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Allosteric Regulation of Glycerol Kinase by Enzyme III^{glc} of the Phosphotransferase System in *Escherichia coli* and *Salmonella typhimurium*

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The mechanism by which enzyme III^{glc} of the bacterial phosphotransferase system regulates the activity of crystalline glycerol kinase from *Escherichia coli* has been studied, and the inhibitory effects have been compared with those produced by fructose-1,6-diphosphate. It was shown that the free, but not the phosphorylated, form of enzyme III^{glc} inhibits the kinase. Mutants of *Salmonella typhimurium* were isolated which were resistant to inhibition by either enzyme III^{glc} (*glpK*^{r} mutants) or fructose-1,6-diphosphate (*glpK*^{i} mutants), and each mutant type was shown to retain full sensitivity to inhibition by the other regulatory agent. Other mutants were fully or partially resistant to regulation by both agents. The two regulatory sites on the kinase are evidently distinct but must overlap or interact functionally. Kinetic analyses have revealed several mechanistic features of the regulatory interactions. (i) Inhibition by both allosteric regulatory agents is strongly pH dependent, with maximal inhibition occurring at ca. pH 6.5 under the assay conditions employed. (ii) Binding of enzyme III^{glc} to glycerol kinase is also pH dependent, the K_i being near 4 μM at pH 6.0 but near 10 μM at pH 7.0. (iii) Whereas fructose-1,6-diphosphate inhibition apparently requires that the enzyme exist in a tetrameric state, both the dimer and the tetramer appear to be fully sensitive to enzyme III^{glc} inhibition. (iv) Inhibition by enzyme III^{glc} (like that by fructose-1,6-diphosphate) is noncompetitive with respect to both substrates. (v) The inhibitory responses of glycerol kinase to fructose-1,6-diphosphate and enzyme III^{glc} show features characteristic of positive cooperativity at low inhibitor concentration. (vi) Neither agent inhibits completely at high inhibitor concentration. (vii) Apparent negative cooperativity with respect to ATP binding is observed with purified *E. coli* glycerol kinase, with glycerol kinase in crude extracts of wild-type *S. typhimurium* cells, and with *glpK*^{r} and *glpK*^{i} mutant forms of glycerol kinase from *S. typhimurium*. These results serve to characterize the regulatory interactions which control the activity of glycerol kinase by fructose-1,6-diphosphate and by enzyme III^{glc} of the phosphotransferase system.

The bacterial phosphotransferase system (PTS) is a complex enzyme system which detects, transports, and phosphorylates a number of carbon sources in bacteria. It also regulates the activity of adenylate cyclase and the uptake of a number of carbohydrates, including glycerol, maltose, melibiose, and lactose (6, 15), in a process which has been termed inducer exclusion (7). Genetic and physiological studies led to the hypothesis that a regulatory protein, termed RPr and thought to be the glucose enzyme III (III^{glc}) of the PTS, directly regulates the activities of the uptake systems (16, 19). Subsequent biochemical investigations established that III^{glc} binds directly to an allosteric regulatory site on the cytoplasmic surface of the lactose permease to inhibit its activity (11). The same is apparently true for the melibiose and maltose permeases (17).

Recently, Postma et al. (12) presented evidence which suggested that the target of III^{glc} action responsible for the inhibition of glycerol uptake is glycerol kinase, the first enzyme of glycerol metabolism, rather than the glycerol facilitator. In vitro experiments at pH 7.5 showed that in crude extracts derived from glycerol-grown wild-type cells of *Salmonella typhimurium*, glycerol kinase was inhibited by III^{glc}. Half-maximal inhibition occurred at ca. 1 mg of III^{glc} per ml (ca. 50 μM). Experiments conducted in vivo have suggested that much lower concentrations should be inhibitory in vitro (1, 9, 18). Consequently, the results of Postma et al. (12) are anomalous.

If glycerol kinase rather than the glycerol permease is the target of PTS-mediated inhibition of glycerol uptake, mutants which specifically render uptake of glycerol resistant to PTS-mediated regulation (17, 20) should possess a glycerol kinase which is altered in the allosteric regulatory site of the enzyme that binds III^{glc}. Since the mutations which render glycerol uptake insensitive to PTS-mediated regulation must map within the structural gene for glycerol kinase (*glpK*), these mutants should be designated *glpK*^{r} *by analogy with the lacY*^{r} and *malK*^{r} mutants isolated previously and characterized by fine structure genetic analyses (14, 15).

Escherichia coli glycerol kinase is a tetrameric enzyme with a molecular weight of 220,000 (23–25). At saturating glycerol and Mg^{2+} concentrations, ATP saturation curves are not hyperbolic but yield double-reciprocal plots with limiting slopes that indicate two apparent K_m values for Mg-ATP, one at ca. 0.1 mM, the other at 0.5 mM (25). The kinase does not exhibit cooperativity with respect to the binding of glycerol. At neutral pH, the enzyme is subject to allosteric inhibition by fructose-1,6-diphosphate (FDP) (21, 25). The K_i value for this compound is ca. 0.5 mM. FDP inhibition is noncompetitive with respect to both substrates, and the percent inhibition is slightly sigmoidal when plotted versus the FDP concentration. Each subunit appears to possess a single binding site for each of the two substrates,

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as well as for the allosteric effector. The tetrameric glycerol kinase can dissociate into the dimeric species. Both the tetramer and the dimer have comparable catalytic activities, but only the tetramer binds the allosteric effector in a fashion which induces the enzyme to undergo a conformational transition to an inactive form (3–5). Mutant strains of *E. coli* in which glycerol kinase is resistant to FDP inhibition are termed *glpK*^{i} (21).

As reported in this communication, we have utilized a kinetic approach with crystalline glycerol kinase to elucidate the mechanism by which III^{glc} regulates the activity of the enzyme, and this inhibitory effect has been compared with that produced by FDP. It is shown that, in agreement with the results of Postma et al. (12), the free, but not the phosphorylated, form of III^{glc} inhibits the kinase. We also characterize mutants of *S. typhimurium* which are resistant to either III^{glc} or FDP inhibition and show that each mutant type retains sensitivity to inhibition by the other regulatory agent. The two regulatory sites are evidently distinct, although they may interact functionally. Kinetic analyses have served to confirm many of the results of de Riel and Paulus and Thorner and Paulus (3–5, 23–25) and have revealed several novel mechanistic features of the regulatory interaction with III^{glc}.

MATERIALS AND METHODS

Growth of bacterial strains and extract preparation. Overnight cultures of strains grown in nutrient broth were used to inoculate 250 ml of nutrient broth containing 0.5% glycerol. Bacteria were grown with shaking at 37°C until late log phase. Cells were harvested by centrifugation and washed three times with ice-cold 50 mM Tris-chloride–1 mM dithiothreitol–0.1 mM phenylmethylsulfonyl fluoride (pH 7.5) (TDP buffer). Cells were resuspended in an equal volume (wt/vol) of TDP buffer and broken by passage through a French press. Whole cells were removed by low-speed centrifugation (5,000 rpm in an SS-34 Sorvall rotor) for 10 min. The crude extract was separated into aliquots and stored at −70°C, where glycerol kinase was stable.

Assay procedures. The standard assay solution used for the determination of glycerol kinase activity contained in a volume of 100 μl (final concentrations): 50 mM buffer at the pH designated in the figure legends and the tables, 10 mM $MgCl_2$, 2.5 mM dithiothreitol, 5 mM ATP, 0.5 mM [^{14}C]glycerol, and variable amounts of glycerol kinase depending on the particular experiment. When cell extracts were used, 5 mM KF was added. Tubes were incubated for 10 min on ice before the addition of [^{14}C]glycerol, and they were then incubated at 37°C for 15 min. Tubes were diluted with 2 ml of ice-cold water and then applied to columns of Bio-Rad AG 1-X2 resin (anion exchanger). They were washed with 3 column volumes of water and eluted two times with 3 ml of 1 M LiCl into scintillation vials. Triton-toluene scintillation fluid (15 ml) was added, and the radioactive samples were counted. Assays for PTS proteins were performed as described previously (21). Protein was determined by the method of Lowry by using bovine serum albumin as the standard.

Protein purification. HPr, enzyme I, and III^{glc} were all purified from *E. coli* P650 to near homogeneity by published procedures (8, 27). HPr and enzyme I were essentially pure by the criterion of slab gel electrophoresis in sodium dodecyl sulfate (26). III^{glc} was estimated to be about 80% pure by sodium dodecyl sulfate gel electrophoresis and high-pressure liquid chromatography gel filtration but was a mixture of the two forms of the enzyme, 75% III^{glc}(slow) (presumed to be native III^{glc}) and 25% III^{glc}(fast) (a proteolytically clipped form of III^{glc}) as defined by Meadow and Roseman and Waygood et al. (8, 26).

Isolation and characterization of *glpK*^{r} and *glpK*^{i} mutants of *S. typhimurium*. Mutants defective for regulation of glycerol kinase were isolated both from *S. typhimurium* SB1475 (*ptsH15*) and SB1476 (*ptsI17*) (19) as follows. Three drops of a stationary-phase culture of one of these bacteria, grown in nutrient broth, were spread on a minimal agar plate containing glycerol (0.2%) and methyl α-glucoside (0.1%). This strain could not grow on these plates because methyl α-glucoside prevented induction of the glycerol catabolic enzymes and inhibited glycerol uptake. Mutation was induced with a crystal of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine as described previously (21). Mutant clones capable of glycerol utilization in the presence of methyl α-glucoside appeared after 2 to 3 days at 37°C. They were purified and tested for their ability to utilize and ferment a variety of carbon sources. Mutants were selected that exhibited the same phenotype as the parental strain except with respect to glycerol utilization.

Two types of glycerol-specific mutants were isolated by this procedure. One type of mutant (represented by strain LJ68 which contains the *glpK*^{2}*261* mutation) grew in media containing glycerol or glycerol plus methyl α-glucoside at the same rate, and it fermented glycerol poorly, as did the parental strain. The other mutant type (represented by strain LJ14, which contains the *glpK*^{i}*252* mutation) fermented glycerol more efficiently than did the parental strain but showed noticeable inhibition of glycerol utilization by methyl α-glucoside. This second mutant type was found to take up [^{14}C]glycerol and to excrete negatively charged radioactive metabolites derived from glycerol at rates that were about threefold greater than those observed with the parental strain when either D,L-lactate or glycerol served as the sole carbon source for growth. The regulatory properties of glycerol kinase in these and other mutant strains were characterized (see the legend to Fig. 6). Other mutants were isolated which exhibited the growth and fermentation properties of strain LJ14 but which were partially or fully resistant to regulation by both FDP and III^{glc} (see below).

Materials. Nutrient broth was from Difco Laboratories. [*U*-^{14}C]glycerol was obtained from ICN Pharmaceuticals. Crystalline *E. coli* glycerol kinase was purchased from Calbiochem-Behring. It was shown to be more than 90% pure by sodium dodecyl sulfate gel electrophoresis (subunit molecular weight, ca. 55,000) and by high-pressure liquid chromatography gel filtration (molecular weight near 220,000). The specific activity of the enzyme used in these studies employing our standard assay conditions was 140,000 μmol of product formed per min per mg of protein at pH 9.0. The specific activity at pH 6.5 was ca. 10% of this value. The crystalline enzyme was stable at 0°C or frozen at −20°C. ATP (Na^+ salt), fructose-1-P, fructose-6-P, FDP, fructose-2,6-diP, and phosphoenolpyruvate (monocyclohexylammonium salt) were all purchased from the Sigma Chemical Co. Bicine [*N*,*N*-bis(2-hydroxyethyl)glycine], HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), and MES [2-(*N*-morpholino)ethanesulfonic acid] buffers were purchased from Calbiochem-Behring. Tris was from Sigma.

RESULTS

pH dependency of glycerol kinase regulation by III^{glc} and FDP. Figure 1 shows the pH dependency of the activity of glycerol kinase. The enzyme exhibits maximal activity at pH 9.0, half-maximal activity at pH 7.5, and virtually no activity

at pH 5. Neither FDP nor III^{glc} were appreciably inhibitory at pH values greater than 7.5, but both were strongly inhibitory at slightly acidic pH. The optimal pH values for inhibition by both agents were between 6.0 and 6.5 (Fig. 1). Although the combined inhibitory effects of the two agents were not strictly additive, the activity of the kinase in the presence of FDP or III^{glc} alone was never more than that in the presence of both agents. Fructose-1-P, fructose-6-P, and fructose-2,6-diP were not inhibitory at a concentration of 5 mM at pH 6.5.

A study of III^{glc} inhibition as a function of both pH and III^{glc} concentration is shown in Fig. 2. It can be seen that as the pH was brought to more acidic values, the affinity of glycerol kinase for the inhibitory protein increased. For example, at pH 7.0 the K_i was ca. 200 μg/ml (10 μM), whereas at pH 6 it was ca. 80 μg/ml (4 μM). This observation provides a partial explanation for the pH curve shown in Fig. 1. It also explains the very low affinity binding observed by Postma et al. (12). These workers conducted their studies at pH 7.5 only. As a result of these observations and the pH curve shown in Fig. 1, we chose MES buffer (pH 6.5) for further studies on the regulation of glycerol kinase by FDP and III^{glc}.

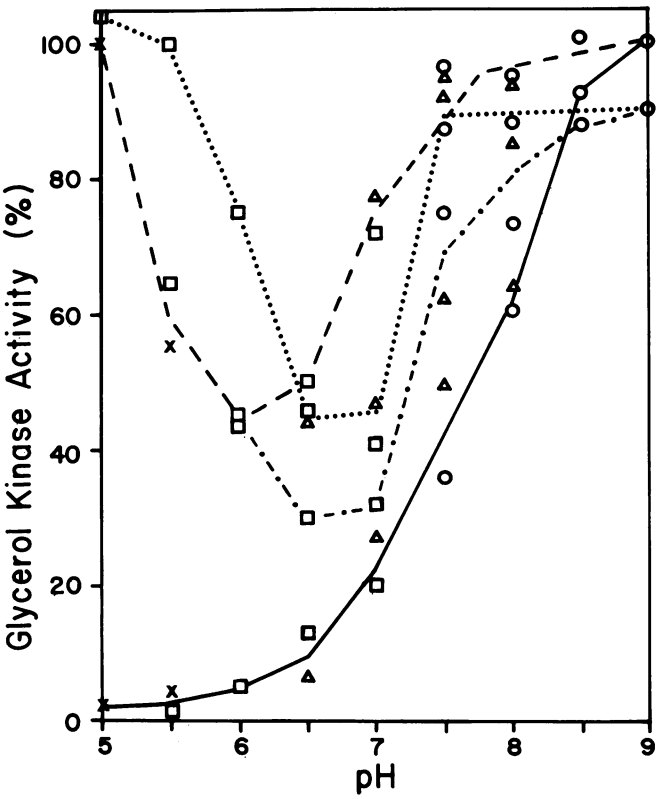

FIG. 1. pH curves for glycerol kinase and its inhibitory responses to FDP and III^{glc}. Symbols indicate the buffer used as follows: ○, Na^+ bicine; △, Na^+ HEPES; □, Na^+ MES; X, Na^+ acetate. The different curves were as follows: total activity relative to the value at pH 9.0 (——), percent residual activity in the presence of III^{glc} (0.4 mg/ml) (– – – –), percent residual activity in the presence of 5 mM FDP (. . . .), and percent residual activity in the presence of both inhibitory agents (– . – . – .). Assays were performed as described in text with crystalline glycerol kinase from *E. coli*. One hundred percent activity at pH 9 corresponds to 140,000 μmol of product formed per min per mg of protein. The specific activity at pH 6.5 was ca. 10% of this value.

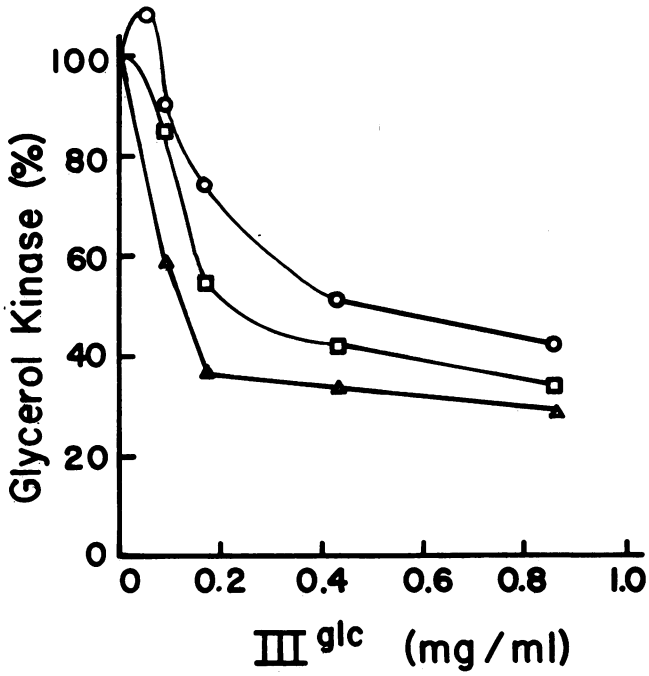

FIG. 2. Effect of pH on the inhibitory response of glycerol kinase to different concentrations of III^{glc}. The inhibitory responses were measured in Na^+ MES buffer at pH 7.0 (○), pH 6.5 (□), and pH 6.0 (△) as described in the text.

It should be noted from the shapes of the curves in Fig. 2 that the inhibiting effect of III^{glc} exhibited apparent cooperativity with respect to III^{glc} concentration. The slight stimulatory effect of low III^{glc} concentrations at pH 7.0 was reproducible but remains unexplained.

Kinetic analysis of III^{glc} inhibition. Figures 3 and 4 show the effects of the inclusion of III^{glc} in the assay solution on the kinetics of glycerol phosphorylation. When kinase activity was studied as a function of glycerol concentration, the K_m for glycerol was 7 μM both in the presence and absence of III^{glc} (Fig. 3). The observation of Thorner and Paulus (23–25) that the enzyme exhibits apparent negative cooperativity with respect to ATP concentration was confirmed (Fig. 4). Moreover, it can be seen that III^{glc} depresses the rates of glycerol phosphorylation to a comparable extent at all ATP concentrations tested. It depresses the V_{max} values, obtained by extrapolating the data both at low and high concentrations of ATP to infinite concentration, without altering the apparent K_m values. Because the experiment shown in Fig. 4 was conducted with saturating concentrations of glycerol (1 mM), the apparent K_m values should approximate the absolute K_m values for ATP. These values, both in the presence and absence of III^{glc}, were 0.06 mM at low ATP concentration and 0.9 mM at high ATP concentration. It should be noted that the Mg^{2+} concentration did not appreciably alter sensitivity to III^{glc} inhibition (data not shown).

Effect of enzyme dissociation on inhibition by FDP and III^{glc}. Thorner and Paulus (24, 25), and de Riel and Paulus (3–5) have shown that glycerol kinase can exist as both a dimer and a tetramer which exhibit comparable catalytic activities. However, only the tetramer is sensitive to inhibition by FDP. To detemine if inhibition by III^{glc} depended on the state of association of the enzyme, glycerol phosphorylation was measured as a function of the glycerol kinase concentration. The glycerol concentration was adjusted ap-

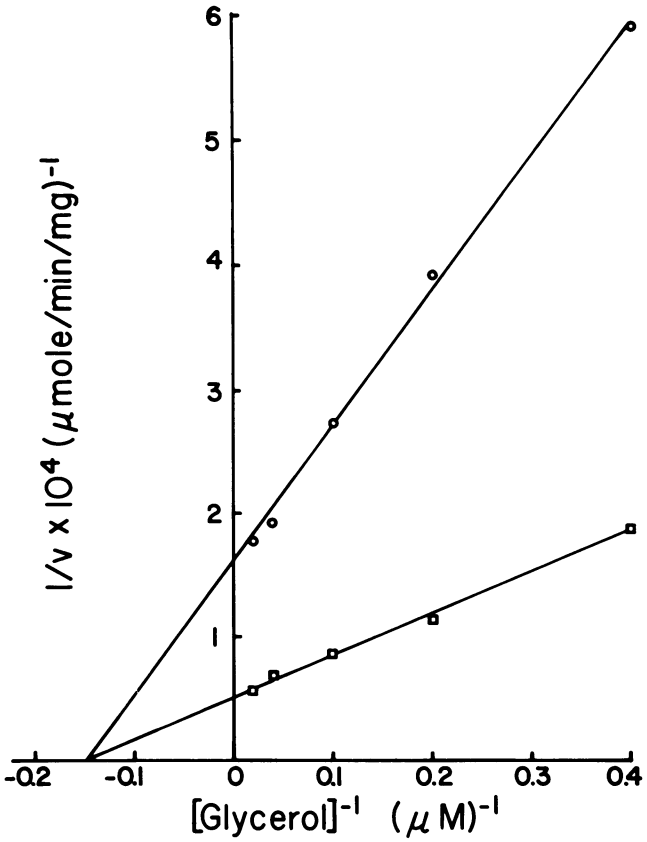

FIG. 3. Double-reciprocal plot (1/v versus 1/glycerol) in the presence or absence of III^{glc} (0.4 mg/ml). K_m values were 7 μM both in the presence (○) and absence (□) of III^{glc}. The concentration of ATP was 5 mM. Consequently, the enzyme was essentially saturated with ATP, and the apparent K_m values approximate the absolute K_m values for glycerol. Assays were performed as described in the text at pH 6.5 in Na^+ MES buffer with crystalline *E. coli* glycerol kinase.

propriately so that the reaction rate was linear with time at each protein concentration tested and so that less than 25% of the substrate was consumed. In Fig. 5 the inhibitory effects of FDP and III^{glc} were studied over a 1,000-fold glycerol kinase concentration range. It can be seen that although inhibition by FDP was completely relieved by enzyme dilution, inhibition by III^{glc} was equally strong at all kinase concentrations tested. It is therefore suggested that the dimer and tetramer are equally sensitive to III^{glc} inhibition and that the mechanism of III^{glc} inhibition differs from that of FDP inhibition. It should be noted, however, that III^{glc} binding might prevent tetramer dissociation under the assay conditions employed. In any case, these results argue against the suggestion of Postma et al. (12) that enzyme dilution was responsible for the low sensitivity of their glycerol kinase preparation to III^{glc} inhibition.

Effect of III^{glc} phosphorylation on glycerol kinase inhibition. Postma et al. (12) reported that phosphorylation of III^{glc} prevented inhibition of glycerol kinase in a crude system at pH 7.5. Table 1 shows that the same is true when crystalline glycerol kinase was studied under optimal assay conditions. The table also shows that although an excess of purified enzyme I and HPr exerted no effect on glycerol kinase activity either in the absence or presence of III^{glc}, the III^{glc} phosphorylating system completely reversed inhibition by III^{glc}.

Analysis of glycerol kinase mutants resistant to either FDP or III^{glc}. Mutants capable of growth on minimal agar medium containing 0.2% glycerol plus 0.1% methyl α-glucoside were isolated from both a leaky enzyme I mutant (*ptsI17*, SB1476) and a leaky HPr mutant (*ptsH15*, SB1475) (19). Several mutants were characterized in which glycerol kinase exhibited altered regulatory properties as described above. The mutants fell into three classes.

One class of mutants was resistant to inhibition by FDP but fully sensitive to inhibition by III^{glc}; a second class was resistant to inhibition by III^{glc} but fully sensitive to inhibition by FDP, and a third class was resistant to inhibition by both allosteric regulatory agents. Some of the mutants showed partial phenotypes, i.e., insensitivity to inhibition by FDP but partial sensitivity to inhibition by III^{glc}. Some of the analyses are shown in Fig. 6. Figure 6A shows the sensitivity of the mutants to inhibition by FDP, whereas Fig. 6B shows the sensitivity of the mutants to inhibition by III^{glc}. It can be seen that glycerol kinase assayed from the parental strain (LT2) was sensitive to inhibition by both FDP and III^{glc}. Strain LJ68 was resistant to inhibition by III^{glc} but fully sensitive to inhibition by FDP. The genetic defect in this strain is therefore designated *glpK*^{r}. By contrast, strain LJ14 is resistant to inhibition by FDP but shows full sensitivity to regulation by III^{glc}. The defect in this strain is therefore designated *glpK*^{i}.

It is also of interest to note that inhibition by FDP does not follow simple saturation kinetics. At low concentrations (<1 mM) inhibition by FDP appears to exhibit positive cooperativity, but even at very high concentrations of FDP, complete inhibition is not observed. These observations were valid for crystalline *E. coli* glycerol kinase, as well as for the enzyme from *S. typhimurium* strains LT2 and LJ68 (Fig.

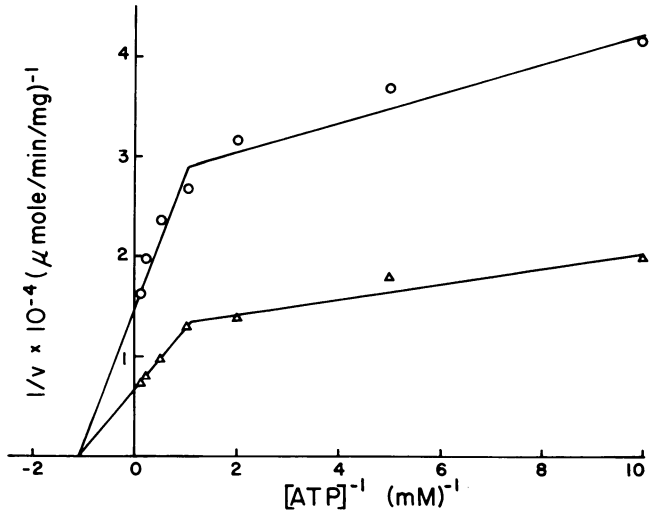

FIG. 4. Double-reciprocal plot (1/v versus 1/ATP) obtained from assays conducted in the presence (○) and absence (△) of III^{glc} (0.4 mg/ml). Extrapolated K_m values were 60 μM and 0.9 mM at low and high concentrations of ATP, respectively, both in the presence and absence of III^{glc}. Very similar plots were obtained when crude extracts of *S. typhimurium* LT2, LJ14, and LJ68 were assayed in the same way. The concentration of glycerol was 0.5 mM. Assays were conducted in Na^+ MES buffer (pH 6.5). In other respects, the assay conditions were as described in the text.

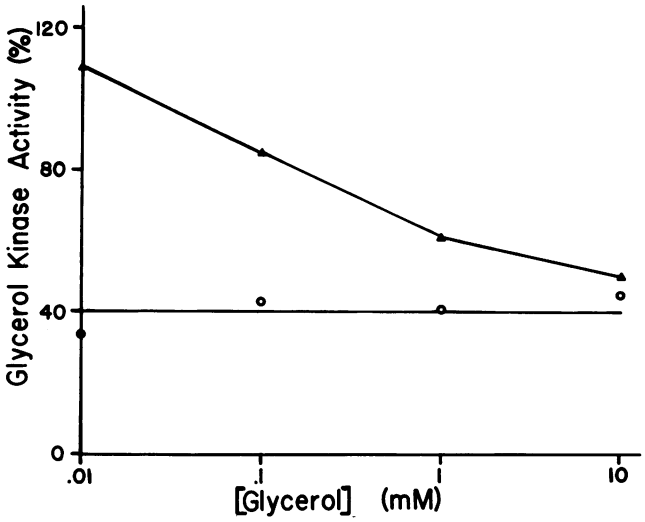

FIG. 5. Inhibitory responses of glycerol kinase to FDP and III^{glc} over a 1,000-fold range of glycerol and glycerol kinase concentrations. Symbols: △, inhibition by 5 mM FDP; ○, inhibition by III^{glc} (0.4 mg/ml). The ATP concentration was maintained at 5 mM. Na^+ MES buffer was used at pH 6.5. The concentrations of enzyme and glycerol were as follows: 0.01 mM glycerol, 0.01 μg of enzyme; 0.1 mM glycerol, 0.1 μg of enzyme; 1.0 mM glycerol, 1.0 μg of enzyme; 10 mM glycerol, 10 μg of enzyme. In other respects the assay was performed as described in the text.

6A). The significance of these observations has yet to be determined.

Finally, as shown in Fig. 4, crystalline glycerol kinase from *E. coli* exhibited apparent negative cooperativity with respect to ATP concentration. We examined the glycerol kinases from *S. typhimurium* LT2, LJ68, and LJ14 and observed that all three *Salmonella* enzymes exhibited apparent negative cooperativity with approximately the same K_m values: 0.2 to 0.3 mM at low concentrations of ATP and 0.8 to 1.0 mM at high ATP concentrations (data not shown). This result shows that apparent negative cooperativity (i) is observed for the *Salmonella* enzyme as well as the *E. coli* enzyme, (ii) is not an artifact resulting from purification, and (iii) is not abolished by the $glpK^i$ or $glpK^r$ mutations.

TABLE 1. Effect of PTS proteins on the inhibition of glycerol kinase by enzyme III^{glc} [a]

Additions	% Activity
None	100
PEP	109
EI + HPr	99
III^{glc}	35
EI + HPr + III^{glc}	37
EL + HPr + III^{glc} + PEP	105

[a] Assays were performed as described in the text in Na^+ MES buffer. The concentrations of PTS proteins were as follows: III^{glc}; 0.4 mg/ml; enzyme I (EI) and HPr, 0.3 IU/ml. The concentration of phosphoenolpyruvate (PEP) was 1.0 mM.

DISCUSSION

In vitro experiments performed in the laboratory of P. Postma showed that in crude extracts derived from glycerol-grown wild-type cells of *S. typhimurium*, glycerol kinase was inhibited by III^{glc}, with half-maximal inhibition occurring at ca. 1 mg of III^{glc} per ml (ca. 50 μM) (12). This concentration of III^{glc} was substantially in excess of that required for half-maximal inhibition of the lactose permease (ca. 200 μg/ml or 10 μM) (10, 11). Since the intracellular concentration of III^{glc} in *E. coli* and *S. typhimurium* is normally ca. 0.5 to 1 mg/ml (22), extrapolation of these in vitro results to the in vivo situation would lead to the prediction that glycerol uptake by whole cells could not be inhibited more than 50% by a sugar substrate of the PTS. Since inhibition in excess of 95% is frequently observed in vivo, it must be concluded that the in vitro assay conditions used by Postma et al. (12) did not mimic the in vivo conditions. The enzyme in the crude extract may not have been in its native state, or a component of the extract may have inhibited binding of III^{glc} to the kinase. It is possible that in the intact cell, glycerol kinase exists in specific association with the glycerol facilitator.

The studies reported here with homogeneous glycerol kinase have provided some clarification regarding these uncertainties. Inhibition of glycerol phosphorylation by both of the two allosteric effectors of glycerol kinase, FDP and III^{glc}, was found to be strongly pH dependent. For example, neither agent was inhibitory at basic pH, but both inhibited at slightly acidic pH values (pH 6.0 to 7.0). At pH 7.0 in

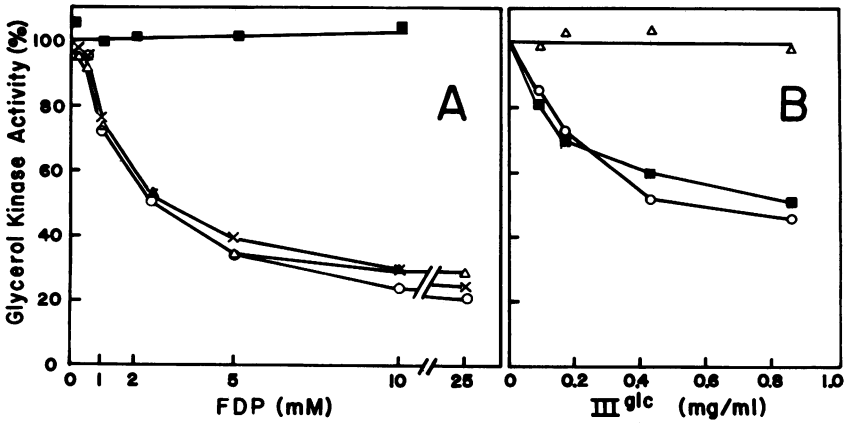

FIG. 6. Effects of FDP (A) and III^{glc} (B) on glycerol kinase activity employing purified *E. coli* glycerol kinase (X); glycerol kinase from *S. typhimurium* LT2 (wild type) (○); *S. typhimurium* mutant LJ14 ($glpK^i$) (□), and *S. typhimurium* LJ68 ($glpK^r$) (△). The standard assay was employed.

TABLE 2. Comparison of the regulatory activities of FDP and III^{glc} of the PTS on glycerol kinase

Characteristic	Characteristics of inhibition by:	
	FDP	III^{glc}
Nature of inhibition	Allosteric; noncompetitive	Allosteric; noncompetitive
pH optimum	~6.5	~6.5
Positive cooperativity	Yes	Yes
Complete inhibition	No	No
Dimer sensitive	No	Yes
Tetramer sensitive	Yes	Yes
glpK^r mutants sensitive	Yes	No
glpK^i mutants sensitive	No	Yes

MES buffer, the K_i of the kinase for III^{glc} was ca. 10 μM, whereas this value decreased to 4 μM when the pH was brought to 6.0. These values reflect much higher affinities than that measured by Postma et al. at pH 7.5 with a crude system. They also reflect higher affinity binding than reported previously for the interaction between III^{glc} and the lactose permease (10, 11). The in vitro results obtained at pH 6 therefore account for the fact that the inhibitory effect of a PTS sugar on glycerol uptake is stronger than that on lactose uptake (1, 18). The in vitro conditions reported here at slightly acidic pH presumably mimic the in vivo conditions. It should be noted, however, that even at pH 6.0, we did not observe inhibition approaching 100%. The in vivo conditions have presumably been only approximated by our in vitro assay conditions.

If glycerol kinase rather than the glycerol permease is the target of PTS-mediated inhibition of glycerol uptake, two predictions can be made. First, phosphorylation of III^{glc} with phosphoenolpyruvate, enzyme I, and HPr should completely reverse the inhibitory effect of III^{glc}, and second, mutants which specifically render uptake of glycerol resistant to PTS-mediated regulation (18, 21) should possess an altered glycerol kinase which is altered in the allosteric regulatory site of the enzyme which functions to bind III^{glc} These predictions have been verified.

Glycerol kinase from a *glpK*^{r} mutant was completely insensitive to regulation by III^{glc} at pH 6.5, the optimal pH for measuring the regulatory interactions. However, the enzyme was fully sensitive to inhibition by FDP. In contrast, glycerol kinase from a *glpK*^{i} mutant was insensitive to regulation by FDP but retained full sensitivity to regulation by III^{glc}. Other mutants were fully or partially insensitive to the inhibitory effects of both agents. These studies therefore establish that the allosteric binding sites on glycerol kinase for FDP and III^{glc} are distinct but functionally overlapping or interacting. The recent cloning of the *glpK* gene (2) should facilitate genetic analyses leading to a detailed understanding of the structural and functional basis for PTS-mediated inhibition of glycerol uptake.

PTS-mediated regulation of glycerol kinase, rather than of the permease, is fully consistent with the inducer exclusion mechanism proposed previously (13, 16). This conclusion results from the fact that the inducer of the glycerol regulon is α-glycerophosphate. Inhibition of either entry or phosphorylation of glycerol should prevent accumulation of the inducer in the cytoplasm.

The results reported here serve to confirm several of the results reported by Thorner and Paulus (23–25) and de Riel and Paulus (3–5). For example, kinetic constants, pH curves, and FDP inhibitory responses reported by these workers were very similar to those observed in our laboratory. Similarly, the apparent negative cooperativity for ATP and the positively cooperative effects that were apparent at low concentrations of FDP were reproduced, and we showed that these characteristics were properties of the crystalline *E. coli* enzyme, as well as the crude wild-type and mutant *S. typhimurium* enzymes. Our studies serve to extend the findings of Paulus and co-workers and of Postma et al. (12) primarily by presenting a detailed analysis of the III^{glc} inhibitory response. We have characterized this interaction and its consequences under a variety of conditions and have isolated and characterized mutants which are specifically altered with respect to the allosteric regulatory sites associated with the kinase. A comparison of the inhibitory responses of glycerol kinase to FDP and III^{glc} is summarized in Table 2.

From the results summarized in this table it can be seen that the inhibitory responses are similar in several respects. Both agents are allosteric inhibitors which function noncompetitively and with similar pH optima. However, the binding sites are clearly different based on the mutant analyses, and the dimeric species which is insensitive to inhibition by FDP appears to be sensitive to inhibition by III^{glc} (Fig. 5). The results duplicated in Fig. 5 might conceivably be explained otherwise if the tetramer binds III^{glc} much more tightly than does the dimer, and III^{glc} promotes association of the dimeric species to the tetramer. This possibility and others must await the development of physical techniques for studying the protein-protein interactions which promote the inhibitory responses to III^{glc}. These studies are presently in progress.

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LITERATURE CITED

1. **Castro, L., B. U. Feucht, M. L. Morse, and M. H. Saier, Jr.** 1976. Regulation of carbohydrate permeases and adenylate cyclase in *Escherichia coli*. J. Biol. Chem. **18**:5522–5527.
2. **Conrad, C. A., G. W. Sterns III, W. E. Prater, J. A. Rheiner, and J. R. Johnson.** 1984. Characterization of a *glpK* transducing phage. Mol. Gen. Genet. **193**:376–378.
3. **de Riel, J. K., and H. Paulus.** 1978. Subunit dissociation in the allosteric regulation of glycerol kinase from *Escherichia coli*. I. Kinetic evidence. Biochemistry **17**:5134–5140.
4. **de Riel, J. K., and H. Paulus.** 1978. Subunit dissociation in the allosteric regulation of glycerol kinase from *Escherichia coli*. II. Physical evidence. Biochemistry **17**:5141–5145.
5. **de Riel, J. K., and H. Paulus.** 1978. Subunit dissociation in the allosteric regulation of glycerol kinase from *Escherichia coli*. III. Role in desensitization. Biochemistry **17**:5146–5150.
6. **Dills, S. S., A. Apperson, M. R. Schmidt, and M. H. Saier, Jr.** 1980. Carbohydrate transport in bacteria. Microbiol. Rev. **44**:385–418.
7. **Magasanik, B.** 1970. Glucose effects: inducer exclusion and repression, p. 189–219. *In* J. R. Beckwith and D. Zipser (ed.), The lactose operon. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
8. **Meadow, N. D., and S. Roseman.** 1982. Sugar transport by the bacterial phosphotransferase system: isolation and characterization of a glucose-specific phosphocarrier protein (III^{glc}) from

Salmonella typhimurium. J. Biol. Chem. **257**:14526–14537.

9. **Nelson, S. O., and P. W. Postma.** 1984. Interactions *in vivo* between III^{Glc} of the phosphoenolpyruvate:sugar phosphotransferase system and the glycerol and maltose uptake systems of *Salmonella typhimurium*. Eur. J. Biochem. **139**:29–34.
10. **Nelson, S. O., J. K. Wright, and P. W. Postma.** 1983. The mechanism of inducer exclusion. Direct interaction between purified III^{glc} of the phosphoenolpyruvate:sugar phosphotransferase system and the lactose carrier of *Escherichia coli*. EMBO J. **133A**:27–32.
11. **Osumi, T., and M. H. Saier, Jr.** 1982. Mechanism of regulation of the lactose permease by the phosphotransferase system in *Escherichia coli*: evidence for protein-protein interaction. Ann. Microbiol. (Inst. Pasteur) **133A**:269–273.
12. **Postma, P. W., W. Epstein, A. R. J. Schuitema, and S. O. Nelson.** 1984. Interaction between III^{Glc} of the phosphoenolpyruvate:sugar phosphotransferase system and glycerol kinase of *Salmonella typhimurium*. J. Bacteriol. **158**:351–353.
13. **Saier, M. H., Jr.** 1977. Bacterial phosphoenolpyruvate:sugar phosphotransferase systems: structural, functional, and evolutionary interrelationships. Bacteriol. Rev. **41**:856–871.
14. **Saier, M. H., Jr.** 1982. The bacterial phosphotransferase system in regulation of carbohydrate permease synthesis and activity, p. 27–32. *In* A. Martonosi (ed.), Membranes and transport, vol. 2. Plenum Pulishing Corp., New York.
15. **Saier, M. H., Jr.** 1985. Mechanisms and regulation of carbohydrate transport in bacteria. Academic Press, Inc., New York.
16. **Saier, M. H., Jr., and B. U. Feucht.** 1975. Coordinate regulation of adenylate cyclase and carbohydrate permeases by the phosphoenolpyruvate:sugar phosphotransferase system in *Salmonella typhimurium*. J. Biol. Chem. **250**:7078–7080.
17. **Saier, M. H., Jr., M. J. Novotny, D. Comeau-. Fuhrman, T. Osumi, and J. D. Desai.** 1983. Cooperative binding of the sugar substrates and the allosteric regulatory protein (enzyme III^{glc} of the phosphotransferase system) to the lactose and melibiose permeases in *Escherichia coli* and *Salmonella typhimurium*. J. Bacteriol. **155**:1351–1359.
18. **Saier, M. H., Jr., and S. Roseman.** 1976. Sugar transport. The *crr* mutation: its effect on repression of enzyme synthesis. J. Biol. Chem. **241**:6598–6605.
19. **Saier, M. H., Jr., R. D. Simoni, and S. Roseman.** 1976. Sugar transport: properties of mutant bacteria defective in proteins of the phosphoenolpyruvate:sugar phosphotransferase system. J. Biol. Chem. **251**:6584–6597.
20. **Saier, M. H., Jr., and C. D. Stiles.** 1975. Molecular dynamics in biological membranes, p. 129. Springer-Verlag, New York.
21. **Saier, M. H., Jr., H. Straud, L. S. Massman, J. J. Judice, M. J. Newman, and B. U. Feucht.** 1978. Permease-specific mutations in *Salmonella typhimurium* and *Escherichia coli* that release the glycerol, maltose, melibiose, and lactose transport systems from regulation by the phosphoenolpyruvate:sugar phosphotransferase system. J. Bacteriol. **133**:1358–1367.
22. **Scholte, B. J., A. R. Schuitema, and P. W. Postma.** 1981. Isolation of III^{Glc} of the phosphoenolpyruvate-dependent glucose phosphotransferase system of *Salmonella typhimurium*. J. Bacteriol. **148**:257–264.
23. **Thorner, J. W.** 1975. Glycerol kinase. Methods Enzymol. **42**:148–156.
24. **Thorner, J. W., and H. Paulus.** 1971. Composition and subunit structure of glycerol kinase from *Escherichia coli*. J. Biol. Chem. **246**:3885–3894.
25. **Thorner, J. W., and H. Paulus.** 1973. Catalytic and allosteric properties of glycerol kinase from *Escherichia coli*. J. Biol. Chem. **248**:3922–3932.
26. **Waygood, E. B., R. L. Mattoo, and K. G. Peri.** 1984. Phosphoproteins and the phosphoenolpyruvate: sugar phosphotransferase system in *Salmonella typhimurium* and *Escherichia coli*: evidence for $III^{mannose}$, $III^{fructose}$, $III^{glucitol}$, and the phosphorylation of $II^{mannitol}$ and $II^{N\text{-acetylglucosamine}}$. J. Cell Biochem. **25**:139–159.
27. **Waygood, E. B., and T. Steeves.** 1980. Enzyme I of the phosphoenolpyruvate:sugar phosphotransferase system of *Escherichia coli*. Purification to homogeneity and some properties. Can. J. Biochem. **58**:40–48.