JOURNAL OF BACTERIOLOGY, Feb. 1993, p. 1087–1094 Vol. 175, No. 4
0021-9193/93/041087-08$02.00/0

# Glycerol Kinase of *Escherichia coli* Is Activated by Interaction with the Glycerol Facilitator

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Received 21 April 1992/Accepted 14 December 1992

**Glycerol transport is commonly cited as the only example of facilitated diffusion across the *Escherichia coli* cytoplasmic membrane. Two proteins, the glycerol facilitator and glycerol kinase, are involved in the entry of external glycerol into cellular metabolism. The glycerol facilitator is thought to act as a carrier or to form a selective pore in the cytoplasmic membrane, whereas the kinase traps the glycerol inside the cell as *sn*-glycerol-3-phosphate. We found that the kinetics of glycerol uptake in a facilitator-minus strain are significantly different from the kinetics of glycerol uptake in the wild type. Free glycerol was not observed inside wild-type cells transporting glycerol, and diffusion of glycerol across the cytoplasmic membrane was not the rate-limiting step for phosphorylation in facilitator-minus mutants. Therefore, the kinetics of glycerol phosphorylation are different, depending on the presence or absence of the facilitator protein. We conclude that there is an interaction between the glycerol facilitator protein and glycerol kinase that stimulates kinase activity, analogous to the hexokinase- and glycerol kinase-porin interactions in mitochondria.**

Glycerol uptake is usually cited as the only example of transport by facilitated diffusion across the *Escherichia coli* inner membrane. Glycerol, like other small uncharged molecules, can cross the cytoplasmic membrane by passive diffusion. However, at low concentrations of glycerol, cells limited to passive uptake have a growth disadvantage (28). It was shown that there is a protein-dependent uptake system for glycerol (29). This uptake system is induced by growth in the presence of glycerol or *sn*-glycerol-3-phosphate (G3P), repressed by growth in the presence of glucose, and constitutive in a *glp* regulon repressor mutant (29). Phosphorylation of cytoplasmic glycerol by glycerol kinase prevents the glycerol from passively exiting the cell and is the first step in metabolism (13). The resulting G3P also serves as a precursor for phospholipid synthesis. It was reported that some polyhydric alcohols, such as ribitol, as well as unrelated small, nonpolar molecules like urea and glycine, are substrates of the glycerol facilitator (14). The transport of these substrates is independent of phosphorylation, because they are not substrates of the glycerol kinase. Because of this broad substrate specificity as well as temperature insensitivity, the glycerol facilitator was described as a channel in the cytoplasmic membrane rather than a specific carrier protein (14).

The gene encoding the glycerol facilitator is located in the 88-min region of the *E. coli* chromosome (2). It is organized in an operon with the structural gene for glycerol kinase (4, 8), with *glpF* being the promoter-proximal gene (33). A third member of the operon, *glpX*, distal to *glpK*, was recently discovered (38). The expression of this operon is catabolite sensitive (11). The *glpF* gene product was identified as a membrane protein with an apparent molecular weight of about 25,000 (33). This is consistent with the calculated molecular weight of 29,727 predicted from the DNA sequence (23). The *glpFKX* operon belongs to the *glp* regulon (most recently reviewed in reference 19). The gene products of the *glp* regulon participate in the uptake and metabolism of glycerol, G3P, and glycerophosphodiesters. The genes and operons of the *glp* regulon are under negative control of the GlpR repressor protein (7, 30). It has recently been reported that *glpF* has a high degree of homology to other integral membrane proteins of bacterial, plant, and animal origin (3). These proteins have been grouped together as a new family of integral membrane proteins, the so-called major intrinsic protein family (25). The functions of most of these proteins are unknown, but because of the similarity to GlpF, roles in solute transport are being considered.

Glycerol kinase is believed to be the pacemaker for the dissimilation of glycerol (40). The active enzyme is a tetramer (35) of identical 56,106-Da subunits (26). The enzyme has been purified (35), and its catalytic properties have been investigated. The $K_m$ for glycerol was 10 $\mu$M, and the $V_{max}$ was 10 $\mu$mol/min/mg of enzyme (36).

We set out to characterize the mechanism of glycerol transport by the facilitator protein, allegedly the simplest of bacterial transport systems and completely different from active transport and phosphotransferase system-mediated transport systems. Our data suggest that effective glycerol uptake and utilization rely on the interaction between the facilitator protein and glycerol kinase, with the phosphorylation reaction being stimulated by this interaction. This activation of GlpK by GlpF would be similar to the hexokinase- and glycerol kinase-porin interactions in mitochondria, in which kinase is activated upon interaction with mitochondrial porin (5). An interaction between GlpF and GlpK has been previously postulated (15).

## MATERIALS AND METHODS

**Bacterial strains.** The bacterial strains used in this study are listed in Table 1. P1 transduction was performed as described by Miller (22). The DNA methods used were from Maniatis et al. (20) and Silhavy et al. (32). Strain GD248 was constructed by using the *glpK*::*lacZ* fusion of strain GD32. The *glpFKX* operon was deleted by using λp1081.1 and the method described by Garrett et al. (12). The deletion was then transduced into a *glpR*$^+$ strain, GD189, selecting for Met$^+$ and screening for a glycerol-minus phenotype. The strain does not grow on glycerol and does not transport

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TABLE 1. Bacterial strains

| Strain[a] | Description | Source or reference |
|---|---|---|
| MC4100 | F$^-$ *araD139* $\Delta$(*argF-lac*)*U169* *rpsL150 relA1 deoC1* *ptsF25 rbsR* | 6 |
| GD32 | MC4100 $\Phi$(*glpK::lacZ*) *glpR* | 33 |
| GD173 | MC4100 *glpF* | 33 |
| GD189 | MC4100 *metB1* | 33 |
| GD202 | MC4100 *glpK* | 33 |
| GD248 | MC4100 $\Delta$*glpFK* | This study |

[a] All strains are derivatives of *E. coli* K-12.

glycerol after transformation with a *glpK*$^+$ plasmid. Neither the facilitator protein nor the kinase is detectable in strain GD248 by Western blots (immunoblots).

**Growth.** For the different transport assays, the strains were grown in minimal medium 9 (M9) (22) containing 0.4% Casamino Acids (Difco Laboratories) with 2.5 mM G3P (5 mM DL-α-G3P; Sigma Chemical Co.) for induction of the *glp* regulon.

**Glycerol transport assays.** Transport assays were performed either by filter assays or by centrifugation through silicon oil (14). These tests were carried out by using logarithmic-phase cells. Cells were washed twice with one culture volume of M9 medium and then resuspended in M9 medium to an optical density at 578 nm of 0.1 in the case of filter assays or 3.0 for the silicon oil test. For the filter assays, [U-$^{14}$C]glycerol (165.8 mCi/mmol; Amersham) was added to the cell suspension to yield a final concentration of 90 nM (not diluted by unlabeled glycerol). At the indicated times, 500-μl samples were transferred to a membrane filter (0.45-μm pore size; Millipore) and vacuum filtered, washed with M9, and scintillation counted in Emulsifier Safe (Canberra Packard). For the silicon oil test, we used double labeling with $^3H_2O$ (5 Ci/ml; Amersham) and [$^{14}$C]glycerol (165.8 mCi/mmol; Amersham) at 0.5 μCi/mol for $^3H_2O$ and 13.5 nCi/ml for [$^{14}$C]glycerol (equal to 90 nM undiluted radiolabeled glycerol). When different chemical concentrations of glycerol were used, mixtures of 90 nM [$^{14}$C]glycerol with various concentrations of unlabeled glycerol were applied. Incubation of the cells with radiolabel was carried out at 10°C. At specified times, 1-ml samples were taken and placed on top of 200 μl of silicon oil (silicon oil AR200; Wacker Chemie; density = 1.04 at 25°C) in a 1.5-ml Eppendorf reaction tube. Centrifugation at 12,000 × *g* for 2 min pelleted the cells. A 50-μl sample of the supernatant was taken and placed in a scintillation vial. The rest of the supernatant and the silicon oil was carefully removed, and the tip of the reaction tube containing the cell pellet was cut off and placed in a scintillation vial containing 0.75 ml of 0.4 M NaOH. After vigorous vortexing, the vials were incubated at 37°C for 1 h. After the addition of 5 ml of scintillation cocktail, the vials were counted by using dual-label counting in a Beckman LS1801.

For the determination of $K_m$ and $V_{max}$ by the centrifugation assay, we used only [$^{14}$C]glycerol, omitting $^3H_2O$. The final concentration of [$^{14}$C]glycerol was 90 nM, with different concentrations of unlabeled glycerol added to achieve the indicated chemical concentration of glycerol. Incubation was performed at room temperature.

**Analysis of metabolites.** To analyze the metabolites and to look for free cytoplasmic glycerol inside the cells, we modified the silicon oil test. We used [$^{14}$C]glycerol (not diluted with unlabeled glycerol) at a concentration of 6.6 μM. Samples were incubated at room temperature. The silicon oil was carefully layered on top of 50 μl of 10% trichloroacetic acid (TCA). After centrifugation of a 1-ml sample of cells through the silicon oil into the TCA, the supernatant and the silicon oil were carefully removed. The pellet in TCA was kept on ice for 15 min and then centrifuged again for 10 min. Samples of the supernatants were analyzed by thin-layer chromatography (solid phase, Silica Gel 60 [Merck]; mobile phase, isopropanol-$H_2O$-$NH_4OH$ [7:2:1]) and then autoradiographed.

**Glycerol kinase assay.** We tested the glycerol kinase activity (13, 28) in cell extracts of different strains. Cells from 250-ml mid-logarithmic-phase cultures were harvested by centrifugation and washed twice with the same volume of M9 medium. The cell pellet was resuspended in 5 ml of 60 mM Tris-HCl (pH 7.5)–10 mM $MgCl_2$ and kept on ice. Sonification was with a Branson Sonic Power Company Sonifier B-12 and a microtip (diameter, 3 mm), five times for 15 s at 50 W, with the extracts chilled in a −20°C ice bath. The extracts were centrifuged at 4°C either for 30 min at 40,000 × *g* or for 1 h at 100,000 × *g*. The pellets were resuspended in 5 ml of buffer and stored at −20°C until further use. Protein concentrations of the supernatants were determined with the Bio-Rad protein assay (Bio-Rad) with bovine serum albumin as a standard. Kinase assays were performed immediately after extraction, because enzyme activity is rapidly lost even in the presence of glycerol, which acts as a stabilizer.

For the glycerol kinase tests according to the method of Richey and Lin (28), the extracts were diluted to a protein concentration of 2 mg/ml. The assay mixture contained 60 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, and 10 mM ATP. To a volume of 220 μl, 50 μl of cell extract was added, and the reaction was started by the addition of 30 μl of glycerol. The concentration of [$^{14}$C]glycerol (165.8 mCi/mmol) was always 0.9 μM, while the concentration of unlabeled glycerol was varied to achieve the final concentrations indicated in Fig. 6. After 15, 30, 45, 60, and 90 s, 50-μl samples of the reaction mixture were applied to a DE-81 filter (Whatman). After 5 s, the filters were placed in a bath of 80% ethanol and afterwards washed twice with water. The filters were dried for 1 h at 70°C and then transferred to scintillation vials. The rates of reaction were determined by measuring the time-dependent linear increase of the retained radioactivity.

**Visualization of glycerol facilitator and kinase.** To control the amounts of kinase and facilitator protein in the mutants, all strains were analyzed with Western blots (37). Proteins from cell fractions were solubilized in sample buffer at 37°C for 1 h and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12.5% acrylamide, 2.6% cross-linking) (SDS-PAGE) (17). The proteins were electrotransferred to a polyvinylidene difluoride membrane (Millipore), probed with anti-GlpF and anti-GlpK antibodies, and then visualized with peroxidase-coupled second antibody and *o*-dianisidine.

## RESULTS

**Transport of glycerol in *glpF*, *glpK*, and *glpF glpK* strains can be differentiated by centrifugation through silicon oil.** In standard filter transport assays with low concentrations of glycerol, there is no apparent difference between the negligible transport rates of mutants lacking the glycerol facilitator and/or glycerol kinase. Measurable transport in this assay is dependent on the presence of both proteins, GlpF

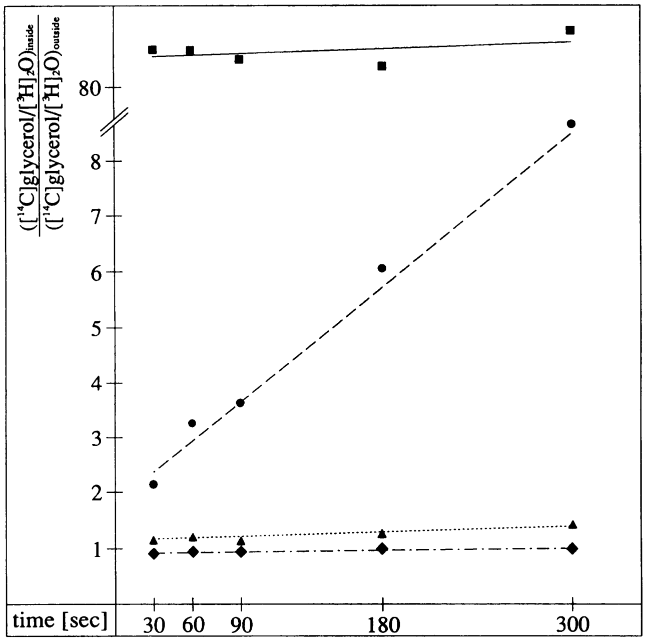

FIG. 1. Glycerol transport behavior of different mutants in the silicon oil test. The lines represent the best fit calculated by linear regression of all values. ■, MC4100 (*glpF*$^+$ *glpK*$^+$); ●, GD173 (*glpF glpK*$^+$); ▲, GD202 (*glpF*$^+$ *glpK*); ◆, GD248 (*glpF glpK*). The concentrations of radiolabel were 0.5 μCi/ml for $^3H_2O$, and 90 nM for [$^{14}$C]glycerol (undiluted stock solution).

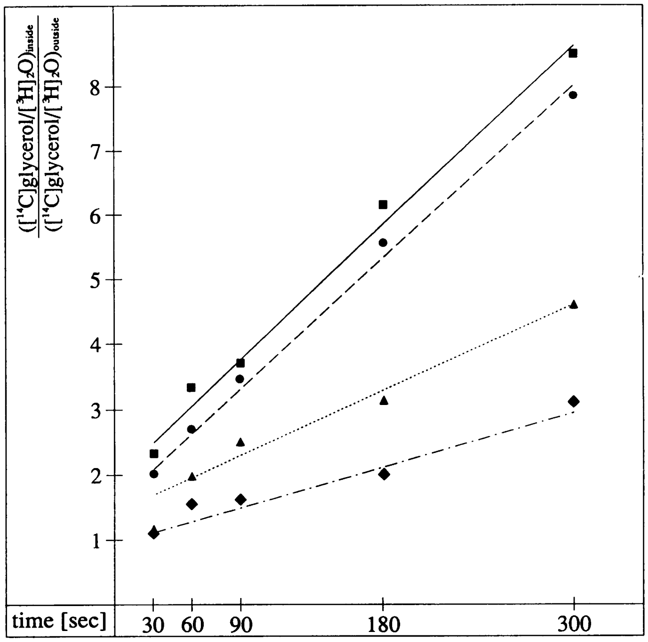

FIG. 2. Glycerol transport behavior of the *glpF* mutant GD173 in the silicon oil test at different glycerol concentrations. The lines represent the best fit calculated by linear regression of all values. The glycerol concentrations were 0.1 (■), 1 (●), 10 (▲), and 100 (◆) μM. The amount of [$^{14}$C]glycerol (undiluted stock solution) was 90 nM in all experiments.

and GlpK. In the case of a mutation in *glpF* or *glpK* or in a double mutant, the signal is too small to be measured. Increasing the cell density results in very long times for filtration and washing, which are not appropriate for transport measurements.

When centrifugation through silicon oil, which necessitates high cell densities, is used, there is a clear difference between equilibration of glycerol across the membrane, measured in *glpK* strains, and slow accumulation due to phosphorylation of the glycerol entering the cell by passive diffusion, measured in a *glpF glpK*$^+$ strain. In addition, $^3H_2O$ is present in the assay and ratios of substrate to water, both intra- and extracellular, are determined such that values slightly over equilibrium are detectable.

The most interesting results came from comparison of a *glpF* mutant with the wild type (Fig. 1). The *glpF glpK*$^+$ strain GD173 showed a much slower accumulation of radiolabel than strain MC4100 (*glpF*$^+$ *glpK*$^+$).

The fact that *glpF* strains show much lower glycerol transport rates has traditionally been attributed to the difference between passive diffusion and facilitated diffusion. In other words, in *glpF* strains passive diffusion was believed to be the rate-limiting step in the metabolism of glycerol.

In our experiments, equilibration of glycerol, indicated by a ratio of 1, was observed in *glpK* strains within the 30 s that elapsed before the first time point, even at the lowest glycerol concentration tested (90 nM). This was independent of the presence or absence of the glycerol facilitator (GD248, *glpF*; GD202, *glpF*$^+$). The fact that *glpK* strains showed equilibration of glycerol within 30 s was the first indication that diffusion of glycerol across the cytoplasmic membrane may not be the rate-limiting step for the observed phosphorylation in the *glpF* mutant. It is clear that the amount of glycerol bound to the cell surface was negligible, because the ratio of $(^{14}C/^3H)_{inside}$ to $(^{14}C/^3H)_{outside}$ would then be >1. Similar rates for passive diffusion of glycerol through the cytoplasmic membrane (half-equilibration times of 10 to 20 s) were obtained by others using other techniques (9).

The *glpF*$^+$ *glpK*$^+$ strain MC4100 showed a ratio of $(^{14}C/^3H)_{inside}$ to $(^{14}C/^3H)_{outside}$ of 80, which was constant over the 5-min sampling period, indicating that almost all of the offered glycerol had been taken up within the initial 30 s.

These measurements were repeated at higher glycerol concentrations, varying the amount of unlabeled glycerol but keeping the amount of [$^{14}$C]glycerol constant. Strain GD173 (*glpF glpK*$^+$) showed a decrease in accumulation of $^{14}$C radiolabel with increasing glycerol concentrations (Fig. 2). Figure 2 shows the ratio of $(^{14}C/^3H)_{inside}$ to $(^{14}C/^3H)_{outside}$. This experiment allowed us to estimate an apparent $K_m$ of about 5 μM for glycerol uptake by strain GD173, reminiscent of the $K_m$ for glycerol kinase of 1.3 μM measured in vitro in cell extracts (13) or 10 μM as measured for the purified enzyme (36). The fact that the uptake of glycerol in strain GD173 is half-maximally saturated at micromolar concentrations that are similar to the $K_m$ of phosphorylation by glycerol kinase in vitro demonstrated that phosphorylation rather than passive diffusion is the rate-limiting step in glycerol uptake under these conditions in a *glpF glpK*$^+$ strain. With this test, the $K_m$ could not be determined accurately because at higher glycerol concentrations the ratio of $^{14}$C to $^3$H declined and increased the error. $K_m$ and $V_{max}$ were determined more accurately, as described below, by using only [$^{14}$C]glycerol at room temperature.

With the wild-type strain, even at a concentration of 0.1 mM glycerol, 70% of the external glycerol was taken up within the first 30 s. Kinetic parameters for MC4100 could

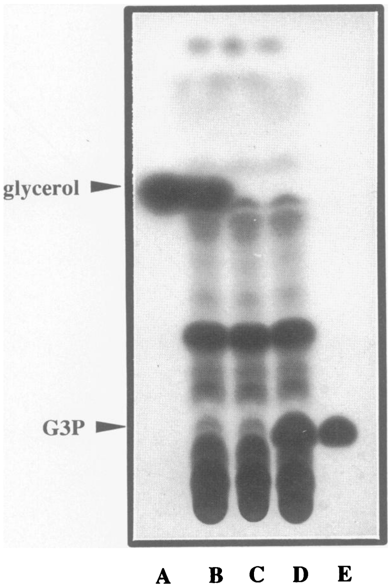

FIG. 3. Thin-layer chromatographic analysis of metabolites after uptake of [$^{14}$C]glycerol. Samples were taken 30 s after the addition of [$^{14}$C]glycerol to the cells and spun through silicon oil into TCA. Ten microliters of the clarified TCA extract was applied to thin-layer chromatography plates. The initial [$^{14}$C]glycerol concentration was 6.6 μM (undiluted stock solution). Lanes are as follows: A, glycerol control; B, MC4100 (*glpF*$^+$ *glpK*$^+$) together with authentic [$^{14}$C] glycerol; C, MC4100; D, MC4100 plus authentic [$^{14}$C]G3P; E, [$^{14}$C]G3P control. The film was exposed for 4 weeks.

therefore not be determined with this method, which necessitates the use of high cell densities.

However, it is obvious that the rate of glycerol uptake (equivalent to phosphorylation) has to be much higher in the *glpF*$^+$ *glpK*$^+$ strain MC4100 than in the *glpF glpK*$^+$ strain GD173. Therefore, either GlpF mediates active transport and most of the label that is accumulated represents free glycerol or GlpF stimulates the activity of glycerol kinase. In order to distinguish between the two possibilities, it was important to look for free glycerol in the cytoplasm of cells rapidly transporting glycerol.

**Analysis of metabolites.** Centrifugation of cells through silicon oil into TCA prevented further metabolism of transported glycerol in addition to separating cells from incubation mixture. Such TCA extracts were subjected to thin-layer chromatography followed by autoradiography. Figure 3 shows that free glycerol could not be detected in the wild-type strain MC4100. In contrast, in strains GD202 and GD248, both lacking glycerol kinase but one containing and the other one not containing GlpF, equal amounts of free glycerol could be detected (Fig. 4, lanes E and F). Thus, even in the absence of GlpF, glycerol can equilibrate through the membrane within 30 s, the time required for the first sampling by this technique. In strain GD173, containing glycerol kinase but lacking GlpF, free glycerol could still be detected, although in smaller amounts than in the glycerol kinase-free strains (Fig. 4, lane D). In addition, metabolic products could be observed in this strain as well. Apart from the much lower total amount of metabolites in the *glpF* strain GD173 than in the wild-type strain MC4100, their patterns appear not entirely identical (Fig. 4, lanes C and D). It seems that the extracts may contain the same labeled components, but their relative compositions are not identical (at least not in one major component). The observation that strain GD173 (*glpF glpK*$^+$) does not contain the same amounts of free glycerol as either strain GD202 or strain GD248 (both lacking *glpK*) could have technical reasons. That is, after the centrifuge is accelerated, the cells enter the silicon oil and are removed from their supply of glycerol. Yet, the internal glycerol kinase is only inactivated after the cells have passed the silicon oil and the enzyme is inactivated by TCA. During this time span, the amount of free glycerol may have been significantly reduced. Alternatively, the rate of phosphorylation by glycerol kinase may approach the rate of diffusion, giving rise to a reduced amount of glycerol compared with that in a strain lacking glycerol kinase.

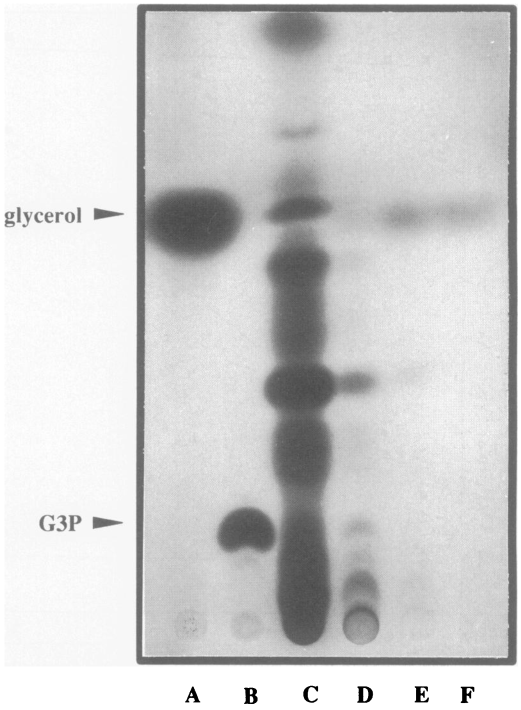

FIG. 4. Different distributions of metabolites in the wild type and the *glpF* mutant. Samples were taken 30 s after the addition of [$^{14}$C]glycerol to the cells and spun through silicon oil into TCA. Ten microliters of the clarified TCA extract was applied to thin-layer chromatography plates. [$^{14}$C]glycerol was present at a concentration of 6.6 μM (undiluted stock solution). Lanes are as follows: A, [$^{14}$C]glycerol control; B, [$^{14}$C]G3P control; C, MC4100 (*glpF*$^+$ *glpK*$^+$); D, GD173 (*glpF glpK*$^+$); E, GD202 (*glpF*$^+$ *glpK*); F, GD248 (*glpK glpF*). The film was exposed for 4 weeks.

If GlpF mediated active transport, significant amounts of labeled glycerol should have been detectable in strain MC4100 (*glpF*$^+$ *glpK*$^+$) while metabolites of G3P should be present in equal relative amounts in both the wild-type and the *glpF* mutant. This was clearly not the case, arguing against active transport.

The labeled metabolite with the highest level of mobility was not identical to glycerol. This can clearly be seen in Fig. 3 for the case in which [$^{14}$C]glycerol was mixed with the TCA extract of the wild-type strain MC4100 (lane B). We conclude that there is very little free glycerol inside wild-type cells that are rapidly transporting glycerol. G3P, the product of the glycerol kinase reaction, was seen in MC4100 only in small amounts when glycerol was given externally at 6.6 μM. When the strain was exposed to 0.1 mM glycerol, the internal G3P spot became more prominent and was subject to turnover at longer intervals (data not shown). Quantitative analysis of the total amount of radiolabel incorporated by the different strains revealed that glycerol was still present in abundance in the incubation medium of strains GD173 (*glpF glpK*$^+$), GD202 (*glpF*$^+$ *glpK*), and GD248 (*glpF glpK*). We found that the wild-type strain MC4100 had taken up 83% of the total glycerol during the 30-s incubation when glycerol was present at a concentration of 6.6 $\mu$M, whereas GD173 had only taken up 1.4%. Therefore, substrate limitation is not the explanation for the absence of G3P among the GD173 reaction products. Assuming that $10^9$ cells have an internal volume of 1 $\mu$l, the amount of glycerol detected in the *glpK* strains corresponds to equilibrium of the substrate.

**Glycerol transport kinetics.** The kinetics of glycerol transport in the *glpF glpK*$^+$ strain GD173 were measured in modified silicon oil transport tests, omitting the $^3H_2O$. The rate of transport was measured at the five different glycerol concentrations indicated in Fig. 5A. The amount of accumulated radioactivity was plotted. The double-reciprocal plot yielded a $K_m$ of 50 $\mu$M and a $V_{max}$ of 0.1 nmol/min/$10^9$ cells. The kinetic parameters were strikingly different in wild-type cells. Since glycerol uptake in wild-type cells was too fast to be measured with the silicon oil test, the transport kinetics for MC4100 were measured with standard filtration assays at the glycerol concentrations indicated in Fig. 5B. A $K_m$ of 5.6 $\mu$M and a $V_{max}$ of 13.1 nmol/min/$10^9$ cells were determined. The $K_m$ is 10-fold lower than that for the *glpF* mutant, and the $V_{max}$ is 130-fold higher.

The observation that half-maximal saturation of glycerol uptake in a *glpF glpK*$^+$ strain occurred at a micromolar concentration of glycerol, in the same concentration range as the $K_m$ of glycerol kinase activity in cell extracts, demonstrated that phosphorylation of glycerol in a *glpF* mutant is not limited by diffusion. Therefore, the $V_{max}$ for in vivo phosphorylation in *glpF* mutants compared with that of a *glpF*$^+$ strain must be due to an effect of GlpF on glycerol kinase, increasing the rate of glycerol phosphorylation significantly. For this conclusion to be correct, the amount of glycerol kinase as well as its kinetic properties, when measured in cellular extracts, must be identical in *glpF* and *glpF*$^+$ strains (see below).

*glpF* mutants grow on glycerol, but the growth $K_m$ is on the order of 5 mM. Under these conditions, the flow rate of glycerol must be close to the $V_{max}$ observed in the wild type. Obviously, this is in contrast to the low $K_m$ and $V_{max}$ values we determined for the *glpF* mutant. This can only mean that in the *glpF* mutant, there exists a second glycerol-utilizing system with a high $K_m$, which is initiated by a reaction other than glycerol phosphorylation. Such a system would escape our measurements, which are limited by workable cell densities.

**Glycerol kinase kinetics.** Glycerol kinase activity in extracts of MC4100 and GD173 was measured after sonification and ultracentrifugation. The kinetic parameters determined were essentially the same for both strains (Fig. 6). GD173 showed a $K_m$ of 27 $\mu$M and a $V_{max}$ of 3.9 nmol/min/mg of protein. For MC4100, a $K_m$ of 20 $\mu$M and a $V_{max}$ of 4.9 nmol/min/mg of protein were determined. Thus, both the mutant and the wild type show the same kinase activity in vitro.

Making the assumption that 1 mg of total cellular protein corresponds to a cell number of 6.6 × $10^9$ (22) and that the soluble fraction comprises approximately 80% of the total protein, the $V_{max}$ is 0.47 nmol/min/$10^9$ cells for strain GD173 and 0.58 nmol/min/$10^9$ cells for strain MC4100. Both values correspond fairly well to the $V_{max}$ determined for glycerol uptake in strain GD173 (0.1 nmol/min/$10^9$ cells) but are well below the $V_{max}$ measured for the wild type (13.1 nmol/min/$10^9$ cells).

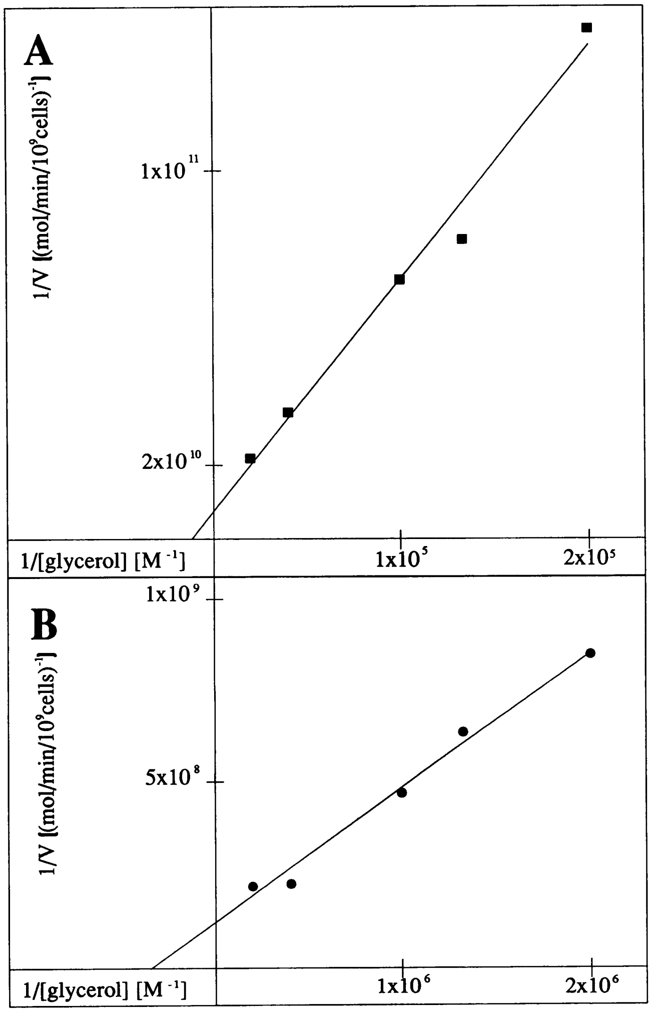

FIG. 5. Lineweaver-Burke plot for glycerol uptake in the *glpF glpK*$^+$ strain GD173 (A) and the *glpF*$^+$ *glpK*$^+$ strain MC4100 (B). Glycerol concentrations used in panel A were 5, 7.5, 10, 25, and 50 $\mu$M. For this test, centrifugation through silicon oil was used. In panel B, the glycerol concentrations were 0.5, 0.75, 1.0, 2.5, and 5.0 $\mu$M. Here, the filtration assay was used. The lines represent the best fit calculated by linear regression of all values.

This confirms our view that the observed difference in glycerol transport kinetics between the wild type and a *glpF* mutant must be the result of stimulation of glycerol kinase activity by its interaction with GlpF.

To verify that the amount of glycerol kinase was the same in the two strains, membrane and soluble cell fractions were analyzed by SDS-PAGE and Western blots probed with specific antibodies (Fig. 7A). Glycerol kinase was present in the same amount in both strains. The distribution between supernatant and pellet was also the same, approximately 2:1. Western blot analysis was also used to show that the wild-type supernatant was free of glycerol facilitator (Fig. 7B). GlpF was indeed present only in the membrane fraction. Centrifugation at 40,000 instead of 100,000 × *g* left

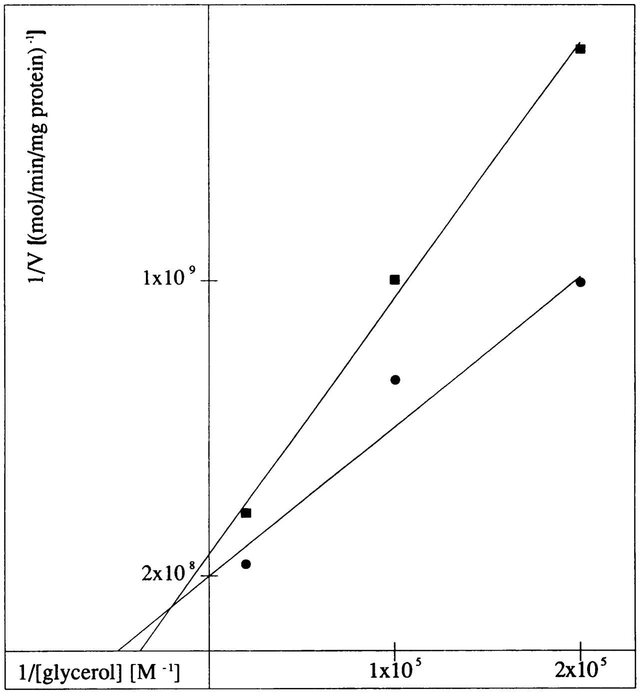

FIG. 6. Lineweaver-Burke plot for glycerol kinase activity of cell extracts from strain MC4100 (*glpF*$^+$ *glpK*$^+$) (●) and strain GD173 (*glpF glpK*$^+$) (■). The lines represent the best fit calculated by linear regression of all values. Glycerol concentrations used were 5, 10, and 50 μM.

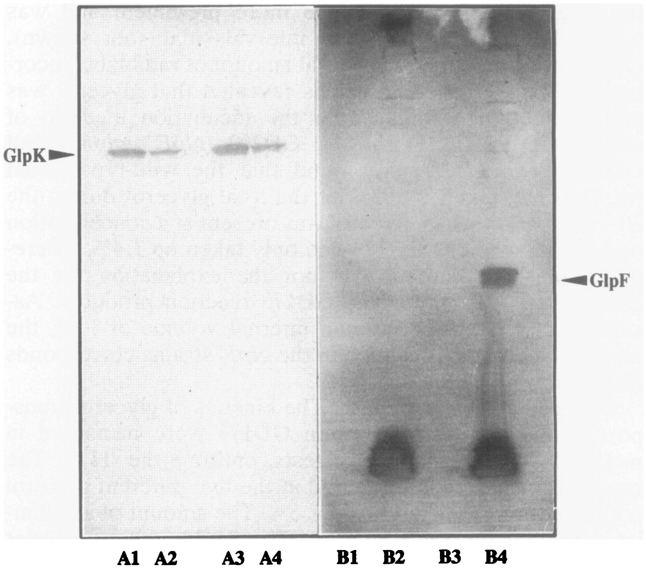

FIG. 7. Glycerol facilitator and glycerol kinase distribution by Western blotting of a 12.5% SDS gel. Samples represent aliquots of supernatant and pellet after ultracentrifugation of sonified cells. Lanes A1 to A4 were done with purified polyclonal anti-GlpK antibodies, and lanes B1 to B4 were done with purified polyclonal anti-GlpF antibodies. The individual lanes are as follows: A1, supernatant of strain GD173 (*glpF glpK*$^+$); A2, pellet of strain GD173; A3, supernatant of strain MC4100 (*glpF*$^+$ *glpK*$^+$); A4, pellet of strain MC4100; B1, supernatant of strain GD173; B2, pellet of strain GD173; B3, supernatant of strain MC4100; B4, pellet of strain MC4100. The label at the bottom of lanes B2 and B4 is due to cross-reaction of antibodies with lipoprotein.

about one-third of the total GlpF protein in the MC4100 supernatant but had no effect on the kinase kinetics. Our interpretation is that the GlpF-GlpK interaction is weak and hence is disrupted by dilution during preparation of the cell extracts. Glycerol kinase kinetics also were not changed by addition of purified GlpF. Attempts to demonstrate the interaction between the glycerol facilitator and glycerol kinase by using a variety of genetic and biochemical methods were unsuccessful.

## DISCUSSION

We conclude that glycerol kinase activity is increased in vivo by the presence of the glycerol facilitator. Comparison of the glycerol transport kinetics of the wild-type strain and the *glpF* mutant shows that both the $V_{max}$ and the $K_m$ of the kinase are changed upon this interaction. The $K_m$ for glycerol was lowered from 50 to 5.6 μM, and the $V_{max}$ increased from 0.1 to 13.1 nmol/min/$10^9$ cells.

Passive diffusion of glycerol was not rate limiting to reach the half-maximal rate of uptake in the *glpF* mutant. After 30 s, the shortest time point, glycerol was equilibrated across the membrane in strains lacking glycerol kinase, whether or not GlpF was present (Fig. 4, lanes E and F). In addition, since there is no significant difference between the $K_m$ of uptake in the *glpF* strain and the $K_m$ of glycerol kinase measured in cellular extracts, there cannot be a diffusion barrier for the in vivo phosphorylation under these conditions.

What then is the explanation for the increased uptake of glycerol in the *glpF*$^+$ strain? No free glycerol was detected in the cytoplasm of wild-type cells transporting glycerol (Fig. 3, lane C), and in cellular extracts, the same kinase kinetics of phosphorylation were found for both the *glpF* and the *glpF*$^+$ strains. Therefore, the observed increased rate of uptake cannot be attributed to active transport by GlpF followed by a constant rate of phosphorylation by glycerol kinase.

Assuming that 1 mg of total cellular protein corresponds to a cell number of $6.6 \times 10^9$ (22) and that the soluble fraction comprises approximately 80% of the total protein, it becomes possible to compare glycerol kinase activity measured in vitro and glycerol transport kinetics measured in vivo. The $K_m$ of glycerol kinase in cellular extracts of both the *glpF* and the *glpF*$^+$ strains was the same (around 30 μM) and corresponds to the $K_m$ of glycerol uptake of the *glpF* mutant (around 50 μM). The $V_{max}$ of glycerol kinase in cellular extracts (0.47 nmol/min/$10^9$ cells for strain GD173 [*glpF*] and 0.58 nmol/min/$10^9$ cells for strain MC4100 [*glpF*$^+$]) is somewhat above the $V_{max}$ of glycerol uptake for the *glpF* mutant GD173 (0.1 nmol/min/$10^9$ cells) but well below the value determined for *glpF*$^+$ strain MC4100 (13.1 nmol/min/$10^9$ cells). Thus, the only explanation for this apparent difference in kinase activity in vitro and in vivo is that glycerol kinase activity is increased by the presence of GlpF.

An interaction between GlpK and GlpF was postulated by Jin et al. (15) and Lin (19). The existence of a GlpF-GlpK interaction awaits biochemical confirmation. Cross-linking experiments as well as attempts to demonstrate a GlpF-dependent association of GlpK with the membrane have not yielded any positive results. Also, the activity of glycerol kinase was not observed to increase in the presence of inverted membrane vesicles containing GlpF. It appears that the kinase-facilitator interaction is weak and is abolished by cell disruption. On the basis of the concentration of glycerol kinase in the cell, the $K_d$ of its binding to GlpF could be as high as 0.1 mM and still be effective. However, such a low affinity would be difficult to detect biochemically.

It has been reported that enzyme $III^{Glc}$ of the phosphotransferase system in its dephosphorylated form exerts inducer exclusion in the glycerol system by reducing the activity of glycerol kinase (27). This effect must be augmented in vivo by the sequestration of glycerol kinase from GlpF.

It is well documented that kinases in mitochondria are activated by interaction with a membrane-bound component. Hexokinase and glycerol kinase interact with mitochondrial porin (5, 21). It was shown that both enzymes occur in soluble and membrane-bound forms that are kinetically different (31). For the mitochondrial glycerol kinase, the $K_m$ for glycerol was lowered, and the $V_{max}$ was increased upon interaction with the porin (16, 31), as we observed for the *E. coli* glycerol kinase. Mitochondrial hexokinase and glycerol kinase were shown to competitively bind to the same structure (10). It is thought that the distribution of these kinases plays a role in regulation of metabolism, because the binding of hexokinase and glycerol kinase to the porin is reciprocal and only in the unbound form are the enzymes sensitive to product inhibition (24). The term "ambiquitous enzyme" was introduced by Wilson (39) to describe proteins that partition reversibly between soluble and membrane-bound forms with distinct kinetics.

The activation of the *E. coli* glycerol kinase by interaction with the glycerol facilitator appears to be analogous to the situation in mitochondria. We propose that GlpF activates glycerol kinase, rendering it insensitive to other effectors such as fructose-1,6-biphosphate (41) or enzyme $III^{Glc}$ (27). The increased catalytic activity would be of advantage for the cell even when external glycerol is abundant, suggesting an important role for the glycerol facilitator not only at low substrate concentrations.

It is surprising that despite the low level of activity of glycerol kinase in *glpF* strains, these strains grow on glycerol. It was reported by Richey and Lin (28) that these strains have a growth disadvantage. Lin (18) reported that strains lacking the glycerol facilitator need about 100-fold more substrate than the wild type (5 mM compared to 50 μM) to grow at a half-maximal rate.

In earlier publications, this was taken as evidence for a function of GlpF in glycerol permeation (28, 29). Our explanation is different and awaits evidence. We argue that growth in a *glpF* mutant is due to an alternate utilization of glycerol that exhibits a growth $K_m$ of 5 mM and is not initiated by phosphorylation. The fact that the distribution of metabolites was different in the wild type and the *glpF* mutant (see Fig. 4) supports the idea that the metabolic routes in these two strains are not identical. For example, the relative proportion of G3P is higher in the *glpF* mutant. We envision that growth in *glpF* mutants is due to the last gene in the *glpFKX* operon, *glpX*. All *glpF* mutants tested so far still expressed *glpX* (38). Therefore, it cannot be excluded that the growth at high glycerol concentrations observed in *glpF* mutants is due to the function of GlpX. Asnis and Brodie (1) have isolated an enzyme that catalyzes oxidation of glycerol to dihydroxyacetone from wild-type *E. coli*. The $K_m$ of this glycerol dehydrogenase for glycerol was determined to be 10 mM (1). The molecular weight was determined to be about 39,000 (34), similar to that of GlpX (38).

The experiments described in this paper have not given any direct evidence for a function of the GlpF protein as a specific facilitator for glycerol. Both *glpF* and *glpF*$^+$ strains equilibrate glycerol across the membrane in the absence of glycerol kinase in less than 30 s. This number allows estimation of a minimal rate of diffusion of glycerol through the membrane. At a 90 nM external concentration, at least $3 \times 10^{-13}$ mol/min/$10^9$ cells diffuses through the membrane, assuming a cell volume of $10^{-12}$ ml. Because passive diffusion is proportional to concentration, at 50 μM glycerol, the apparent $K_m$ of glycerol uptake in the *glpF* mutant, the minimal rate would only be 0.15 nmol/min/$10^9$ cells. This rate is high enough to easily satisfy the low phosphorylation capacity of glycerol kinase in the mutant (0.05 nmol/min/$10^9$ cells at 50 μM) but insufficient for the phosphorylation capacity in the wild type, which is close to 13.1 nmol/min/$10^9$ cells, the $V_{max}$ of the system.

Thus, our results do not exclude the possibility that GlpF in addition to stimulating glycerol kinase also functions as a specific pore facilitating the diffusion of glycerol. If it were possible to increase the phosphorylating capacity of glycerol kinase in a *glpF* mutant to levels observed in the wild type or higher by increasing the amount of GlpK at least 130-fold, there might well be a limitation of glycerol diffusion and the postulated facilitator function of GlpF could be directly demonstrated.

## ACKNOWLEDGMENTS

We appreciate the efforts of B. Hirner and C. Seiter, who sought genetic and biochemical evidence for a GlpF-GlpK interaction to no avail. We thank H. Schramke for construction of strain GD248, M. Anton, E. Bremer, and K. Heller for helpful discussion, E. Bakker for introducing us to the silicon oil method, and P. Postma and J. van der Vlag for the anti-GlpK antibodies.

This work was supported by the Deutsche Forschungs Gemeinschaft (SFB 156) and the Fonds der Chemischen Industrie.

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