,2 (25), there is no connection between this compound and the glpQ-encoded phosphodiesterase. In preliminary experiments, hydrolysis of the sn-1,2-isomer by the phosphodiesterase was not detected.

The phosphodiesterase did not hydrolyze bis(p-nitrophenoxy)phosphate, a substrate for periplasmic cyclic phosphodiesterase of E. coli (26). Thus, the enzyme recognizes the glycerophospho moiety and apparently does not hydrolyze other types of phosphodiester bonds.

**The glpT-encoded Active Transport System Has No Affinity for the Related Glycerophosphodiesterases.** The glpT system exhibits some flexibility with respect to substrate specificity, mm; retaining not only glycerol-3-P, but also its phosphonate analogs 3,4-dihydroxybutyl-1-phosphonate, the antibiotic fosfomycin, glyceraldehyde-3-P, and inorganic phosphate (with low affinity). To see if the transport system recognizes glycerophosphodiesters, the experiment shown in Table VI was done. Accumulation of [$^{14}$C]glycerol-3-P by washed cells was determined as soon as possible after addition of the labeled substrate yielding 2 mol of glycerol-3-P/mol of substrate. The apparent $V_{max}$ was determined in the presence or absence of glycerophosphoethanolamine or glycerophosphocholine. In the glpT-glpQ strain DL291/pB13 (Table VI), in this strain, the glycerophosphodiesterases had no inhibitory effect on transport, even though present at greater than 1,000-fold excess over [$^{14}$C]glycerol-3-P. These results show that the glpT-encoded transport system has no apparent affinity for these glycerophosphodiesterases.

Results showing that glycerophosphodiesterases are not accumulated via other transport systems and subsequently hydrolyzed were obtained using the glycerol-3-P auxotrophic strain BB2636 (pBS26) and its Δ(glpT-glpa)593 gyrA derivative, TL86. Glycerophosphodiesters derived from E. coli phospholipids when present at 0.2 mM complemented the glycerol-3-P auxotrophy in BB2636 but not in TL86.

**DISCUSSION**

These results have clarified the relationship between the glpT-encoded active transport system for glycerol-3-P and the periplasmic protein encoded by glpQ. Action of the glpQ-encoded phosphodiesterase on extractophospholipid glycerophosphodiesterases provides glycerol-3-P which is readily accumulated via the glycerol-3-P permease present in the cytoplasmic membrane (Fig. 1). Organization of the genes encoding these two intimately related functions into a transcriptional unit provides for co-regulation of their expression independent of the other glp operons whose operators have differing affinities for the 3-glpr repressor and are more or less sensitive to catabolite repression (1).

Because of its promotor-distal location in the operon, glpQ could be lost by mutation without affecting the remaining members of the glp regulon. Preservation of glpQ indicates that the cell may be frequently exposed to glycerophosphodiesterases. Indeed, such phosphodiesterases are the degradation products of compounds which are abundant in organic material (e.g. glycerophospholipids and teichoic and lipoteichoic acids). In addition, glycerophosphodiesterases could be the products of reactions localized in the bacterial cell envelope. Various enzyme activities capable of yielding glycerophosphodiesterase from phospholipids are present in the E. coli cell envelope (21-23, 27), although their physiological significance is incompletely understood.

It is interesting to note that the components of the glp regulon are especially well suited for utilization of glycerophosphoglycerol. In this case, action of the periplasmic phosphodiesterase yields glycerol-3-P plus glycerol, both of which are readily assimilated via the previously defined pathways.

**Acknowledgments**—We would like to thank Anke Middendorf for skillful technical assistance, E. P. Kenney for the gift of cyclic sn-

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**Table V**

<table>
<thead>
<tr>
<th>Substrate specificity of glycerophosphodiesterase</th>
<th>Substrate</th>
<th>$K_a$ (mM)</th>
<th>$V_{max}$ (units/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerophosphoethanolamine</td>
<td>0.2</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>Glycerophosphocholine</td>
<td>0.2</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Glycerophosphoglycerol</td>
<td>0.2</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>Bis(glycerophospho)glycerol</td>
<td>0.4</td>
<td>27</td>
<td></td>
</tr>
</tbody>
</table>

*Units/mg of cold osmotic shock protein from DL291/pGS31.

* Determined using the chemically synthesized equimolar mixture of cyclic sn-2,3- and sn-1,2-phosphoglycerol.

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**Table VI**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Plasmid genotype</th>
<th>Addition</th>
<th>Transport rate (μM)</th>
</tr>
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<tbody>
<tr>
<td>pBS1</td>
<td>glpT+glpQ+</td>
<td>None</td>
<td>12</td>
</tr>
<tr>
<td>pBS1</td>
<td>glpT+glpQ+</td>
<td>Glycerophosphoethanolamine</td>
<td>2</td>
</tr>
<tr>
<td>pBS1</td>
<td>glpT+glpQ+</td>
<td>Glycerophosphocholine</td>
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</tr>
<tr>
<td>pBS1</td>
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<td>Glycerophosphoglycerol</td>
<td>2</td>
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<tr>
<td>pBS1</td>
<td>glpT+glpQ+</td>
<td>Glycerophosphoethanolamine</td>
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<tr>
<td>pBS1</td>
<td>glpT+glpQ+</td>
<td>Glycerophosphocholine</td>
<td>15</td>
</tr>
</tbody>
</table>

*Expressed as picomole of [14C]glycerol-3-P accumulated per min by 1 x 10^9 cells.

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Periplasmic glpQ-encoded Phosphodiesterase of E. coli

1(3),2-phosphoglycerol, and E. C. C. Lin for the suggestion that the periplasmic GLPT (GlpQ)-protein may be an enzyme which hydrolyzes this compound.

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Periplasmic glycerophosphodiester phosphodiesterase of Escherichia coli, a new enzyme of the glp regulon.
T J Larson, M Ehrmann and W Boos