Cloning and Sequencing of two Enterococcal glpK Genes and Regulation of the Encoded Glycerol Kinases by Phosphoenolpyruvate-dependent, Phosphotransferase System-catalyzed Phosphorylation of a Single Histidyl Residue*

(Received for publication, February 18, 1997, and in revised form, March 24, 1997)

Véronique Charrier‡, Ellen Buckley§, Derek Parsonage§, Anne Galinier‡, Emmanuelle Darbon‡, Michel Jaquindes, Eric Forests, Josef Deutscher‡, and Al Claiborne§

From the ‡Institut de Biologie et Chimie des Protéines, CNRS, 7 passage du Vercors, F-69367 Lyon Cedex 07, France, §Department of Biochemistry, Wake Forest University Medical Center, Winston-Salem, North Carolina 27157, and ¶Institut de Biologie Structurale, CNRS, 41 avenue des Martyrs, F-38027 Grenoble Cedex 1, France

The glpK genes of Enterococcus casseliflavus and Enterococcus faecalis, encoding glycerol kinase, the key enzyme of glycerol uptake and metabolism in bacteria, have been cloned and sequenced. The translated amino acid sequences exhibit strong homology to the amino acid sequences of other bacterial glycerol kinases. After expression of the enterococcal glpK genes in Escherichia coli, both glycerol kinases were purified and were found to be phosphorylated by enzyme I and the histidine-containing protein of the phosphoenolpyruvate:glycose phosphotransferase system. Phosphoenolpyruvate-dependent phosphorylation caused a 9-fold increase in enzyme activity. The site of phosphorylation in glycerol kinase of E. casseliflavus was determined as His-232. Site-specific mutagenesis was used to replace His-232 in glycerol kinase of E. casseliflavus with an alanyl, glutamate, or argynyl residue. The mutant proteins could no longer be phosphorylated confirming that His-232 of E. casseliflavus glycerol kinase represents the site of phosphorylation. The His\(^{232} \rightarrow\) Arg glycerol kinase exhibited an about 3-fold elevated activity compared with wild-type glycerol kinase. Fructose 1,6-bisphosphate was found to inhibit E. casseliflavus glycerol kinase activity. However, neither EI\(\text{A}^{\text{Glc}}\) from E. coli nor the EI\(\text{A}^{\text{Glc}}\) domain of Bacillus subtilis had an inhibitory effect on glycerol kinase of E. casseliflavus.

Glycerol uptake in Gram-negative and Gram-positive bacteria is mediated by the glycerol diffusion facilitator, an integral membrane protein of 30 kDa, catalyzing the rapid equilibration of concentration gradients of glycerol across the cytoplasmic membrane. The intracellular glycerol is converted to glycerol-3-P by the enzyme glycerol kinase that uses ATP as phosphoryl donor. Glycerol-3-P is not a substrate of the glycerol diffusion facilitator and hence remains entrapped in the cell, where it is further metabolized. Phosphorylation of glycerol by glycerol kinase provides the driving force for the uptake of glycerol, as it creates a constant imbalance of the glycerol concentrations inside and outside the cell. It was therefore not surprising that glycerol kinase is the target of several regulatory mechanisms.

Mutants of Gram-positive and Gram-negative bacteria defective in one of the general components of the phosphotransferase system (PTS), enzyme I (6–10), or histidine-containing protein (HP\(\text{Pr}\)) (11), had lost the ability to grow on glycerol as the sole carbon source, although glycerol is not transported by the PTS. EI\(\text{A}^{\text{Glc}}\) of the PTS has been shown to inhibit glycerol kinase activity in Escherichia coli (12) and Salmonella typhimurium (13) by allosteric interaction leading to reduced uptake of glycerol. P-EI\(\text{A}^{\text{Glc}}\) is not able to interact with glycerol kinase. The phosphorylatable His-90 in EI\(\text{A}^{\text{Glc}}\) (previously referred to as His-91) (14) is buried in the center of the interphase in the EI\(\text{A}^{\text{Glc}}\)-glycerol kinase complex, and phosphorylation at His-90 of EI\(\text{A}^{\text{Glc}}\) has been proposed to prevent the formation of the complex by electrostatic repulsion (15).

Glycerol kinase of Gram-negative as well as Gram-positive bacteria is allosterically inhibited by fructose 1,6-bisphosphate (FBP) (4, 12, 16). However, in Gram-positive bacteria such as Enterococcus faecalis (17) or Bacillus stearothermophilus (18), PTS-dependent regulation of glycerol kinase was found to occur by phosphoenolpyruvate (PEP)-dependent phosphorylation at the N-3 position of a histidyl residue (4). This reaction is catalyzed by the general PTS proteins enzyme I and HPr. Phosphorylation of glycerol kinase, which was found to be reversible, i.e. P-glycerol kinase is able to phosphorylate HPr, increased glycerol kinase activity about 10-fold (4). By using polyclonal antibodies against glycerol kinase of E. faecalis it has been demonstrated that during growth on glycerol mainly the active, phosphorylated form of glycerol kinase was present in the cells (19). However, if a rapidly metabolizable PTS substrate was present in the growth medium in addition to glycerol, mainly unphosphorylated, less active glycerol kinase was found in the cells. It was therefore predicted that phosphorylation of glycerol kinase is used to regulate glycerol uptake and metabolism in response to the presence or absence of a PTS substrate in the growth medium (19).

The abbreviations used are: PTS, phosphotransferase system; cre, catabolite-responsive element; FBP, fructose 1,6-bisphosphate; HPr, histidine-containing protein; IPTG, isopropyl 1-thio-β-D-galactopyranoside; ESI, electrospray ionization; MS, mass spectrometry; MALDI, matrix assisted laser desorption ionization; PAG, polyacrylamide gel; PCR, polymerase chain reaction; PEP, phosphoenolpyruvate; GlpO, α-glycerophosphate oxidase; contig, group of overlapping clones; NTA, nitrilotriacetic acid; bp, base pair(s); kb, kilobase pair(s).

1 The costs of publication of this article were defrayed in part by the European Community Biotech Program (Contract No. BI02-CT92-0137 and BI04-CT96-0380) and by the CNRS (to J. D.), and National Institutes of Health Grant GM-35394 and National Science Foundation Grant INT-9400123 (to A. C.). 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.

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1 To whom correspondence should be addressed: Institut de Biologie et Chimie des Protéines, CNRS, 7 passage du Vercors, F-69367 Lyon Cedex 07, France. Tel.: (33) 4 72 72 26 77; Fax: (33) 4 72 76 90 50; E-mail: jcl@ibcp.fr.

2 J. Deutscher and M. Steinmetz, unpublished results.
We have cloned and sequenced the glpK genes, which encode glycerol kinase, from Enterococcus casseliflavus and E. faecalis. We found that His-232 of glycerol kinase from E. casseliflavus is the site of PEP-dependent phosphorylation and that mutations affecting His-232 lead to the loss of phosphorylation and activation of glycerol kinase.

**EXPERIMENTAL PROCEDURES**

**Materials**—Isopropyl 1-thio-β-D-galactopyranoside (IPTG) was purchased from 5Prime-3Prime, Inc., or from TEBU, France, and agarose was from FMC Bioproducts. y-[35S]ATP was purchased from Amersham, Farnham, France.

**Bacterial Strains**—E. casseliflavus (ATCC 12755) was grown with shaking at 37 °C in M17 medium (20) supplemented with 1% (w/v) glucose (GM17). Stationary phase E. casseliflavus was harvested in the same buffer containing 15 mg/ml tetracycline and appropriate antibiotics when transformed with plasmids: ampicillin at 50–100 μg/ml or chloramphenicol at 50 μg/ml.

**glpK Oligonucleotide Design and PCR Amplification**—A GAP alignment of the protein sequences for glycerol kinase from E. coli (22) and Bacillus subtilis (23) indicated that the B. subtilis sequence from Ile-76 to Thr-85 is absolutely conserved between the two species. This consensus sequence was used to design the gk1 primer, using enterococcal codon usage data (24).

\[ \text{glk1: } 5'\text{-ATT GGA ATT ACA AAT CAA CGA GAA AC-3'} \]
\[ C \text{ C T C C} \]

**Ile-Gly-Ile-Thr-Asn-Gln-Arg-Glu-Thr**

**Scheme I**

The gko1 (sense) and gko3 (antisense) oligonucleotide primers were then designed from the α-glycerophosphate oxidase (GlpO) N-terminal and Gpo3 peptide sequences (25).

**Genomic DNA** from E. casseliflavus was subjected to amplification by polymerase chain reaction (PCR) using either gko1 plus gko3 or glk1 plus gko3 primer combinations; the 0.9- and 2.4-kb PCR products obtained from these reactions, respectively, were cloned into either pCRII (Invitrogen) or pCR-Script SK(−) (Stratagene) vector. The plasmid containing the 2.4-kb gko3 PCR product was used to identify restriction sites within the insert.

**Preparation and Analysis of Genomic DNA**—Chromosomal DNAs were prepared from E. casseliflavus and E. faecalis by the methods of Caparon and Scott (26) and Anderson and McKay (27), respectively, after overnight growth in GM17. Southern blots were probed with digoxigenin-labeled DNA using a Genius kit from Boehringer Mannheim. A subgenomic E. casseliflavus library was constructed using a size-fractionated (4.5-kb) pool of HindIII-digested chromosomal DNA in pWKS30 (28), transferred to competent XLI-Blue cells (29). Colonies were screened by hybridization with a digoxigenin-labeled probe of the 2.4-kb gko3 PCR product and the 1.1-kb CloI fragment. Possible positive clones were confirmed by restriction digest and by PCR analysis of plasmid DNA.

**DNA Sequencing of glpK Clones**—A 3.5-kb HindIII/PstI fragment from the E. casseliflavus glpK clone pGO60 was subcloned into the plasmid pMOB, to allow sequencing using the TN1000 transposon system (30). Isolates were chosen which had transposon insertions at approximately 200-bp intervals. Specific oligonucleotides were used to elucidate sequence ambiguity in cases where only one DNA strand was harvested. Overall, the entire glkK locus as contained within the pMOB subclone (pGLP07) was sequenced on both strands. Contig assembly, GAP alignments, and database searches were carried out using the GCG suite of DNA analysis programs (31).

The glkK gene from E. faecalis 26487 was cloned using a similar subgenomic approach. A Southern blot of E. faecalis genomic DNA was probed with a digoxigenin-labeled DNA fragment containing the entire E. casseliflavus glpK gene, to identify a 1.6-kb EcoRI/BamHI fragment for cloning. Sequence analysis of this EcoRIBamHI clone in pMOB (pGLP12) indicated that it lacked approximately 150–180 bp corresponding to the 5′-end of the gene. Genomic Southern combined with the partial sequence available indicated that a 0.7–0.8-kb DraI fragment would complete the glpK coding sequence. A pool of appropriately sized DraI fragments, generated by digestion of chromosomal DNA, was circularized with T4 DNA ligase. This circularized DNA was then used as the template to PCR amplify the 5′-end of the glkK gene using two divergent primers designed from the known sequence of pGLP12. The resulting PCR product was cloned into pBluescript, and the H232A, H232E, and H232R mutants were generated in this plasmid using the MutS-H polymerase mutagenesis kit (Amersham Corp.). The plasmid expression plasmid for wild-type glpK was constructed by subcloning the 2-kb EcoRI/PstI fragment containing the structural gene and ribosome-binding site into the T7 expression vector pOXO4 (32) cut with XhoI (made blunt end) and PstI. The recombinant glycerol kinase was expressed in E. coli JM109DE3 by growing cells in Luria Bertani medium containing 30 μg/ml chloramphenicol at 37 °C until they had reached an A600 of about 1. IPTG was subsequently added to a final concentration of 0.5 mM and cells were incubated for an additional 4 h at 37 °C. The mutant proteins were expressed following the protocol developed for the wild-type enzyme. To create an expression vector for E. faecalis glpK, the 5′-end of the gene was PCR-amplified using a primer derived from the glpK sequence downstream of the internal EcoRI site and primer EFGKRB, which included an immediately upstream of the glpK ribosome-binding site: GCGTCTGGAGGAGAATTATCGATGGCAGAAG (the XhoI site is in italics and the initiation codon of glpK is underlined). The resulting 237-bp PCR product was digested with XhoI and EcoRI and ligated into the pGLP12 clone digested with the same restriction enzymes. The complete E. faecalis glpK gene with its ribosome-binding site was excised by digestion with XhoI and BamHI and ligated into pXO4 to generate the plasmid pEFGLPK3. The PCR-generated fragment of glpK was sequenced to confirm the subcloning. This plasmid was transformed into E. coli BL21DE3 for expression of the E. faecalis glycerol kinase after induction with IPTG, which gave better results than the strain JM109DE3 that we used for expression of glpK from E. casseliflavus.

**Purification of E. casseliflavus and E. faecalis Glycerol Kinase**—Cells were harvested by centrifugation after induction with IPTG and washed with 20 mM Tris/HCl buffer, pH 8, before they were resuspended in the same buffer containing 6 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride, and 240 units/ml of benzonuclease (Merck). Cells were subsequently disrupted by two passages through a French pressure cell at 15,000 p.s.i. Cell debris was removed by centrifugation and the glycerol kinase was loaded onto a DEAE column (Whatman DE-52, 2.5 × 7 cm) equilibrated with 20 mM Tris/HCl buffer, pH 8.0. Proteins were eluted with a gradient of 0–1 M NaCl in this buffer. Glycerol kinase of E. casseliflavus and E. faecalis eluted at around 0.6 M NaCl. Glycerol kinase containing fractions were pooled, concentrated and dialyzed against 20 mM Tris/HCl, pH 8, containing 1 mM EDTA (disodium salt) and 1 mM dithiothreitol by using Vivaspin vials (Vivascience, France). The dialyzed solution was loaded onto a Q Sepharose (Fast Flow) column (Pharmacia Biotech Inc.) equilibrated with 20 mM Tris/HCl, pH 8.0. Proteins were eluted with a gradient of 0–1 M NaCl in the above buffer. Glycerol kinase eluted as a single peak and was more than 90% pure. The glycerol kinase pool was desalted after chromatography on a Sephadex G-25 column equilibrated with 50 mM ammonium bicarbonate, lyophilized, and stored at −20 °C.

**Enzymatic Test of Glycerol Kinase Activity**—Glycerol kinase activity was determined by using a coupled spectrophotometric assay carried out in 100 mM glycine/hydrazine buffer, pH 8.8, as described in Deutscher and Sauerwald (4). To measure the effect of fructose 1,6-biphosphate or EIAl₄⁺ from E. coli or B. subtilis on glycerol kinase activity, these compounds were included in the reaction mixture at the indicated concentrations. EIAl₄⁺ was purified from E. coli as described in Dorshuc et al. (14) and the B. subtilis EIAl₄⁺ domain was prepared as described in Reizer et al. (33). To measure the effect of phosphorylation on glycerol kinase activity, glycerol kinase (2.5 μg) was incubated with PEP, MgCl₂, enzyme I (0.5 μg), and HPr (1 μg) and after 20 min incubation at 37 °C, one-fifth of the reaction mixture was used for the glycerol kinase assay, the remaining four-fifths were loaded on a denaturing 7.5% polyacrylamide (7.5%) gel and stained with silver nitrate (PAG) to determine the degree of phosphorylation of glycerol kinase.

**Phosphorylation of Glycerol Kinase**—To determine the site of phosphorylation in glycerol kinase, the enzyme isolated from E. casseliflavus was phosphorylated using enzyme I and HPr from B. subtilis. Both PTS proteins were synthesized with a His tag attached to the N-terminus (34). This allowed a rapid, one-step purification of the two general PTS.
Proteins. Glycerol kinase (75 μg) was incubated with enzyme 1 (10 μg) and HPr (5 μg) in 90 μL of 50 mM Tris/HCl, pH 8.0, containing 10 mM MgCl₂ and 5 mM PEP at 37 °C for 20 min. After incubation, most of the enzyme 1 and HPr was removed by adding a small amount of Ni-NTA resin in the incubation mixture which was subsequently removed by centrifugation. A 3-μl aliquot was taken from the assay mixture and loaded on a denaturing 7.5% PAGE to determine the approximate degree of phosphorylation of glycerol kinase. Trypsin (3 μg) was subsequently added to the P-glycerol kinase preparation which was incubated for a further 3 h at 37 °C. Tryptic peptides of glycerol kinase were obtained by incubating glycerol kinase with enzyme 1 and HPr in the absence of PEP but using otherwise the same procedure described for P-glycerol kinase. The tryptic peptides of glycerol kinase and P-glycerol kinase were subsequently analyzed by mass spectrometry. Phosphorylation of wild-type glycerol kinase or of the mutant derivatives for analytical purposes or with [32P]PEP was carried out by incubating 3 μg of glycerol kinase for 20 min at 37 °C with 0.5 μg of enzyme 1 from Staphylococcus carnosus or B. subtilis and 1 μg of HPr (B. subtilis) in 50 mM Tris/HCl buffer containing 10 mM MgCl₂ and either 10 mM PEP or 0.5 μM [32P]PEP (0.1 μCi) in a total volume of 20 μL. [32P]PEP was prepared from γ-[32P]ATP as described in Roosien et al. (35).

Mass Spectrometry—Liquid chromatography coupled to electrospray ionization mass spectrometry (ESI-MS) was performed using a SCIEX API III+ triple quadrupole mass spectrometer (Perkin-Elmer Sciex Instrument, Thornhill, Canada) equipped with a nebulizer-assisted electrospray (ionspray) source. Liquid chromatography was directly coupled to ESI-MS using a 140B syringe pump system (Applied Biosystems, Foster City, CA). Tryptic digests of glycerol kinase or P-glycerol kinase were loaded onto a Brownlee reverse phase C18 column (5 μm, 1 mm × 100 mm, Applied Biosystems) run with a flow rate of 50 μL/min. Using a Valco T; a split of 1/3 was applied such that approximately 15 μL/min was directed to the mass spectrometer. The remaining effluent was diverted to an Applied Biosystems 785 UV detector monitoring at 214 nm. Peptide separation was achieved using a linear gradient of 0–100% acetonitrile (containing 0.1% trifluoroacetic acid) with a slope of 1%/min. Mass spectra (0–100% acetonitrile) were acquired with a 2-ms dwell time per step of 0.9 Da.

Matrix-assisted laser desorption ionization mass spectra (MALDI-MS) of peptides derived from glycerol kinase or P-glycerol kinase were recorded on a RETOF (time of flight) instrument from Perseptive Biosystems (Framingham, MA). A solution of 2,5-dihydroxybenzoic acid dissolved in 70% acetonitrile was used as a matrix. A 1-μl aliquot of the tryptic peptide mixture, containing 15 pmol of digested protein, was mixed with 1 μL of the matrix solution, and half of it was deposited on the target and dried. Spectra were recorded from 128 laser shots (nitrogen laser, 337 nm) with an accelerating voltage of 20,000 V in a linear mode with a 50-ns pulsed source delay extraction. The instrument was calibrated using human insulin and horse hemoglobin as standards.

RESULTS

Cloning of the glpK Genes from E. casseliflavus and E. faecalis—The glpk gene in B. subtilis precedes the glpD locus encoding the FAD-dependent α-glycerophosphate dehydrogenase (23), and sequence analyses of four peptides (including Gpo3) isolated from the E. casseliflavus α-glycerophosphate oxidase (GpoO) indicated identities of 47–62% with corresponding segments of the B. subtilis Gpo3 (25). To test for the possible linkage of the glpK and glpO loci in E. casseliflavus, an oligonucleotide primer (glk1) was designed based on a GAP alignment of the B. subtilis (23) and E. coli (22) glk loci. The PCR product was also mapped in P-CR-Script with a series of restriction digests (25); both the 1.1-kb ClaI fragment corresponding to glpK and the 2.4-kb PCR product were used as probes in the glpK clone described.

Based on the results of Southern analyses, genomic DNA was digested with HindIII, and fragments of 4–5 kb were used to generate a subgenomic library in the low copy number plasmid pWSK30 (28). XL-1 Blue transformants were screened by hybridization with the glpKO probe. From approximately 120 recombinant colonies screened, a single positive clone (containing plasmid pGLP06) was isolated. The identity of the insert of pGLP06 with part of the E. casseliflavus glp operon was confirmed by restriction analysis based on the known PstI and KpnI sites (25) and by PCR analysis with both glk and gpo primers.

Nucleotide Sequence of the glpK Genes—The 3.5 kb HindIII/PstI fragment of the pGLP06 insert was subcloned into the pMOB vector for transposon-facilitated sequencing (30), and complete nucleotide sequence data were obtained on both strands. The composite sequence includes 3511 bp that account for the entire glpK coding sequence (1521 bp) as well as a partial open reading frame of 467 bp following glpK, which corresponds to the 5′-end of the glpO gene encoding α-glycerophosphate oxidase. An intergenic region of only 3 bp separates the glpK stop codon from the glpO start codon; the glpK ribosome-binding site (AGGGAG) is identified 14 bp upstream of the initiation codon.

The E. faecalis glpK gene was sequenced as described under “Experimental Procedures.” On the basis of the N-terminal protein sequence for the E. faecalis 26487 glycerol kinase (AEEKYMAIDQGTTSSRA) the ATG codon corresponding to the start codon was recognized immediately, although the initiating Met is removed post-translationally in the E. faecalis enzyme. The E. faecalis glpK encodes a polypeptide of 501 amino acids with a calculated molecular weight of 55,445 (including Met-1), similar to the value of 55 kDa reported previously for the purified E. faecalis glycerol kinase (17).

Fig. 1 presents a CLUSTAL alignment for the glyk genes from E. casseliflavus and E. faecalis (this work) and two other Gram-positive sources (B. subtilis (23), Streptococcus pyogenes (36)) as well as two Gram-negative sources (E. coli (22) and Hemophilus influenzae (37)). The Mycoplasma genitalium glycerol kinase sequence (38) exhibited less homology to the above sequences and was therefore not included in this figure. GAP alignments reveal that the enterococcal sequences are strongly conserved (56–63% identity) as compared with both the E. coli and B. subtilis sequences. Among the absolutely conserved segments are the ADP pyrophosphate binding site (Asp-10, Arg-17, Gly-266, Thr-267, and Gly-411), elements of the glycerol binding site (Arg-83, Glu-84, Thr-103, Tyr-135, Asp-245, and Phe-270), and residues which interact with the ADP adenosine moeity (Ile-313, Glu-314, Ala-412, and Asn-415) (see Fig. 1). These functional assignments are derived from the 2.6-Å crystal structure of the regulatory complex of EIIAGlc and E. coli glycerol kinase (15).

Expression of the glpK Genes from E. casseliflavus and E. faecalis in E. coli and Purification of the Corresponding Glycerol Kinases—To overproduce glycerol kinase in E. coli, the glpK genes were cloned into the expression vector pOXO4 (32) as described under “Experimental Procedures.” The resulting plasmid containing glpK of E. casseliflavus was called pGLPK02 and contained the complete glpK gene and 467 bp of the following glpO gene, but was missing the glp promoter (see Fig. 2A). In this plasmid, expression of the glpK gene was controlled by the T7 promoter. The plasmid containing glpK of E. faecalis was called pEFLPK3. The plasmid pGLPK02 was transformed into the E. coli strain JM109DE3 and plasmid pEFLPK3 was transformed into the E. coli strain BL21DE3. In both E. coli strains the T7 RNA polymerase is expressed from an IPTG-inducible promoter. Transformants of

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3 H. E. Meyer and J. Deutscher, unpublished results.
**Fig. 1. Multiple alignment of amino acid sequences of glycerol kinases derived from four Gram-positive and two Gram-negative bacteria.**

**E. coli** (E. coli) stands for **E. casseliflavus** and **E. faecalis**. The glycerol kinase amino acid sequences of these two bacteria were derived from the DNA sequence of the corresponding gkp genes determined within this work (GenBank™ accession nos. U94355 for **E. casseliflavus** gkp and U94356 for **E. faecalis** gkp). The **B. subtilis** (B. subtilis) sequence was taken from **H. influenzae** (H. influenzae) sequence (36). The glycerol kinase sequences of the two Gram-negative bacteria **E. coli** (E. coli) and **H. influenzae** (H. influenzae) were taken from Pettigrew et al. (22) and Fleischmann et al. (37), respectively. The shaded sequence of **E. casseliflavus** glycerol kinase corresponds to the tryptic peptide 21 which contains the phosphorylatable His-232, indicated by an arrow 232.**

**JM109DE3** was overproduced to a 55-kDa protein (Fig. 2C, lane 2), which was barely synthesized in cells which were not induced with IPTG (Fig. 2C, lane 1). The molecular weight of the overexpressed protein is in good agreement with the molecular weight of 55,811 calculated for the 55-kDa protein. Similarly, expression of glycerol kinase from **E. faecalis** could be induced with IPTG in strain BL21DE3 carrying plasmid pEPLPK3 (data not shown).

**Crude extracts of glycerol kinase overproducing cells were prepared as described under “Experimental Procedures.”** Glycerol kinase from **E. casseliflavus** or **E. faecalis** was subsequently purified by ion exchange chromatography on two anion exchange columns, DEAE cellulose DE52 and Q-Sepharose Fast Flow. The elution profile from the second column obtained by SDS-polyacrylamide gel electrophoresis (Fig. 2) is shown in Fig. 2B for glycerol kinase from **E. casseliflavus**. The fractions corresponding to the peak indicated by an arrow contained glycerol kinase activity. These fractions were pooled, and the glycerol kinase preparation was found to be more than 95% pure and was judged by SDS-polyacrylamide gel electrophoresis (Fig. 2C, lane 3). Between 10 and 15 mg of **E. casseliflavus** glycerol kinase and about 5 mg of **E. faecalis** glycerol kinase were obtained from a 1-liter culture.

**PEP-dependent Phosphorylation of Glycerol Kinase—**As glycerol kinase isolated from **E. faecalis** has been shown to be phosphorylated by PEP, enzyme I, and HPr of the PTS (17), we tested whether the purified, enterococcal glycerol kinases isolated from **E. coli** can also be phosphorylated by these PTS proteins. Three techniques have been described to demonstrate PEP-dependent phosphorylation of glycerol kinase from **E. faecalis** (4, 17): phosphorylation with [32P]PEP followed by separation on a SDS-PAG and autoradiography or separation of glycerol kinase and P-glycerol kinase on either SDS or on nondenaturing PAGs. Using [32P]PEP we could demonstrate that recombinant glycerol kinase from **E. casseliflavus** and **E. faecalis** isolated from **E. coli** becomes phosphorylated by PEP, enzyme I, and HPr (Fig. 3, A and B, lanes 3). If either enzyme I or HPr were absent, no radioactive band corresponding to phosphorylated glycerol kinase was observed. The presence of P-glycerol kinase in Fig. 3C, lane 3, on which a sample containing glycerol kinase or enzyme I, HPr, MgCl2, and PEP had been loaded, corresponded to P-glycerol kinase. An identical result has previously been described for glycerol kinase purified from **E. faecalis**. Although glycerol kinase purified from **E. faecalis** has been demonstrated to migrate faster than P-glycerol kinase, no separation of phosphorylated and unphosphorylated forms of both recombinant glycerol kinases could also be separated on a SDS-PAG. The protein band migrating just above the band of P-glycerol kinase on either SDS or on nondenaturing PAGs. Using [32P]PEP we could demonstrate that recombinant glycerol kinase from **E. casseliflavus** and **E. faecalis** isolated from **E. coli** becomes phosphorylated by PEP, enzyme I, and HPr (Fig. 3, A and B, lanes 3). If either enzyme I or HPr were absent, no radioactive band corresponding to phosphorylated glycerol kinase was observed. The presence of P-glycerol kinase in Fig. 3C, lane 3, on which a sample containing glycerol kinase or enzyme I, HPr, MgCl2, and PEP had been loaded, corresponded to P-glycerol kinase. An identical result has previously been described for glycerol kinase purified from **E. faecalis**. Although glycerol kinase purified from **E. faecalis** has been demonstrated to migrate faster than P-glycerol kinase, no separation of P-glycerol kinase from P-glycerol kinase could be observed. The presence of P-glycerol kinase in the samples...
Induction of synthesis of glycerol kinase with IPTG and the purity of the arrow in therefore not due to expression in on a nondenaturing PAG in response to phosphorylation is therefore not due to expression in a SDS-PAG (10%) stained with Coomassie Brilliant Blue (C).

The phosphorylation assay using either 0.5 μM [32P]PEP (0.1 μCi) or 10 μm PEP contained in a total volume of 20 μl of 50 mm Tris HCl, pH 7.4, 10 mm MgCl2 and varying mixtures of the following proteins: 2 μg of glycerol kinase from E. casseliflavus or from E. faecalis, 0.5 μg of enzyme I, 1 μg of HPr. The assay mixtures were incubated for 20 min at 37 °C before SDS sample buffer was added to terminate the reaction. Samples containing [32P]PEP were separated on a denaturing 12.5% PAG which was subsequently exposed to autoradiography (A and B), whereas samples containing PEP were separated on a denaturing 7.5% PAG, which was stained with Coomassie Brilliant Blue (C). The samples loaded on the different lanes contained the following proteins. A, glycerol kinase from E. casseliflavus and B, glycerol kinase from E. faecalis: lane 1, enzyme I; lane 2, enzyme I and HPr; lane 3, enzyme I, HPr, and glycerol kinase; lane 4, glycerol kinase; lane 5, enzyme I and glycerol kinase; lane 6, HPr and glycerol kinase. C, glycerol kinase from E. casseliflavus: lane 1, molecular weight standards; lane 2, glycerol kinase; lane 3, enzyme I, HPr, and glycerol kinase; lane 4, enzyme I and glycerol kinase; lane 5, HPr and glycerol kinase. GlpK stands for glycerol kinase and P-GlpK for P-glycerol kinase.

P-glycerol kinase preparation. The obtained peptides were either directly analyzed by MALDI-MS or separated by high performance liquid chromatography on a reverse phase column and on-line detected by ESI-MS. The mass spectra obtained with MALDI-MS for tryptic digests of glycerol kinase and partially phosphorylated glycerol kinase are shown in Fig. 4, A and B, respectively (the P-glycerol kinase preparation, the spectrum of which is shown in Fig. 4B, contained about 40% phosphorylated enzyme before cleavage with trypsin). Peaks corresponding to the calculated mass of all expected tryptic fragments exhibiting a mass larger than 1000 Da could be detected. The two spectra are identical with the exception of one peak exhibiting a molecular mass of 3300.69 Da being present in Fig. 4B, but being absent in Fig. 4A (see insets of Fig. 4, A and B). A peak which differs from the peak present only in Fig. 4B by about 80 Da, the mass of the phosphoryl group, can be found in Fig. 4A (3249.47 Da) and in Fig. 4B (3250.73 Da). The ratio of the intensities of the two peaks exhibiting a molecular mass of 3250.73 and 3300.69 Da in Fig. 4B is about 2:1 (see insets of Fig. 4B), which is in good agreement with the estimated 40% P-glycerol kinase being present in the analyzed sample. The peaks exhibiting a molecular mass of 3249.47 and 3250.73 Da in Fig. 4, A and B, respectively, correspond to the tryptic peptide 21 that extends from amino acid Ser-230 to Lys-259 in the sequence of glycerol kinase (Fig. 1) and which has a calculated mass of 3252.66 Da. To further confirm that peptide 21 carries the phosphoryl group, tryptic digests obtained from glycerol kinase and P-glycerol kinase were separated by reverse phase chromatography, and peptides were on-line detected by ESI-MS in the effluent. The tryptic peptide 21 derived from glycerol kinase was found to elute at 55%...
Acetonitrile. Peaks corresponding to molecules carrying 2, 3, or 4 positive charges could be detected (data not shown). No other peaks exhibiting a molecular mass close to 3252 Da or a fraction of this value (divided by 2, 3, or 4) could be detected in the effluent of the reverse phase column. Peptide 21 was found to elute at the same position when a tryptic digest of partially phosphorylated glycerol kinase, containing 60% unphosphorylated glycerol kinase, was separated on the reverse phase column. However, an additional peptide was found to coelute with peptide 21, which differed in the 2-, 3-, and 4-fold charged state from peptide 21 exactly by the mass calculated for the mass of the phosphoryl group in a 2-, 3-, or 4-fold charged molecule (80 divided by 2, 3, or 4) (data not shown). These results strongly suggest that phosphorylation of glycerol kinase occurs at an amino acid residue present in peptide 21. Peptide 21 contains a single histidyl residue which corresponds to position 232 in E. casseliflavus. As glycerol kinase from E. faecalis has been shown to be phosphorylated at the N-3 position of a histidyl residue (4), it was most likely that His-232 of E. casseliflavus is the site of phosphorylation in glycerol kinase. MALDI-MS of tryptic fragments obtained with the three mutant proteins (Fig. 5B). These data confirm that His-232 is the site of phosphorylation in glycerol kinase.

**Glycerol Kinase Activity in Unphosphorylated, Phosphorylated, and Mutant Glycerol Kinases**—The effect of the mutations on glycerol kinase activity was measured in the three mutant proteins and compared with wild-type glycerol kinase. Using saturating substrate concentrations, glycerol kinase exhibited an activity of 5.6 nmol of product formed/min/μg of protein (kcat = 308/min) (Table I). Phosphorylation of glycerol kinase with PEP, enzyme I, and HPr stimulated glycerol kinase activity about 3-fold (Table I). However, as only about 30% of glycerol kinase was present in the phosphorylated form as judged by SDS-polyacrylamide gel electrophoresis, complete phosphorylation of glycerol kinase would cause a 9-fold stimulation of activity, which is similar to what has been reported for glycerol kinase from E. faecalis (10-fold stimulation) (4). The H232A mutant protein was found to exhibit an enzyme activity almost identical to the wild-type protein, whereas the H232E mutant protein had a 2.5-fold lower and the H232R mutant glycerol kinase a 3.4-fold higher activity compared with the wild-type enzyme (Table I). None of the mutant proteins were stimulated after incubation with PEP, enzyme I and HPr (data not shown).
Effect of FBP and EIIAGlc on E. casseliflavus Glycerol Kinase Activity—Glycerol kinase from E. coli was found to be inhibited by allosteric interaction with FBP and EIIAGlc (12). A single mutation replacing Gly-304 of E. coli glycerol kinase with a seryl residue has recently been demonstrated to prevent inhibition of glycerol kinase by both effectors (39). FBP inhibited also glycerol kinase of E. casseliflavus. The presence of 10 mM FBP caused a 7-fold decrease of E. casseliflavus glycerol kinase activity and the \( K_i \) value of FBP was calculated to be 1.9 mM; this compares with the value of 7 mM determined with the E. faecalis enzyme (4). In contrast, no inhibitory effect on E. casseliflavus glycerol kinase activity was observed, when purified EIIAGlc of E. coli or purified EIIAGlc domain of B. subtilis, which is part of the enzyme II**C** complex (11), was added to the assay mixture. The presence of 1 mg/ml EIIAGlc of E. coli inhibited E. coli glycerol kinase activity about 3.5-fold (12). The S. typhimurium EIIANag domain which forms the C-terminal domain of the enzyme IICBa specific for N-acetylglucosamine and which strongly resembles EIIAGlc was also found to be able to inhibit S. typhimurium glycerol kinase activity similar to EIIAGlc (40). However, neither E. coli EIIAGlc at concentrations up to 2 mg/ml nor B. subtilis EIIAGlc domain at concentrations up to 0.7 mg/ml exerted an inhibitory effect on glycerol kinase of E. casseliflavus (data not shown).

DISCUSSION

Glycerol kinase is the key enzyme of glycerol uptake and metabolism in bacteria (3). It catalyzes the first step of glycerol metabolism and provides the driving force for glycerol uptake by converting intracellular glycerol to glycerol-3-P which is not a substrate for the glycerol diffusion facilitator. Mutants of Gram-positive and Gram-negative bacteria defective in enzyme I (6–10) or HPr (11) of the PTS could not grow on glycerol as sole carbon source. In Gram-negative bacteria, glycerol kinase activity was found to be regulated by a mechanism involving phosphorylation/dephosphorylation of EIIAGlc. Unphosphorylated EIIAGlc interacts with glycerol kinase and inhibits its activity, whereas no inhibitory effect of P-EIIAGlc could be detected (12). The complex formed between E. coli glycerol kinase and EIIAGlc was crystallized and its structure has been determined (15). The interaction of the two proteins involves the amino acids Arg-402 and Pro-472 to Tyr-481 located in the C-terminal part of glycerol kinase and the active site of EIIAGlc including the phosphorylatable His-90 (14). However, no inhibition of glycerol kinase by EIIAGlc nor an activation by P-EIIAGlc seems to occur in B. subtilis. Mutants deleted for the 3' end of ptsG, encoding the EIIAGlc domain, ptsH and the 5' end of ptsI did not grow on glycerol as sole carbon source (11). As the absence of EIIAGlc in wild-type and ptsHI strains did not restore growth on glycerol, it was concluded that EIIAGlc plays no role in regulation of glycerol kinase activity in Gram-positive bacteria. This assumption was further confirmed by the in vitro activity assays presented in Table I. Neither EIIAGlc from E. coli nor the EIIAGlc domain of B. subtilis had an inhibitory effect on glycerol kinase from E. casseliflavus at concentrations corresponding to or exceeding the intracellular concentrations of these proteins.

Nevertheless, glycerol kinase of Gram-positive bacteria was also found to be regulated by PTS proteins. Enzyme I and HPr, the general proteins of the PTS, phosphorylate glycerol kinase from E. faecalis in a PEP-requiring reaction (17). Glycerol kinase activity is about 10-fold stimulated by this phosphorylation (4). As the amount of P-glycerol kinase present in the cells was diminished by the presence of a PTS sugar in the growth medium, it was concluded that glycerol kinase activity in Gram-positive bacteria is regulated by this phosphorylation reaction catalyzed by enzyme I and HPr (19). Phosphorylation of E. faecalis glycerol kinase occurs at the N-3 position of a histidyl residue. After cloning the gpk gene from E. casseliflavus, overproducing the encoded glycerol kinase and carrying out mass spectrometry of glycerol kinase and P-glycerol kinase, we have identified His-232 in this enzyme to be the site of PEP-dependent phosphorylation.

An amino acid sequence comparison with known glycerol kinase sequences derived from Gram-positive and Gram-negative bacteria revealed that His-232 and its surrounding are well conserved within glycerol kinases of the presented Gram-positive bacteria, whereas an equivalent of His-232 is absent from glycerol kinases of Gram-negative bacteria (Fig. 1). The amino acid composition of a labeled tryptic peptide isolated from [32P]P-glycerol kinase of E. faecalis (4) is in good agreement with the amino acid composition of the peptide extending from position 229 to 262, confirming that His-231, the equivalent of His-232 of E. casseliflavus glycerol kinase, is the site of phosphorylation in glycerol kinase of E. faecalis. It is interesting to note that the PEP-dependent site of phosphorylation in the enterococcal glycerol kinases corresponds to a location in

![FIG. 5. Phosphorylation of wild-type and His322→Ala, His322→Arg mutant glycerol kinases from E. casseliflavus.](image)

**TABLE I**

| Enzyme activity \( \times 10^{-3} \) (units/mg) | P-GlpK | GlpK | GlpK/H232A | GlpK/H232E | GlpK/H232R |
|---|---|---|---|---|---|
| P-GlpK | 5.6 | 14.7 | 5.4 | 2.3 | 19.2 |
| *a* The amount of phosphorylated glycerol kinase present in the assay mixture was estimated by SDS-polyacrylamide gel electrophoresis to be 30%.  
*b* Enzyme activities are expressed as nmoles of product formed/min/mg of protein. |
the E. coli enzyme within subdomain IA (Fig. 6), which is separated from the EIIA^{Glc} binding site by a deep and narrow interdomain cleft. Several of the residues in this location (Gly-230 to Arg-236) could not be identified in the electron density map of the E. coli glycerol kinase. The distance between Ile-229 and Asp-245 (Cα–Cα) is 21 Å in the E. coli enzyme, which suggests that the positive effect of phosphorylation of His-232 in the enterococcal glycerol kinase (10-fold increase in $k_{cat}$ with no change in either $K_m$ value) is mediated via elements of protein structure within the equivalent subdomain. The crystal structure of the complex between E. coli glycerol kinase and EIIA^{Glc} (15) indicates that the primary contact region includes Arg-402 and Pro-472 to Tyr-481 from subdomain IIC of the kinase. The sequence alignment of Fig. 1 indicates that these residues are not conserved in the enzyme from Gram-positive sources. By contrast, the catalytic residues identified in the E. coli enzyme are absolutely conserved within the enterococcal glycerol kinases (Figs. 1 and 6). As the glycerol kinases from Gram-positive and Gram-negative bacteria exhibit strongly conserved sequences (more than 50% identity) it is likely that the corresponding three-dimensional structures are very similar.

The bacterial glycerol kinase sequences exhibit also homology (about 30% identical amino acids) to sequences of eukaryotic glycerol kinases such as human glycerol kinase and glycerol kinase from Saccharomyces cerevisiae, although the S. cerevisiae glycerol kinase carries N- and C-terminal extensions and is therefore much longer (710 amino acids) compared with the human and bacterial enzymes with about 500 amino acids. However, none of the eukaryotic glycerol kinases contains an equivalent of the phosphorylatable His-232, conserved in glycerol kinases of Gram-positive bacteria. In contrast, the Gram-positive bacterium M. genitalium contains the phosphorylatable histidyl residue, but the surrounding amino acids are not conserved. The consensus sequence (Y/F)HF(Y/F)G of the glycerol kinase phosphorylation site is changed to NHWSS in position 231 to 234 of the M. genitalium glycerol kinase sequence, although this enzyme exhibits more than 37% sequence identity when compared with glycerol kinase sequences of other Gram-positive bacteria.

The importance of His-232 or its equivalent in regulation of glycerol kinases of Gram-positive bacteria was also confirmed by the finding that among 5 revertants which were isolated from a B. subtilis ΔptsGHI strain and which were able to grow on glycerol as sole carbon source, one was affected at His-230 which is the equivalent to His-232 of E. casseliflavus glycerol kinase (see Fig. 1) (His^{232} → Arg mutation) and two were affected at the nearby Phe-232 (Phe^{232} → Ser mutation) (41). The two other mutations were also located in the B. subtilis glpK gene and affected amino acids in the domain containing His-230. Interestingly, one of these amino acids (Glu-67 in glycerol kinase of E. casseliflavus) is conserved only in glycerol kinases of Gram-positive bacteria. The other amino acid (Phe-137 in glycerol kinase of E. casseliflavus and Phe-136 in glycerol kinase of E. coli) is located next to the conserved Tyr-135/136 in the glycerol binding site and is conserved in all glycerol kinase sequences (see Fig. 1 and Fig. 6). The H230R and the F232S mutations of glpK of B. subtilis were thought to cause structural changes similar to those evoked by PEP-dependent phosphorylation and hence to increase glycerol kinase activity allowing growth on glycerol minimal medium even in the absence of enzyme I and HPr. To test this assumption, we replaced the phosphorylatable His-232 in glycerol kinase of E. casseliflavus with an arginine. We found that this mutation increased E. casseliflavus glycerol kinase activity 3.4-fold. Although phosphorylation has a stronger stimulatory effect than the H232R mutation, this increase in glycerol kinase activity might be sufficient to allow the B. subtilis ΔptsGHI strain to grow on glycerol as sole carbon source.

Despite two completely different mechanisms of PTS-dependent regulation of glycerol kinase activity in Gram-positive and Gram-negative bacteria, the phenotype of mutants defective in the general proteins of the PTS, enzyme I and HPr, was found to be identical. ptsH and ptsI mutant strains of Gram-positive and Gram-negative bacteria are unable to use glycerol as sole carbon source (6–11). In ptsH or ptsI mutants of Gram-negative bacteria, EIIA^{Glc} cannot be phosphorylated, and it exerts a permanent repressive effect on glycerol kinase activity, whereas in corresponding mutants of Gram-positive bacteria glycerol kinase cannot be phosphorylated and activated by PEP-dependent, enzyme I- and HPr-catalyzed phosphorylation. By contrast, the mechanism of FBP-dependent regulation of glycerol kinase (16) seems to be identical for glycerol kinases from Gram-positive and Gram-negative bacteria, as similar inhibitory effects of this glycolytic intermediate can be observed for glycerol kinases derived from Gram-positive and Gram-negative bacteria. These findings might be explained by assuming that during evolution the inhibitory effect of FBP on glycerol kinase evolved before bacteria separated into Gram-positive and Gram-negative bacteria, whereas the more sophisticated regulation of glycerol kinase activity by the PTS evolved after this separation.

Similar to EIIA^{Glc} in Gram-negative bacteria (42), HPr seems to be the central regulatory protein controlling carbon metabolism in Gram-positive bacteria. It regulates not only glycerol kinase activity by reversible phosphorylation of a histidyl residue, it plays also a major role in carbon catabolite repression. EIIA^{Glc} has been reported to regulate adenyl cyclase activity in Gram-negative bacteria (42). In Gram-positive bacteria, HPr can be phosphorylated at Ser-46 (43), and seryl-phosphorylated HPr allows the catabolite control protein CcpA, a member of the LacI-GalR family of repressors, to bind to the catabolite responsive element (cre), an operator-like sequence present in most catabolite repression-sensitive operons (44, 45). The presence of a cre-like sequence in front of the glpK
gene in *E. casseliflavus* suggests that expression of the *glp* operon might also be regulated by seryl-phosphorylated HPr and the catabolite control protein CcpA.

HPr of *B. subtilis* regulates also the activity of the transcriptional activator LevR (46) and the antiterminators SacT (47) and LicT\(^5\) by PEP-dependent, enzyme I-requiring protein phosphorylation. LevR and LicT seem to be phosphorylated at more than one histidyl residue (46). In addition to the regulatory phosphorylation recognized by HPr could be detected. It is therefore likely that HPr recognizes a certain structural element present presumably in all proteins phosphorylated by histidyl-phosphorylated HPr.

Acknowledgments—We thank W. Hengstenberg for providing us with purified enzyme I from *S. carnosus* and J. Reizer for purified EIIA\(^4\) domain of *B. subtilis.*

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