Escherichia coli Glycerol Kinase: Role of a Tetramer Interface in Regulation by Fructose 1,6-Bisphosphate and Phosphotransferase System Regulatory Protein III^{glc} †

Wei Zhang Liu,‡ Rick Faber,§,‖ Michael Feese,§ S. James Remington,§ and Donald W. Pettigrew*,‡

Department of Biochemistry & Biophysics, Texas A&M University, College Station, Texas 77843-2128, and Institute of Molecular Biology and Departments of Chemistry and Physics, University of Oregon, Eugene, Oregon 97402

Received April 29, 1994; Revised Manuscript Received June 13, 1994®

ABSTRACT: *Escherichia coli* glycerol kinase (EC 2.7.1.30; ATP:glycerol 3-phosphotransferase) is a key element in a signal transduction pathway that couples expression of genes required for glycerol metabolism to the relative availability of glycerol and glucose. Its catalytic activity is inhibited by protein–protein interactions with III^{glc}, a phosphotransferase system protein, and by fructose 1,6-bisphosphate (FBP); each of these allosteric effectors constitutes a positive signal that glucose is available. Loss of glucose inhibition of glycerol metabolism was used to screen for regulatory mutants of glycerol kinase after hydroxylamine mutagenesis of the cloned *glpK* gene. Two mutant enzymes were identified and shown by DNA sequencing to contain the mutations alanine 65 to threonine (A65T) and aspartate 72 to asparagine (D72N). Initial velocity studies show the mutations do not significantly affect the catalytic properties, hence active-site structures, of the enzymes. Both mutations decrease inhibition by FBP; A65T eliminates the inhibition while D72N appears to decrease the affinity for FBP and the extent of the inhibition. However, neither mutation significantly affects inhibition by III^{glc}. Gel-permeation chromatography studies show that both of the mutations alter the dimer–tetramer assembly reaction of the enzyme and the effect of FBP in increasing the molecular weight. The effects of the mutations on the assembly reaction are consistent with the locations of these two amino acid residues in the X-ray structure, which shows them to be associated with an α-helix that constitutes one of the two subunit–subunit interfaces within the tetramer. Thus, mutations that affect the dimer–tetramer assembly of glycerol kinase decrease the regulation by FBP but do not alter regulation by III^{glc}. These results suggest that the allosteric mechanism used to regulate the activity of glycerol kinase *in vivo* may be dependent on its concentration.

Glycerol kinase (EC 2.7.1.30; ATP:glycerol 3-phosphotransferase) catalyzes the rate-limiting step in glycerol utilization by *Escherichia coli* (Zwaig et al., 1970). The product of the reaction, G3P,[1] has at least two functions: after oxidation, it enters glycolysis at the level of the triose phosphates, serving as a carbon source; and it is the inducer for the expression of the elements of the *glp* regulon, one of which is the *glpK* gene coding for glycerol kinase (Lin, 1976). Thus, glycerol kinase is an element in a signal transduction pathway that couples information about carbon source availabilty to expression of the gene products needed for carbon source metabolism. Glycerol kinase activity in this pathway is modulated at the genetic level by control of transcription (Lin, 1976) and at the protein level by inhibition by III^{glc} of the PTS (Postma et al., 1984; Novotny et al., 1985) and the glycolytic intermediate, FBP (Zwaig & Lin, 1966). Each of the allosteric effectors constitutes a positive signal that glucose is available; and inhibition by the effector blocks inducer synthesis, preventing unneeded expression of proteins for glycerol metabolism. Cells which lack the allosteric control mechanisms are killed by glycerol if it is added to cultures that have induced expression of the *glp* regulon elements (Zwaig et al., 1970).

Investigations of these allosteric control mechanisms are motivated by their role in regulating a physiologically important signal transduction pathway. Control by III^{glc} is dependent on its state of phosphorylation in a mechanism termed inducer exclusion, which has been reviewed recently (Meadow et al., 1990; Postma et al., 1993). The crystal structure of the complex of glycerol kinase with the unphosphorylated form of III^{glc} has been determined (Hurley et al., 1993), and the association of the two proteins forms a novel intermolecular binding site for Zn(II) (Feese et al., 1994). A proposed mechanism for FBP regulation is based on a dimer–tetramer assembly reaction which was demonstrated for glycerol kinase (DeRiel & Paulus, 1978a) and postulates that FBP binds to and inhibits the tetrameric form of the enzyme (DeRiel & Paulus, 1978b). We report here the identification and characterization of two mutant glycerol kinases, designated A65T and D72N, which were identified after random chemical mutagenesis by utilizing a screen for loss of glucose control of glycerol metabolism that was described by Zwaig and Lin (1966). The effects of the mutations on the molecular weight and regulatory properties of glycerol kinase are consistent with a role of the dimer–tetramer assembly reaction

† Supported by Grants GM 42618 (S.J.R.) and GM 49992 (D.W.P.) from the National Institutes of Health and by the Texas Agricultural Experiment Station Expanded Research Areas, 1989–1991, and Research Enhancement Program 1991–1993.

* To whom correspondence should be addressed.

‡ Texas A&M University.

§ University of Oregon.

‖ Present address: Department of Biochemistry, Massey University, Palmerston North, New Zealand.

® Abstract published in *Advance ACS Abstracts,* August 1, 1994.

[1] Abbreviations: bp, base pairs; FBP, fructose 1,6-bisphosphate; G3P, *sn*-glycerol 3-phosphate; NEM, *N*-ethylmaleimide; PTS, phospho*enol*pyruvate:sugar phosphotransferase system; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Mutant proteins are designated by using the single-letter abbreviation for the amino acids and the notation, e.g., A65T, meaning that alanine, the wild-type amino acid residue at position 65, is replaced by threonine.

0006-2960/94/0433-10120$04.50/0

in FBP regulation but not in III^{glc} regulation, and their effects on the dimer–tetramer assembly reaction are consistent with their locations in the structure.

MATERIALS AND METHODS

Materials. Chemicals and enzymes are purchased from Sigma Chemical Co., St. Louis, MO, unless otherwise indicated. Restriction enzymes were obtained from either New England Biolabs or Promega Corp. [1,3-$^{14}C_2$]glycerol was purchased from New England Nuclear. Purified *E. coli* III^{glc} was provided by Drs. Norman D. Meadow and Saul Roseman of the Department of Biology, The Johns Hopkins University.

Plasmids. The plasmid used for the source of the *glpK* gene in these studies, pWT165, was constructed by cloning the *Hin*dIII fragment that contains the gene from the RFM13 DNA that was used in the sequencing of the gene (Pettigrew et al., 1988) into the vector pHG165 (Stewart et al., 1986). Successful recombinants were identified by screening on MacConkey–glycerol–ampicillin plates.

Bacterial Strains and Media. E. coli strains RY1693 (Hfr PK3 *thr-1 leuB6 thi1 lacY1 azi-15 tonA21 supE44*) and TB1 (*ara* Δ(*lac–proAB*) *rpsL φ80 lacZΔM15 hsdR*(r_k^-, m^{k+})) (Baldwin et al., 1984) were obtained from Ry Young of the Department of Biochemistry & Biophysics, Texas A&M University. *E. coli* E.C.C. Lin strain 27 (Lin, 1976) were obtained from the *E. coli* Genetic Stock Center at the Department of Biology, Yale University.

E. coli strain DG1 was constructed in this laboratory to provide a *glpK* deletion background for mutagenesis work; the strain previously used as a background, JJ161 (Conrad et al., 1984), reportedly produces wild-type amounts of immunologically reactive but catalytically inactive glycerol kinase upon induction by glycerol (Cozzarelli & Lin, 1966). The strain DG1 was constructed by crossing a plasmid-borne copy of the *glpK* gene containing a deletion into the chromosome of strain TB1. A deletion in the cloned *glpK* gene was constructed by digesting pWT165 with *Eco*RV, which cleaves at nucleotide 204 generating a blunt end, and *Dra*III, which cleaves at nucleotide 469. The overhanging end of the *Dra*III cleavage site was filled in with T4 DNA polymerase, and the plasmid was circularized by blunt-end ligation following the protocols in Maniatis et al. (1982). Competent cells of strain TB1 were prepared according to Maniatis et al. (1982) and transformed with the ligation reaction. Successful transformants were identified by growth on LB–ampicillin plates. Twelve colonies were chosen and plasmids were purified by the alkaline lysis method (Maniatis et al., 1982) after overnight growth in LB–ampicillin. The putative deletion constructs were screened for absence of cleavage by *Eco*RV; four isolates were obtained. These four isolates were further characterized with respect to cleavage by *Eco*RV, *Eco*RI, *Dra*III, *Bam*HI, and *Hin*dIII. None of the plasmids was cleaved by *Dra*III or *Eco*RV, and the cleavage products from digestion by the other enzymes produced DNA fragments of the expected sizes. Both *Bam*HI and *Eco*RI gave a single fragment of 5915 bp, and *Hin*dIII gave two fragments of 3380 and 2535 bp. The size of the cloning vector pHG165 is 3374 bp (Stewart et al., 1986). The size of the *Hin*dIII fragment that bears the intact *glpK* gene is 2800 bp (Pettigrew et al., 1988); the *Eco*RV–*Dra*III cleavage and fill procedure should remove 263 bp, generating a *Hin*dIII fragment of 2537 bp and making the total predicted length of the construct to be 5911 bp. The construct, pDP1, contains the first 204 bp at the 5′ end and the last 1032 bp at the 3′ end of the *glpK* gene, and the reading frame is shifted one base at the ligation junction.

The *glpK* deletion was moved into the chromosome by transforming RY1693 (Hfr PK3) with pDP1. The transformed strain was mated with strain TB1. Exconjugates were selected by plating on MacConkey–glycerol plates with ampicillin and streptomycin sulfate (100 μg/mL). Twelve white colonies (Glc^-) were chosen as candidates for plasmid integration events into *glpK*. These candidates were passaged four times by growth to saturation in LB medium without antibiotics to allow segregation and loss of the plasmid. The cultures were plated on MacConkey–glycerol medium. White colonies obtained after overnight incubation at 37 °C were screened for Amp^R, and two Amp^S isolates, designated DG1 and DG2, were selected for further study. For both strains, a Glc^- phenotype on MacConkey–glycerol plates is complemented by the plasmid pWT165, carrying *glpK*. The two strains appeared to be identical to one another, and strain DG1 was used for all subsequent work. The deletion in the chromosomal copy of the *glpK* gene in strain DG1 was verified by Southern blotting. Subsequent experiments showed that strain DG1 retains α-complementation to the *lacZΔM15* mutation.

LB medium was made according to Maniatis et al. (1982). MacConkey agar base was a product of Difco and was prepared according to the manufacturer's instructions with carbon sources added to a final concentration of 1%, unless otherwise indicated. Ampicillin was added when indicated to a final concentration of 75 μg/mL.

Mutagenesis and Identification of Putative Glucose-Control Mutants. Random mutagenesis was performed by using hydroxylamine. Purified pWT165 plasmid DNA was incubated for 2 h at 70 °C with 28 mg/mL hydroxylamine in 0.125 M potassium phosphate, pH 6, and 2.5 mM EDTA. The hydroxylamine was removed by using a spun column of Sephadex G-25 in TE buffer (Maniatis et al., 1982). The mutagenized plasmid DNA was used to transform competent cells of E.C.C. Lin strain 27, which was used because it is $glpK^-$ and $glpR^-$, constitutively expressing the remaining elements of the *glp* regulon. The transformed cells were screened for putative glucose-control mutants by using the procedure described by Zwaig and Lin (1966). The transformation reactions (50 μL) were plated on MacConkey–ampicillin agar containing 1% glucose and 1 μM [1,3-$^{14}C_2$]glycerol (1 Ci/mol). After overnight incubation at 37 °C, the colonies were blotted with sterile filter paper, and the blots were placed on X-ray film for 48 h for autoradiography. Putative mutants were identified from dark spots on the autoradiograph, indicative of their ability to concentrate the radioactive glycerol even in the presence of glucose. As a positive control, cells of E.C.C. Lin strain 43, a regulatory mutant that was identified by using this screen (Zwaig and Lin, 1966), were plated separately; all colonies of this strain gave dark spots on the autoradiographs. As an additional control, strain 27 cells that were transformed with pWT165 that was either untreated or carried through a mock mutagenesis procedure without hydroxylamine were plated; no dark spots were obtained for any colonies on these plates.

Sequencing of the Mutants. The *Hin*dIII fragment bearing each of the mutants was excised from the respective plasmid and ligated into the *Hin*dIII site of M13mp19. Both orientations of each inserted fragment were isolated, as determined by a complementary test using M13mp19 containing the wild-type gene in known orientation and verified by DNA sequencing. The DNA sequence of each fragment was determined by using the dideoxynucleotide method with the Sequenase enzyme from U.S. Biochemicals and [α-^{35}S]-thio-2′-dATP from New England Nuclear. A series of

oligonucleotide primers that hybridize at intervals of about 250 bp along the *glpK* gene was used for the sequencing. Both DNA strands were sequenced using both dGTP and dITP reactions.

Enzyme Activity Assays and Kinetics Studies. The enzyme activity of glycerol kinase was measured by using the previously described ADP-coupled spectrophotometric assay (Pettigrew et al., 1990) at pH 7 and 25 °C with additions as indicated in the figure legends and tables. Initial velocity studies were performed and analyzed as described previously (Pettigrew et al., 1990). One unit of enzyme corresponds to the conversion of 1 μmol of substrate to product/min under the stated conditions. For these studies, glycerol was removed from the purified glycerol kinases by either dialysis or Sephadex G-25 column chromatography using 0.1 M triethanolamine hydrochloride buffer, pH 7.0.

For the initial screening procedures, crude extracts were prepared by using centrifugation to collect the bacterial cells after overnight growth in LB–ampicillin. Following removal of the spent medium, the cells were resuspended in a volume of standard buffer at pH 7 (Pettigrew et al., 1990) equal to the two-thirds of the volume of the culture taken for the centrifugation. The cells were disrupted by sonication, and the extracts were clarified by centrifugation. Glycerol kinase activity was determined by using 5–10 μL of an appropriate dilution of the extract to initiate the coupled reaction in a total volume of 1 mL.

Gel-Permeation Chromatography. Glycerol kinase samples of 0.2 mg of protein in 0.5 mL of 0.1 M triethanolamine hydrochloride buffer at pH 7.0, 2 mM glycerol, with or without 2 mM FBP, were applied to a Bio-Gel 0.5m column (1.3 × 30 cm) equilibrated with the same buffer. The flow rate was maintained by using an LKB Microperpex peristaltic pump attached to the column outlet, and 0.5-mL fractions of the effluent were collected by using an Isco fraction collector. The column and fraction collector were operated at ambient temperature (~21 °C). The elution position of glycerol kinase was determined by measuring glycerol kinase activity with the ADP-coupled spectrophotometric assay with 3 mM ATP and 2 mM glycerol. The columns were calibrated by using the following standards with the indicated molecular weights: blue dextran to determine the void volume, glycylglycine to determine the total volume, catalase (232 000), aldolase (148 000), bovine serum albumin (66 000), ovalbumin (45 000), and myoglobin (17 000). Elution volumes were determined by absorbance measurements of the fractions. Partition coefficients were determined from the observed elution volumes of the standards and glycerol kinases as described by Ackers (1970). A calibration curve was constructed by plotting the logarithm of the molecular weight of the protein standard *versus* the experimentally determined partition coefficient. Apparent molecular weights of the glycerol kinases were determined from the calibration curve.

RESULTS

Identification of Glucose-Control Mutants of Glycerol Kinase. Putative glucose-control mutants of glycerol kinase were generated by using the hydroxylamine mutagenesis procedure that is described under Materials and Methods. A total of 215 transformants were obtained in two separate mutagenesis experiments; of these, 46 colonies were identified as putative mutants by the autoradiographic screen and chosen for further characterization. The putative mutants were grown overnight in LB–ampicillin, and extracts that were prepared from these overnight cultures were used for further screening. Glycerol kinase activity and inhibition by 10 mM FBP were assessed by assaying enzyme activity by using an ADP-coupled spectrophotometric assay (Pettigrew et al., 1990), and expression of glycerol kinase was assessed by using SDS–PAGE.

Two putative mutants, designated 51 and 191, showed less FBP inhibition than the wild-type enzyme while producing the same level of enzyme, as assessed by specific activity and SDS–PAGE. These putative mutant *glpK* genes were sequenced as described under Materials and Methods. In each case, the sequence of the mutant gene differed from that of the wild-type gene (Pettigrew et al., 1988) by only a single nucleotide. For 51, the nucleotide change is G193A, changing codon 66 from GCG to ACG, which translates to the amino acid mutation A65T.[2] For 191, the nucleotide change is G214A, changing codon 73 from GAT at AAT, which translates to the amino acid mutation D72N. Each of the nucleotide mutations is consistent with the C to T base transition expected for hydroxylamine mutagenesis (Drake, 1970) occurring in the complementary DNA strand.

The remaining putative mutants displayed the same degree of inhibition by FBP as the wild-type enzyme. The expression of glycerol kinase in these cells was increased markedly, as indicated by increased specific activity in the enzyme assays and by SDS–PAGE. It appears that the increased level of glycerol kinase results in false positives. Results of initial mutagenesis studies in which we used a different plasmid, pCJ102 (Conrad et al., 1984), which constitutively expresses higher enzyme levels, are consistent with this hypothesis; all of the colonies produced putative glucose-control mutants with this plasmid.

Purification of Glycerol Kinase Glucose-Control Mutants. The plasmids bearing the mutant *glpK* genes were moved from strain 27 to strain DG1 for enzyme expression and purification. Both of the mutant glycerol kinases were purified from this strain by using previously described protocols (Faber et al., 1989). For the purification of the A65T glycerol kinase, an additional purification step using hydrophobic chromatography on phenyl-Sepharose, with a gradient from 20% to 70% ethylene glycol in 0.1 M triethanolamine hydrochloride buffer, pH 7, 2 mM glycerol, and 1 mM β-mercaptoethanol, was used. SDS–PAGE of the purified enzymes showed them to be greater than 95% homogeneous glycerol kinase (not shown). Two independent purifications of each mutant glycerol kinase were performed. Preliminary studies showed that the functional properties were the same for both preparations of each mutant enzyme. Most of the results that are reported here were obtained with the second preparation of each enzyme.

Initial Velocity Studies. Initial velocity studies were performed in the forward reaction direction by using the ADP-coupled spectrophotometric assay that is described under Materials and Methods. Results of the initial velocity studies are shown in the double-reciprocal plots in Figure 1. Similar results are obtained for both mutant glycerol kinases; at a low concentration of glycerol (0.015 mM), substrate inhibition by ATP is observed, while apparent substrate activation by ATP is observed at higher glycerol concentrations. These results contrast those obtained for the wild-type enzyme, where apparent activation by ATP is observed at all glycerol concentrations (Pettigrew et al., 1990).

The initial velocity data were analyzed by fitting the data obtained at ATP concentrations ≤0.1 mM, as done previously

[2] The amino acid residues are numbered as they appear in the wild-type purified protein, from which the N-terminal methionine residue is removed after translation; thus, amino acid residue *i* in the numbering of the protein corresponds to codon *i* + 1 in the *glpK* gene (Pettigrew et al., 1988).

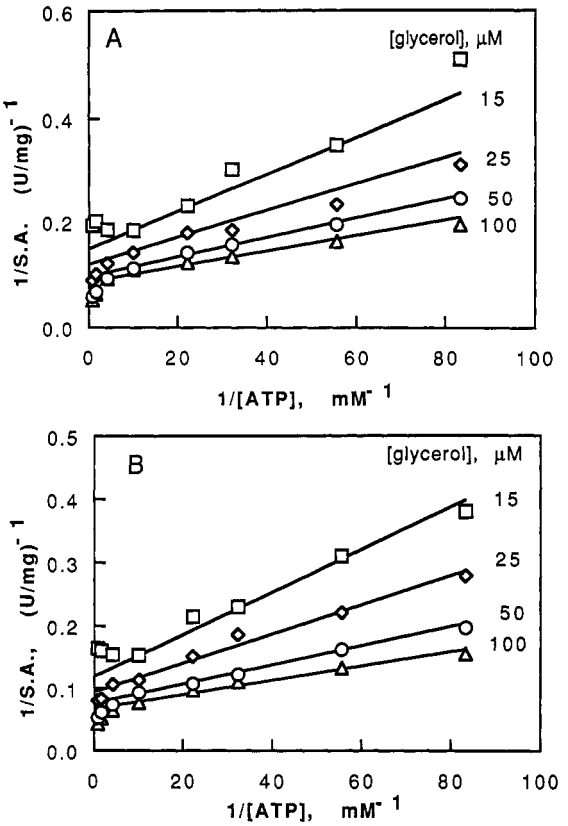

FIGURE 1: Double-reciprocal plot of the ATP dependence of the initial velocity for mutant glycerol kinases A65T and D72N. Initial velocities were measured by using the ADP-coupled spectrophotometric assay that is described under Materials and Methods at pH 7, 25 °C, and 0.44 μg/mL mutant glycerol kinase. Glycerol concentrations are indicated on the figures. The points are the experimental data, and the lines show the fit of the data at [ATP] ≤ 0.1 mM. (A) Glycerol kinase A65T. (B) Glycerol kinase D72N.

Table 1: Steady-State Kinetics Parameters for Mutant Glycerol Kinases[a]

enzyme	V_{max} (units/mg)	K_a (μM)	K_b (μM)	K_{ia} (μM)
A65T	13.6 ± 1	15 ± 4	15 ± 4	34 ± 17
D72N	17.3 ± 1	4 ± 3	18 ± 4	34 ± 14
wt[b]	15.3 ± 0.3	8.4 ± 0.7	4.9 ± 1.2	86 ± 25

[a] Steady-state kinetics parameters were estimated from fits of the initial velocity data in Figure 1 as described under Materials and Methods. Substrate a is ATP and substrate b is glycerol. [b] Parameters for the wild-type glycerol kinase from Pettigrew et al. (1990).

for the wild-type enzyme (Pettigrew et al., 1990). The kinetic constants obtained from the fits are summarized and compared to those obtained for the wild-type enzyme in Table 1. The V_{max} is little altered by the mutations, showing that the conformation of the active site in the ternary complex is essentially the same as that of the wild-type enzyme. The mutations A65T and D72N have modest effects on the substrate Michaelis and dissociation constants. The Michaelis constants appear to be unaffected or increased about 2–4-fold while the dissociation constants are decreased about 2-fold. As a consequence of these changes, the dissociation constant is about 2 times larger than the Michaelis constant, in contrast to the case of the wild-type enzyme, for which the dissociation constant is 10 times larger than the Michaelis constant. These kinetic results indicate that the mutations A65T and D72N have small effects on the active-site structure of glycerol kinase. This result is consistent with the similar specific activities obtained for the mutants and wild-type enzyme in the crude extracts that were used in the initial screening procedures.

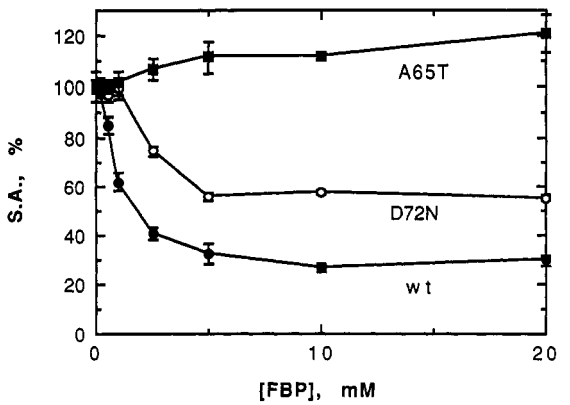

FIGURE 2: FBP inhibition of wild-type (wt) and mutant glycerol kinases. The specific activities of glycerol kinases at pH 7, 25 °C, 0.44 μg/mL, 0.1 mM glycerol, 2 mM ATP, and the indicated concentrations of FBP were determined by using the ADP-coupled spectrophotometric assay that is described under Materials and Methods. The results are expressed as a percentage relative to the specific activity at 0 mM FBP, values for which (units per milligram) are as follows: wt, 26.9; A65T, 22.8; D72N, 23.5. The points show the mean of at least three determinations and the error bars show the standard deviation. The results obtained for each enzyme are labeled in the figure.

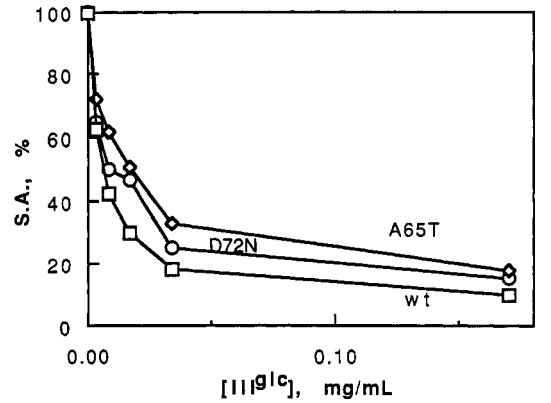

FIGURE 3: III^{glc} inhibition of wild-type (wt) and mutant glycerol kinases. The specific activities of glycerol kinases at pH 7, 25 °C, 2 mM glycerol, 2 mM ATP, 0.1 mM $ZnCl_2$, and the indicated concentrations of III^{glc} were determined by using the ADP-coupled spectrophotometric assay that is described under Materials and Methods. The results are expressed as a percentage relative to the specific activity at 0 mg/mL III^{glc}, values for which (units per milligram) are as follows: wt, 17.2; A65T, 7.3; D72N, 6.8. The enzyme concentrations (micrograms per milliliter) were as follows: wt, 0.5; A65T, 0.87; D72N, 0.5. The results obtained for each enzyme are labeled in the figure.

FBP and III^{glc} Regulatory Properties. Effects of these mutations on regulation of glycerol kinase by FBP and III^{glc} are shown in Figures 2 and 3, respectively. The results that are presented in Figure 2 show that the two mutations have different effects on FBP inhibition of glycerol kinase. The mutation by D72N appears to decrease the affinity for FBP and to decrease the extent of inhibition. The mutation A65T appears to not only abolish FBP inhibition under these assay conditions but also result in weak activation by FBP. It is important to note that the decreased extent of inhibition observed at 10 mM FBP with the purified mutant enzymes is consistent with the results obtained with the crude preparations that were used in the initial screening procedures.

On the other hand, neither mutation has much effect on III^{glc} regulation, as shown in Figure 3. This result is consistent with genetics studies showing that mutants that lack one or the other, or both, modes of regulation can be isolated (Novotny et al., 1985). Analysis of these data indicates that III^{glc} inhibition of D72N is indistinguishable from the wild-type

Table 2: Effect of FBP on the Molecular Weight of Mutant Glycerol Kinases[a]

enzyme	molecular weight	
	no FBP	2 mM FBP
A65T	115 000	115 000
D72N	115 000	178 000
wt	158 000	178 000

[a] Apparent molecular weights were estimated by gel-permeation chromatography on Bio-Gel 0.5m as described under Materials and Methods.

glycerol kinase, and an apparent inhibition constant for III^{glc} of about 0.3 μM is obtained. For the A65T glycerol kinase, a value of about 0.9 μM is obtained for the inhibition constant. The maximum extent of inhibition, about 95% under these conditions, is the same for all three enzymes.

Effects of the Mutations on the Molecular Weight of Glycerol Kinase. Results of gel permeation chromatography studies that are presented in Table 2 show that the mutations A65T and D72N, which affect FBP inhition, also affect the apparent molecular weight of glycerol kinase as well as the effect of FBP on the apparent molecular weight. The subunit molecular weight of glycerol kinase is 56 000 (Pettigrew et al., 1989). In the absence of FBP, the molecular weight of the wild-type glycerol kinase is greater than that of the dimer, which is consistent with the dimer–tetramer assembly reaction of glycerol kinase (DeRiel & Paulus, 1978a). However, the apparent molecular weight of both of the mutant glycerol kinases corresponds to that of the dimer. This observation suggests that the mutations interfere with the tetramer assembly reaction, shifting the equilibrium toward the dimer. The decreased apparent molecular weight of the mutant glycerol kinases may also reflect increased interactions with the chromatography support medium, leading to an increased elution volume. However, similar results were obtained with two different support media, Sephadex G-200 and Bio-Gel 0.5m, which suggests that this explanation is unlikely.

The mutations A65T and D72N have different effects on the response of the apparent molecular weight to FBP. Addition of FBP results in an increase in the apparent molecular weights of the wild-type enzyme and the D72N mutant, and the molecular weights are both increased to the same value. The result obtained for the wild-type enzyme is consistent with the results described by DeRiel and Paulus (1978a). However, FBP has little or no effect on the molecular weight of the A65T mutant glycerol kinase. This result is consistent with a decreased ability for tetramer formation, decreased affinity for FBP, or both.

DISCUSSION

Effects of the Mutations on the Catalytic Properties and ATP Regulation of Glycerol Kinase. The mutations A65T and D72N have little effect on the catalytic properties of glycerol kinase. These mutations appear to have only small effects on the V_{max}, Michaelis constants, or substrate dissociation constants. The net result of the apparent small changes in the Michaelis and substrate dissociation constants is closer agreement between these constants; for the mutant glycerol kinases, the substrate dissociation constants are twice the Michaelis constants, while for the wild-type enzyme, the dissociation constants are 10 times the Michaelis constants. The primary difference between the catalytic properties of these mutant glycerol kinases and the wild-type enzyme is the observation of apparent substrate inhibition by MgATP at the lowest glycerol concentration. There are several possible explanations for this observation, but these cannot be evaluated from the present data. The mutations do not alter the regulatory behavior with respect to ATP at higher glycerol concentrations; i.e., the apparent substrate activation by ATP is not affected. Retention of the apparent substrate activation by ATP observed for the mutation A65T indicates that assembly to the tetramer is not required for the ATP regulation, which is consistent with its previously reported lack of protein concentration dependence (Thorner & Paulus, 1973) and indicates that it is an inherent property of the dimer.

The observation that these mutants do not appear to have a large effect on the catalytic properties of glycerol kinase is consistent with the similar specific activities of the dimer and tetramer postulated by deRiel and Paulus (1978b). According to that postulate, the conformational changes that are associated with tetramer assembly have little or no effect on the catalytic properties. The mutant glycerol kinases A65T and D72N show that the converse is true; namely, structure changes that affect the dimer–tetramer assembly reaction have little or no effect on the catalytic properties.

Effects of the Mutations on the Enzyme Structure. The effects on the dimer–tetramer assembly reaction determined by gel-permeation chromatography are consistent with structural changes. The absence of significant effects on the catalytic properties suggests that the structure of the active-site region is not much affected by either of the mutations and is consistent with localized conformational changes in the region of the mutation.

The effects of the mutations on the dimer–tetramer assembly reaction are consistent with the locations of the two amino acid residues in the crystal structure of glycerol kinase. The dimer–tetramer contact in the tetramer consists of interactions between a subunit in one dimer and each of the subunits in the other dimer (Hurley et al., 1993). One of these contacts is formed between the symmetry-related O and Z subunits by a loop and α-helix consisting of amino acid residues 321–333, while the other is formed by α-helices from each monomer, residues 49–68, that pack in an antiparallel fashion to form the only contact between O and X subunits in the tetramer. Amino acid residue A65 is located in this α-helix, while amino acid residue D72 is located just C-terminal to it. Figure 4 shows closeup views of this region of the structure for one of the crystal forms of the wild-type enzyme (R. Faber and S. J. Remington, manuscript in preparation). Since this region constitutes a significant portion of the contacts between the dimers, mutations here are expected to affect the dimer–tetramer assembly reaction. Since each mutant subunit contains the amino acid change, there are two mutations in each contact, i.e., four mutations per tetramer. Due to the small interacting surfaces in this tetramer contact, even small perturbations of the structure can be expected to affect the assembly of the tetramer because each amino acid residue contributes a significant portion of the interactions. Thus, small, local conformational changes that do not affect the active site could affect the tetramer assembly reaction.

Examination of Figure 4 (top) shows that mutation of A65 to T would increase the size of the amino acid side chain at each end of the interacting helices. This is expected to block sterically the interactions between the helices, preventing tetramer formation. This does not appear to be the case for the D72N mutation, however. Figure 4 (bottom) shows that D72 is located in a loop that connects the tetramer interface α-helix to a strand of β-conformation which is part of the central β-sheet of domain IA of the protein (Hurley et al., 1993). The carboxylate group of D72 is near the carboxylate group of E2 and the ϵ-amino group of K232. Thus, conversion of the carboxylate to an amide seen for the D72N mutation

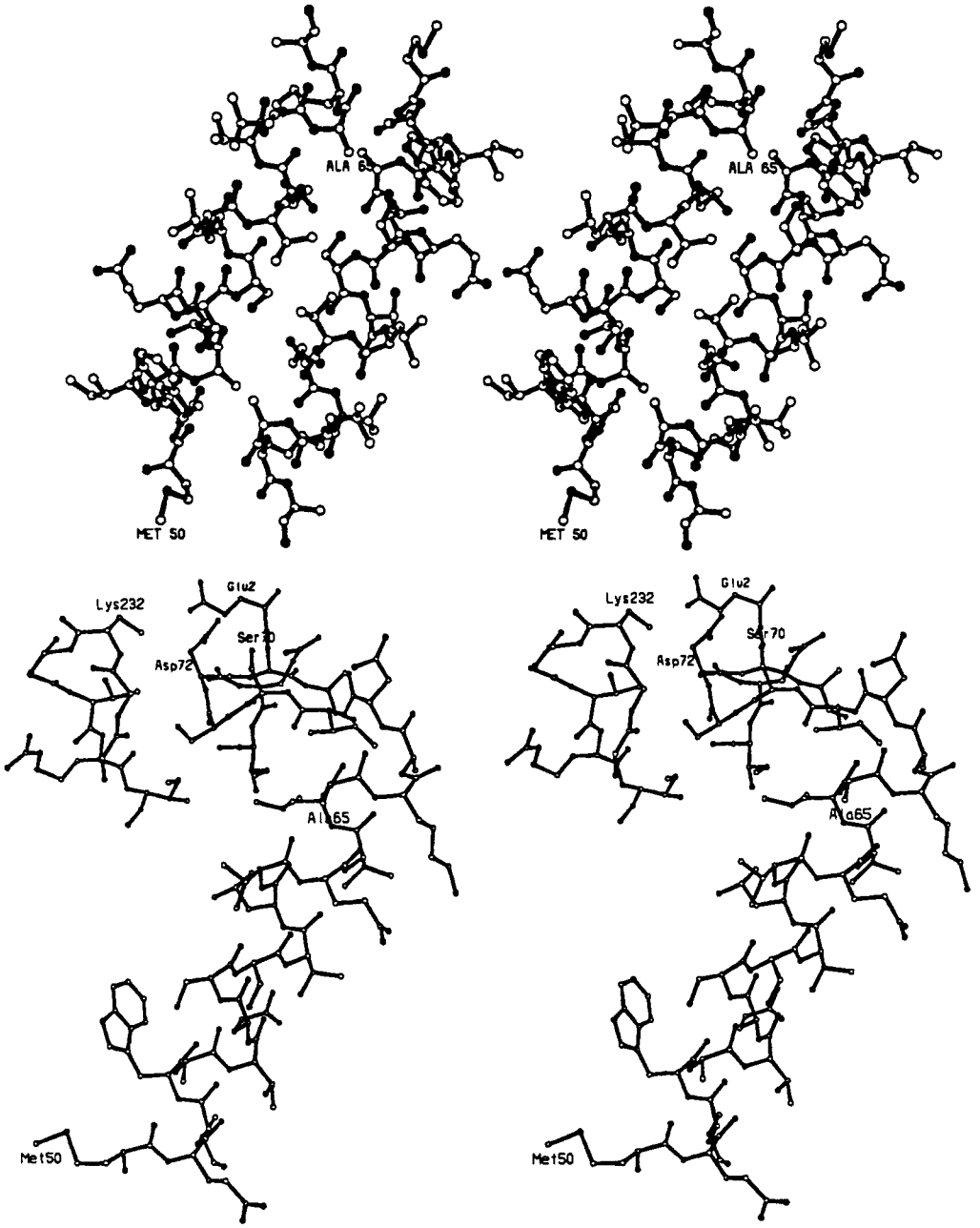

FIGURE 4: Stereoviews of the regions of glycerol kinase around the sites of mutation. These views were constructed from coordinates obtained for the complex of glycerol kinase with glycerol and MgADP (R. Faber and S. J. Remington, manuscript in preparation). (Top panel) A65 and tetramer interface α-helix interactions. This view is approximately normal to the O–X interface, showing the interactions between the α-helices from each subunit, where the α-helix from one subunit is on the left-hand side with the N-terminus at the bottom, labeled MET 50, and the other α-helix is on the right-hand side with the N-terminus at the top. The β-carbon of A65 in the left-hand helix is labeled. (Bottom panel) Region around D72. This view is approximately normal to that shown in the top panel, showing one of the α-helices from the perspective of its neighboring subunit in the tetramer; the relation of this view to that shown in the top panel can be seen by comparing the orientations of W53. Residue D72 and nearby charged residues E2 and K232, as well as A65, are labeled. Residue K232 is truncated at its γ-carbon.

is expected to perturb significantly the electrostatic interactions in this region. The observation that the D72N mutation alters tetramer assembly but does not affect the catalytic properties indicates that the perturbation of the electrostatic environment affects the structure of the tetramer interface α-helix but does not affect the structure of the central β-sheet. Direct demonstration of the effects of these mutations on enzyme structure will be provided by crystallographic studies.

Effects of the Mutations on the Allosteric Regulation of Glycerol Kinase. The effects of the mutations are consistent with a role for the dimer–tetramer assembly reaction in the regulation of FBP and suggest that tetramer assembly is required for FBP inhibition. These conclusions are based on the relations between the results of the gel-permeation chromatography experiments and the FBP inhibition experiments. Each of the mutations appears to shift the dimer–tetramer equilibrium toward the dimer, by stabilizing the dimer, destabilizing the tetramer, or both. This is shown by the smaller molecular sizes of each of the mutant glycerol kinases relative to the wild-type enzyme in the absence of FBP. The molecular sizes of both A65T and D72N glycerol kinases correspond to the dimer in the absence of FBP, while the molecular size of the wild-type enzyme is significantly larger than that of the dimer. Addition of FBP shifts the molecular size of the D72N enzyme to that of the wild-type enzyme, while it does not affect the size of the A65T enzyme. This suggests that the A65T mutation has a greater effect on the dimer–tetramer equilibrium than does the D72N mutation, which is consistent with the structure shown in Figure 4.

The effects of these mutations on inhibition of the catalytic activity by FBP are consistent with the results of the gel-permeation chromatography studies. FBP binding to the wild-type enzyme shifts the equilibrium strongly toward the tetramer (DeRiel & Paulus, 1978a). However, FBP binding is unable to shift the dimer–tetramer equilibrium completely to the tetramer at the low enzyme concentrations that are typically used in the ADP-coupled assay (~0.5 μg/mL). This accounts for the observation that the maximum extent of inhibition in these assays is about 75%; i.e., about 25% of the enzyme remains in the active dimer and/or tetramer forms under these conditions. For the D72N mutation, the shift in the dimer–tetramer equilibrium toward the dimer is reflected as a decrease in the maximum extent of inhibition in the assay to about 50%.

The result that is obtained for the A65T mutant may provide insight into the relations between FBP binding and the dimer–tetramer assembly reaction. According to the model that was developed by DeRiel and Paulus (1978b), the dimer does not bind FBP. The gel-permeation chromatography results show that the dimer–tetramer equilibrium is strongly shifted toward the dimer by the mutation A65T, to the extent that no tetramer is formed even at a protein concentration that is nearly 2 orders of magnitude greater than that in the activity assay. Thus, the dimer is the only form expected at the low concentrations of enzyme in the activity assay. For this mutation, no inhibition is observed in the assay; indeed a slight activation is observed (Figure 2), suggesting the dimer does bind FBP.

Neither of the mutations significantly affects inhibition by III^{glc}. This shows that regulation by III^{glc} is independent of regulation by FBP, which is consistent with results of genetics studies (Novotny et al., 1985). In addition, it indicates that III^{glc} regulation does not involve the dimer–tetramer assembly reaction; i.e., regulation by III^{glc} is not dependent on glycerol kinase concentration, as shown by Novotny et al. (1985). This result has important implications for control of enzyme activity *in vivo*; it indicates that III^{glc} inhibition of glycerol kinase can occur at the low uninduced level of the enzyme, which may not be the case for FBP control. It is tempting to speculate that, in fact, these two different inhibitors operate under two different glycol kinase concentration regimes, with III^{glc} providing control in uninduced cells and FBP providing control in fully induced cells. The results oriented here are consistent with this postulate.

It is relevant to ask how these mutants, which retain normal III^{glc} regulation, could be identified by the screen for loss of glucose control of glycerol metabolism; in particular, why was glycerol incorporation not inhibited by III^{glc} even in the absence of FBP inhibition? The lack of III^{glc} inhibition results from the use of the multicopy plasmid-borne copy of the *glpK* gene. This results in higher levels of glycerol kinase expression; the uninduced level of glycerol kinase in these cells is at least 4 times higher than the basal level in the parent strain TB1. Under these conditions, the limiting amount of dephospho-III^{glc} is titrated by the higher glycerol kinase levels. This provides a teleological explanation for the two independent mechanisms for glucose control of glycerol kinase activity; regulation by FBP provides the means to effectively inhibit the activity at higher enzyme concentrations in the face of limiting III^{glc}. However, the false positives obtained in our initial mutagenesis experiments using plasmid pCJ102 show that both control mechanisms are rendered inoperative by excess glycerol kinase. This observation shows that FBP does not completely inhibit glycerol kinase under these conditions, suggesting that the concentration of FBP in the cell is not sufficient to saturate the enzyme and/or the enzyme·FBP complex retains partial catalytic activity. The importance of its *in vivo* concentration in the allosteric regulation of glycerol kinase is also indicated by the ability of the screen to identify the D72N mutation despite its relatively modest effects on FBP inhibition. Thus, at the lower concentration of enzyme that is obtained with the uninduced pWT165 plasmid, the small effects of the D72N mutation on FBP inhibition are sufficient to permit detection of loss of glucose control of glycerol metabolism, while this is not the case at the higher enzyme levels obtained from constitutive expression with the pCJ102 plasmid.

ACKNOWLEDGMENT

We thank Sandie Scarborough and Shari Fernandez for expert technical assistance, Drs. Ry Young, Mike Benedik, and Greg Shipley for helpful discussions and for assistance in the construction of strain DG1, and Drs. Paul Fitzpatrick and David Giedroc for helpful comments on the manuscript.

REFERENCES

Ackers, G. K. (1970) *Adv. Protein Chem. 24*, 343–446.
Baldwin, T. O., Berends, T., Bunch, T. A., Holzman, T. F., Rausch, S. K., Shamansky, L., Treat, M. L., & Ziegler, M. M. (1984) *Biochemistry 23*, 3663–3667.
Conrad, C. A., Stearns, G. W., III, Prater, W. E., Rheiner, J. A., & Johnson, J. R. (1984) *Mol. Gen. Genet. 193*, 376–378.
Cozzarelli, N. R., & Lin, E. C. C. (1966) *J. Bacteriol. 91*, 1763–1766.
de Riel, J. K., & Paulus, H. (1978a) *Biochemistry 17*, 5141–5146.
de Riel, J. K., & Paulus, H. (1978b) *Biochemistry 17*, 5134–5140.
Drake, J. W. (1970) *The Molecular Basis of Mutation*, p 150, Holden-Day, San Francisco, CA.
Faber, H. R., Pettigrew, D. W., & Remington, S. J. (1989) *J. Mol. Biol. 207*, 637–639.
Feese, M., Pettigrew, D., Meadow, N., Roseman, S., & Remington, S. J. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 3544–3548.
Hurley, J. H., Faber, H. R., Worthylake, D., Meadow, N. D., Roseman, S., Pettigrew, D. W., & Remington, S. J. (1993) *Science 259*, 673–677.
Lin, E. C. C. (1976) *Annu. Rev. Microbiol. 30*, 535–578.
Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning. A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harber, NY.
Meadow, N. D., Fox, D., & Roseman, S. (1990) *Annu. Rev. Biochem. 59*, 497–542.
Novotny, M. J., Frederickson, W. L., Waygood, E. B., & Saier, M. H., Jr. (1985) *J. Bacteriol. 162*, 810–816.
Pettigrew, D. W., Ma, D.-P., Conrad, C. A., & Johnson, J. R. (1988) *J. Biol. Chem. 263*, 135–139.
Pettigrew, D. W., Yu, G.-J., & Liu, Y. (1990) *Biochemistry 29*, 8620–8627.
Postma, P., Epstein, W., Schuitema, A., & Nelson, S. (1984) *J. Bacteriol. 158*, 351–353.
Postma, P. W., Lengeler, J. W., & Jacobson, G. R. (1993) *Microbiol. Rev. 57*, 543–594.
Stewart, G. S. A. B., Lubinsky-Mink, S., Jackson, C. G., Cassel, A., & Kuhn, J. (1986) *Plasmid 15*, 172–181.
Thorner, J. W., & Paulus, H. (1973) *J. Biol. Chem. 248*, 3922–3932.
Zwaig, N., & Lin, E. C. C. (1966) *Biochem. Biophys. Res. Commun. 22*, 414–418.
Zwaig, N., Kistler, W. C., & Lin, E. C. C. (1970) *J. Bacteriol. 102*, 753–759.