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A Single Amino Acid Change in *Escherichia coli* Glycerol Kinase Abolishes Glucose Control of Glycerol Utilization In Vivo†

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***Escherichia coli* glycerol kinase (EC 2.7.1.30; ATP:glycerol 3-phosphotransferase) is a key element in glucose control of glycerol metabolism. Its catalytic activity is inhibited allosterically by the glycolytic intermediate, fructose 1,6-bisphosphate, and by the phosphotransferase system phosphocarrier protein, III^{Glc} (also known as IIA^{Glc}). These inhibitors provide mechanisms by which glucose blocks glycerol utilization in vivo. We report here the cloning and sequencing of the *glpK22* gene isolated from E. C. C. Lin strain 43, a strain that shows the loss of glucose control of glycerol utilization. DNA sequencing shows a single missense mutation that translates to the amino acid change Gly-304 to Ser (G-304-S) in glycerol kinase. The effects of this substitution on the functional and physical properties of the purified mutant enzyme were determined. Neither of the allosteric ligands inhibits it under conditions that produce strong inhibition of the wild-type enzyme, which is sufficient to explain the phenotype of strain 43. However, III^{Glc} activates the mutant enzyme, which could not be predicted from the phenotype. In the wild-type enzyme, G-304 is located 1.3 nm from the active site and 2.5 nm from the III^{Glc} binding site (M. Feese, D. W. Pettigrew, N. D. Meadow, S. Roseman, and S. J. Remington, Proc. Natl. Acad. Sci. USA 91:3544–3548, 1994). It is located in the same region as amino acid substitutions in the related protein DnaK which alter its catalytic and regulatory properties and which are postulated to interfere with a domain closure motion (A. S. Kamath-Loeb, C. Z. Lu, W.-C. Suh, M. A. Lonetto, and C. A. Gross, J. Biol. Chem. 270:30051–30059, 1995). The global effect of the G-304-S substitution on the conformation and catalytic and regulatory properties of glycerol kinase is consistent with a role for the domain closure motion in the molecular mechanism for glucose control of glycerol utilization.**

Glycerol kinase (EC 2.7.1.30; ATP:glycerol 3-phosphotransferase) catalyzes the rate-limiting step in glycerol utilization by *Escherichia coli* (25). Its catalytic activity is regulated at the protein level by inhibition by two allosteric effectors, the glucose-specific phosphocarrier protein of the phospho*enol*pyruvate:sugar phosphotransferase system, III^{Glc} (also known as IIA^{Glc}) (13, 18), and the glycolytic intermediate, fructose 1,6-bisphosphate (FBP) (26). Control by III^{Glc} is dependent on its state of phosphorylation in a mechanism termed inducer exclusion, which has been reviewed recently (12, 19, 20). These effectors provide the basis for glucose inhibition of glycerol utilization. In wild-type cells, this inhibition prevents synthesis of glycerol 3-phosphate, which is the inducer for the elements of the *glp* regulon. We are investigating the molecular basis of these allosteric control mechanisms by which glycerol kinase functions in a signal transduction pathway that modulates gene expression in response to carbon source availability. The crystal structure of the complex of glycerol kinase with the unphosphorylated form of III^{Glc} has been determined (8), and the association of the two proteins forms a novel intermolecular binding site for Zn(II) (5). A proposed mechanism for FBP regulation postulates that FBP binds to and inhibits the tetrameric form of the enzyme (3). We have recently shown that amino acid substitutions in the tetramer interface which decrease the extent of tetramer formation also decrease FBP inhibition and that FBP and III^{Glc} inhibition can operate independently (10).

We report here the cloning and sequencing of the *glpK22* gene from E. C. C. Lin strain 43. The phenotype for this mutant is the loss of glucose inhibition of glycerol utilization, resulting in simultaneous utilization of both carbon sources (26). Initial characterization of strain 43 showed that it produces a glycerol kinase that is insensitive to inhibition by 3 mM FBP, a result which demonstrated the role of FBP in glucose regulation of glycerol utilization. However, the role of III^{Glc} in diauxic growth was not known at that time, and the effect of III^{Glc} on this mutant was unknown. The substitution in the protein that is deduced from the gene sequence is Gly-304 to Ser (G-304-S). Purification and characterization of the mutant glycerol kinase show that this single amino acid substitution has a global effect on the kinase. It affects the catalytic, regulatory, and conformational properties of the enzyme. This broad range of effects is consistent with the location of G-304 in the structure of the wild-type enzyme and suggests a central role for a domain movement mechanism in the catalytic and regulatory properties of glycerol kinase.

MATERIALS AND METHODS

Materials. Chemicals and enzymes were purchased from Sigma Chemical Co. (St. Louis, Mo.) unless otherwise indicated. Restriction enzymes were obtained from either New England BioLabs or Promega Corp. III^{Glc} was purified as described previously (14). *E. coli* DG1 [*ara*Δ(*lac-proAB*) (ϕ80 *lac*ZΔM15) *rpsL glpK202 hsdR4*] was constructed in this laboratory to provide a *glpK* deletion background (10). E. C. C. Lin strain 43 (CGSC 5511) containing the *glpK22* allele was obtained from the *E. coli* Genetic Stock Center at the Department of Biology, Yale University.

Cloning and sequencing of the *glpK22* gene. The *glpK22* gene was cloned by complementing the glycerol-negative phenotype of strain DG1 with restriction

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fragments of chromosomal DNA from strain 43. Molecular biology procedures were performed as described by Maniatis et al. (11), except as noted. Chromosomal DNA was isolated from strain 43 by using the procedure of Silhavy et al. (21). The purified chromosomal DNA was digested with *Hin*dIII or with *Eco*RI and ligated into the corresponding restriction site of plasmid pHG165 (22). Competent cells of strain DG1 were transformed and plated on MacConkey agar-glycerol-ampicillin to screen for complementation of the glycerol-negative phenotype. For DNA sequencing, the *Hin*dIII fragment bearing the *glpK22* gene was excised from plasmids purified from the glycerol-positive cells and ligated into the *Hin*dIII site of M13mp19. Both orientations of the inserted fragment were isolated, as determined by a complementarity test with a *glpK* gene of known orientation and verified by DNA sequencing. The DNA sequences of the sense and antisense strands were determined by using the dideoxy nucleotide method with the Sequenase enzyme from U.S. Biochemicals and [α-^{35}S]thio-2′-dATP from New England Nuclear as described elsewhere (10). Both dGTP and dITP reactions were run in parallel to permit reading of the sequence through regions of compressions.

Luria-Bertani (LB) medium was made as described by Maniatis et al. (11). The 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.

Purification of the G-304-S glycerol kinase. The wild-type and G-304-S glycerol kinases were purified as described previously (4). For the G-304-S glycerol kinase, additional protein bands were seen by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the preparation after the first two chromatography columns. An additional purification step was therefore necessary, with the use of hydrophobic chromatography on phenyl-Sepharose with a gradient from 20 to 70% ethylene glycol in 0.1 M triethanolamine-HCl buffer (pH 7)–2 mM glycerol–1 mM β-mercaptoethanol. SDS-PAGE of the purified enzyme showed that it was greater than 95% pure glycerol kinase (data not shown). Three independent purifications were done, and the catalytic and regulatory properties of the different preparations were the same.

Enzyme activity assays and kinetics studies. The enzyme activity of glycerol kinase was determined by using an ADP-coupled spectrophotometric assay (16) at pH 7 and 25°C with additions as indicated in the figure legends and tables. For these assays, glycerol is routinely removed from the enzyme by either dialysis or Sephadex G-25 chromatography after the crystalline enzyme is dissolved (10, 16). However, for the G-304-S glycerol kinase, removal of glycerol resulted in rapid, irreversible inactivation of the enzyme, accompanied by its precipitation. Consequently, the concentration of glycerol was maintained at ≥1 mM, and, for assays at the lowest concentration of glycerol, no glycerol other than that in the enzyme solution was added. The reactions were initiated by the addition of the enzyme to the cuvettes after thermal equilibration. Under these conditions, the enzyme retains its activity, and its steady-state kinetic properties are well behaved. One unit of enzyme converts 1 μmol of substrate to product per min under the stated conditions.

Initial-velocity studies were performed and analyzed as previously described (16). Kinetic constants were obtained by fitting the data obtained at [ATP] of ≤3 mM to the following equation:

$$v = \frac{V_{max}[ATP][glycerol]}{[ATP][glycerol] + K_{atp}[glycerol] + K_{glycerol}[ATP] + K_{iatp}K_{glycerol}}$$

where v is the initial velocity, V_{max} is the maximum velocity, K_{atp} and $K_{glycerol}$ are the Michaelis constants for the substrates, and K_{iatp} is the dissociation constant for ATP. In this range of ATP concentrations, the data are well described by the equation. At higher concentrations of ATP, substrate inhibition is obtained, and those data were omitted from the analysis. This is equivalent to the method used previously in analyzing the kinetics of the wild-type enzyme, which show apparent activation by ATP (16).

Nonlinear least-squares fitting of the data to the equations was performed by using NonLin for Macintosh, written by R. J. Brenstein, Robelko Software, Carbondale, Ill.

In the studies of the III^{Glc} regulation, several control experiments were performed to verify the role of III^{Glc} in the activation of the G-304-S enzyme. The apparent activation is not due to a contaminant in the III^{Glc} preparation because no activity is obtained in the absence of glycerol kinase. Furthermore, it does not represent a simple stabilization of the G-304-S enzyme. The activity of the enzyme is not altered by incubation for 15 min in the assay without either Zn(II) or III^{Glc} prior to initiation of the reaction by the addition of ATP. Incubation with Zn(II) results in a small increase in activity, followed by a slow decrease, which is not significant in the time scale of the enzyme-initiated reactions. The small increase in activity due to Zn(II) alone is not reflected in the data presented because the data for each curve are normalized to the activity obtained at 0 μM III^{Glc} but with all the remaining assay components present. If both Zn(II) and III^{Glc} are included in the incubation, the large increase in activity discussed below is obtained.

Gel permeation chromatography. The apparent molecular weights of glycerol kinase samples of 0.2 mg of protein in 0.5 ml of 0.1 M triethanolamine-HCl buffer at pH 7.0–2 mM glycerol, with or without FBP, were determined by gel permeation chromatography as described previously (10). The elution positions of the enzymes were determined either by continuously monitoring the A_{280} of the column effluent or by assays of enzyme activity in fractions of the effluent. Both methods yielded the same apparent molecular weights for both wild-type and the G-304-S glycerol kinase.

Proteolysis studies. Incubation mixtures containing glycerol kinase at 0.1 mg/ml in 0.1 M triethanolamine HCl (pH 7) were prepared, with other additions as indicated. The digestions were initiated by the addition of *N*-α-*p*-tosyl-L-lysine chloromethylketone (TLCK)-α-chymotrypsin to a final concentration of 5 μg/ml and incubated at room temperature. At the indicated times, aliquots of the reaction mixture containing 1 μg of glycerol kinase were removed, and the reaction was quenched with 1 mM phenylmethylsulfonyl fluoride. An equal volume of 2× SDS-PAGE sample buffer was added, and the samples were heated in a boiling water bath for 3 min. The entire sample was loaded onto an SDS-PAGE gel (10% separating gel). After electrophoresis was completed, the gels were stained with Coomassie blue to visualize the proteins. For quantitation, the destained gels were scanned by using a Hewlett-Packard Scan Jet IIcx scanner with the program SigmaGel by Jandel Scientific Software, and the scans were analyzed by using the National Institutes of Health program Image 1.59. Pseudo-first-order plots were constructed by plotting the log of the ratio of the integrated band intensity at time t to that at time zero versus time. For both glycerol kinases, the rate of disappearance of the 56-kDa band, i.e., the subunit, follows pseudo-first-order kinetics. Because of the instability of the G-304-S enzyme on the removal of glycerol, glycerol was included in all the digests for both enzymes. Glycerol does not alter the kinetics or cleavage pattern for the wild-type enzyme. No cleavage occurs during incubation of glycerol kinase and III^{Glc} separately or together in the absence of chymotrypsin. The effect of III^{Glc} on the cleavage of either glycerol kinase requires the presence of Zn(II) at the concentrations of III^{Glc} used in these experiments. With III^{Glc} in the absence of Zn(II), the cleavage kinetics and pattern are the same as in the absence of III^{Glc}, showing that the decreased rate in the presence of Zn(II) and III^{Glc} is not due to titration of the chymotrypsin. Finally, neither the kinetics nor pattern of cleavage of either glycerol kinase is affected by Zn(II) alone.

Nucleotide sequence accession member. The sequence of the *glpK22* gene is found in GenBank under accession no. U41468.

RESULTS

Sequence of the *glpK22* gene. Glycerol-positive colonies were obtained with DNA fragments from both the *Hin*dIII and *Eco*RI restriction digests of the chromosomal DNA from strain 43. Agarose gel electrophoresis of restriction digests of the plasmids purified from the respective glycerol-positive cells showed insert sizes of about 3 kb for the *Hin*dIII fragment and about 10 kb for the *Eco*RI fragment. The size of each of these fragments agrees well with that predicted by the DNA sequence of this region (17). The *Hin*dIII fragment was sequenced as described in Materials and Methods. The sequence of the *glpK22* allele showed a single nucleotide change, Gua-913-Ade, from the sequence of the *glpK* gene (15). This nucleotide change results in mutation of codon 305 from GGT to AGT, which translates to the amino acid change G-304-S in the protein. (The amino acid residues are numbered as they appear in the wild-type purified protein, from which the N-terminal Met residue is removed after translation; thus, amino acid residue n in the numbering of the protein corresponds to codon $n+1$ in the *glpK* gene [15].)

Properties of the G-304-S glycerol kinase. The catalytic, regulatory, and physical properties of the purified G-304-S glycerol kinase were characterized as described in Materials and Methods. Table 1 shows the kinetic constants obtained from the fits of the initial-velocity data as well as those obtained previously for the wild-type enzyme (16) for comparison. The G-304-S substitution significantly alters substrate binding. The apparent V_{max} is only slightly altered by the G-304-S substitution, but the substrate affinity is decreased substantially. The substrate dissociation constant, K_{iS}, is increased 12-fold for ATP and 30-fold for glycerol. The decreased substrate affinity is reflected in the Michaelis constants, which are increased 100-fold for ATP and 25-fold for glycerol. The consequence of the decreased substrate affinity is decreased catalytic efficiency that is shown by the smaller values of k_{cat}/K_m, 100-fold for ATP and 30-fold for glycerol, obtained for the G-304-S enzyme. The dissociation constants for

TABLE 1. Steady-state kinetics parameters for G-304-S glycerol kinase

Enzyme	V_{max} (U/mg)	K_{ATP} (μM)	$K_{glycerol}$ (μM)	K_{iATP} (μM)	k_{cat}/K_{ATP} (M^{-1} s^{-1})	$k_{cat}/K_{glycerol}$ (M^{-1} s^{-1})
G-304-S	12.7 ± 1.3	830 ± 200	126 ± 40	1024 ± 400	1.4×10^4	9.4×10^4
Wild type[a]	15.7 ± 0.3	8.4 ± 0.7	4.9 ± 1.2	86 ± 25	1.7×10^6	3×10^6

[a] Parameters for the wild-type glycerol kinase are taken from reference 16.

the substrates are equal to the respective Michaelis constants. For the wild-type enzyme, the substrate dissociation constant is 10 times larger than the Michaelis constant, which indicates that some step after formation of the ternary complex, e.g., product release, is at least partially rate limiting. The agreement between the substrate Michaelis and dissociation constants obtained for the G-304-S enzyme indicates that this is no longer the case. Thus, the amino acid change affects both substrate binding and the rates of steps that occur after the formation of the ternary complex, suggesting that the rate-limiting step in the kinetic mechanism may be changed and the agreement of the V_{max} values may be fortuitous.

Regulatory behavior of the G-304-S glycerol kinase with respect to FBP is shown in Fig. 1. The concentration of FBP required for inhibition of the G-304-S enzyme is much higher than that for the wild-type enzyme, which is consistent with the screen that was used to isolate the mutant and with its initial characterization (25, 26). The apparent affinity for FBP is decreased at least 20-fold in the mutant enzyme, so that 20 mM FBP is required to inhibit the mutant to the same extent that <2 mM FBP inhibits the wild-type enzyme.

We have shown previously that FBP inhibition is related to the apparent molecular weight of the enzyme (10). The apparent molecular weights of the wild-type and G-304-S glycerol kinases were determined by gel permeation chromatography. The subunit molecular weight of glycerol kinase is 56,000 (15). In the absence of FBP, the apparent molecular weight of the G-304-S glycerol kinase (89,000) is less than that of the wild-type enzyme (158,000). The decreased apparent molecular weight of the mutant glycerol kinase may also reflect increased interactions with the chromatography support medium, leading to an increased elution volume. However, the same results were obtained with two different support media, Sephadex G-200 and Bio-Gel A-0.5m. The addition of 2 mM FBP increases the apparent molecular weight of the wild-type glycerol kinase (178,000) but has no effect on the G-304-S enzyme. This is consistent with the lack of inhibition of the G-304-S enzyme by 2 mM FBP (Fig. 1). However, the results in Fig. 1 show that inhibition is obtained at higher concentrations of FBP. Similarly, the apparent molecular weight of the G-304-S glycerol kinase (108,000) is increased by 10 mM FBP.

The regulatory behavior of the G-304-S glycerol kinase with respect to III^{Glc} has not been described previously. The effect of III^{Glc} on the catalytic activity of wild-type and G-304-S glycerol kinases is shown in Fig. 2. For the wild-type enzyme, we showed that Zn(II) increases the apparent affinity for III^{Glc} by binding to the intermolecular Zn(II) site that is formed on association of the two proteins (5). Assays were therefore performed without and with the addition of Zn(II). Binding of III^{Glc} has a different effect on each enzyme. The activity of the wild-type enzyme is inhibited, while that of the G-304-S enzyme is increased. The lines that are drawn through the datum points show the fit for hyperbolic binding of III^{Glc} to a single site on each subunit of glycerol kinase, which is consistent with the structure of the complex (8). The parameters obtained from the fit show that binding of III^{Glc} gives 95% inhibition of the wild-type enzyme but gives twofold, i.e., 100%, activation of the G-304-S enzyme. For the wild-type enzyme, the apparent dissociation constants for III^{Glc} binding are 15 μM in the absence of Zn(II) and 0.6 μM with 0.1 mM Zn(II), values which agree with our previous report (5). For the G-304-S enzyme, the corresponding values are 110 and 9 μM. Thus, the G-304-S substitution decreases the apparent affinity for III^{Glc} binding by 10- to 20-fold. Zn(II) increases the affinity for III^{Glc} for both enzymes about 15-fold. However, Zn(II) does not alter the extent of the inhibition or activation.

In the related protein DnaK, conformational change due to ATP binding affects cleavage by proteases (9). The effect of ATP binding on the conformations of the G-304-S and wild-type glycerol kinases was examined by using proteolysis by chymotrypsin. The results of those experiments are shown in Fig. 3. Only two protein bands other than the 56-kDa band corresponding to the subunit are observed in the digests, one of about 41 kDa and one of about 17 kDa. (The smaller band is not shown in the figure.) The wild-type enzyme is cleaved more slowly under these conditions. Both ATP and III^{Glc} appear to prevent its cleavage as indicated by the absence of the 41-kDa band. However, quantitation of the rate of disappearance of the 56-kDa band shows that while ATP prevents its disappearance, III^{Glc} does not affect its rate of disappearance. No other protein bands are seen in the digest with III^{Glc}. Thus, although III^{Glc} does not appear to alter the rate of cleavage, it may alter the pattern of cleavage of the wild-type enzyme. The G-304-S enzyme is cleaved more rapidly, and its rate of cleavage is decreased greatly by either ATP or III^{Glc}. These results show that the conformations of the two enzymes with bound glycerol are different. However, both enzymes undergo a conformational change upon binding of ATP or III^{Glc}.

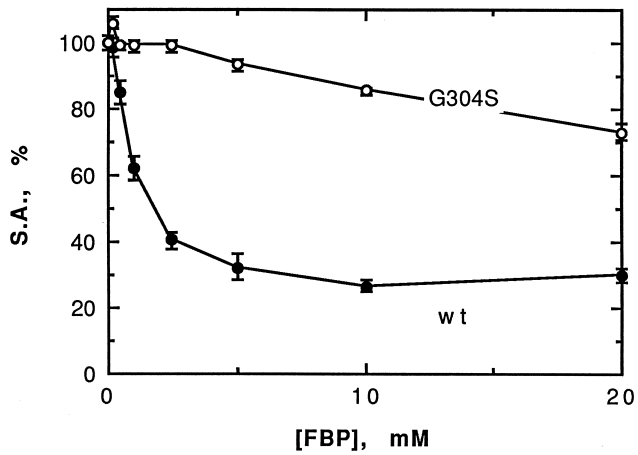

FIG. 1. FBP inhibition of wild-type (wt) and G-304-S glycerol kinases. The specific activities were determined at pH 7, 25°C, 0.44 μg of glycerol kinase per ml, 0.1 mM glycerol, 2 mM ATP, and the indicated concentrations of FBP as described in Materials and Methods. The results are expressed as percentages relative to the specific activities (S.A.) at 0 mM FBP of the wild-type glycerol kinase (26.9 U/mg) and the G-304-S mutant (10.4 U/mg). The values are the means for at least three determinations, and the error bars show the standard deviation.

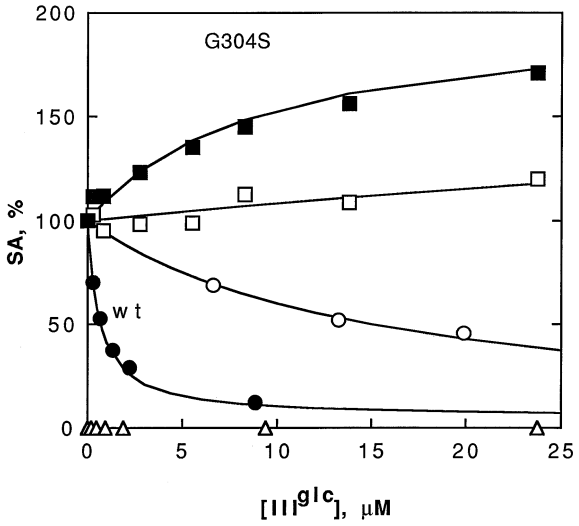

FIG. 2. III^{Glc} inhibition of wild-type (wt) and G-304-S glycerol kinases. The specific activities were determined as described in the legend to Fig. 1, except that the glycerol concentration was 2 mM. The results are expressed as percentages relative to the specific activities (SA) at 0 III^{Glc} of wild type (17.2 U/mg) and G-304-S (18 U/mg). Circles, wild type; squares, G-304-S; open triangles, no glycerol kinase in the assay; closed symbols indicate that 0.1 mM Zn(II) was added to the assay. The lines that are drawn through the datum points were calculated for hyperbolic binding of III^{Glc} to the enzyme with the following dissociation constants: wild type, 15 μM; wild type + Zn(II), 0.6 μM; G-304-S, 110 μM; and G-304-S + Zn(II), 9 μM. For the calculated lines, 95% inhibition was used for the wild-type enzyme and twofold activation was used for G-304-S.

DISCUSSION

The results presented here identify a single point mutation in the structural gene for *E. coli* glycerol kinase which results in the loss of glucose repression of glycerol utilization in vivo and allows the simultaneous utilization of both carbon sources (26). The mutation is a single nucleotide change that results in the substitution of serine for glycine at residue 304 in glycerol kinase. This change affects the regulation by both FBP and III^{Glc}; neither allosteric effector inhibits the mutant enzyme under conditions that result in strong inhibition of the wild-type enzyme. The catalytic and regulatory properties of the G-304-S glycerol kinase are consistent with, and sufficient to explain, the phenotype of strain 43. However, the activation of the G-304-S enzyme by III^{Glc} is completely unexpected and could not be predicted from the phenotype.

The G-304-S substitution globally affects the functional properties and conformation of glycerol kinase. While it has little effect on V_{max}, it decreases the affinity for binding both substrates (12-fold for ATP and 30-fold for glycerol), decreases the catalytic efficiency for both substrates (100-fold for ATP and 30-fold for glycerol), appears to alter the rate-limiting step in the catalytic mechanism, reduces the apparent affinity for FBP at least 20-fold, and converts the effect of III^{Glc} from inhibition to activation while decreasing the apparent affinity for III^{Glc} 10- to 20-fold. The substitution alters the conformation as determined by proteolysis, stability in the absence of glycerol, and the apparent molecular weight. On the other hand, the G-304-S glycerol kinase appears to be expressed normally, and its specific activity differs little from that of the wild-type enzyme. Thus, the G-304-S glycerol kinase is able to attain a conformation that is not too different from that of the wild-type enzyme. This conclusion is supported by the proteolysis results. While the rate of cleavage is greater for the G-304-S enzyme, the pattern of peptides seen in SDS-PAGE is the same as that for the wild type. In addition, the rate of cleavage is decreased by binding of ATP for both enzymes, suggesting that they undergo a similar conformational change. The substitution of Ser for Gly produces the smallest increase in the volume of the side chain possible, about 0.03 nm^3 (the same volume increase would be obtained with Ala) (2). The global effect that this change has on the properties of the enzyme suggests that the substitution affects a region that is of central importance in the conformation and functional properties of the enzyme. The location of G-304 in the structure of the wild-type enzyme and comparison of the effects of the G-304-S substitution to the results obtained for related proteins indicate that this is indeed the case and suggest a plausible molecular basis for one aspect of glucose control of glycerol utilization.

The location of G-304 in the crystal structure of the wild-type glycerol kinase-III^{Glc} complex (5) is shown in Fig. 4. The site of the substitution is 1.3 nm from the active site and 2.5 nm from the III^{Glc} binding site. G-304 is located in the β sheet of domain II (domain nomenclature is as given in reference 8). The strand in which it is located directly interacts or connects with two other elements of secondary structure that are involved in binding of both substrates; the interactions between these protein elements and the substrates have been described previously (8). Movement of either or both of these neighboring elements of secondary structure that could result from the substitution is consistent with the effects on substrate binding.

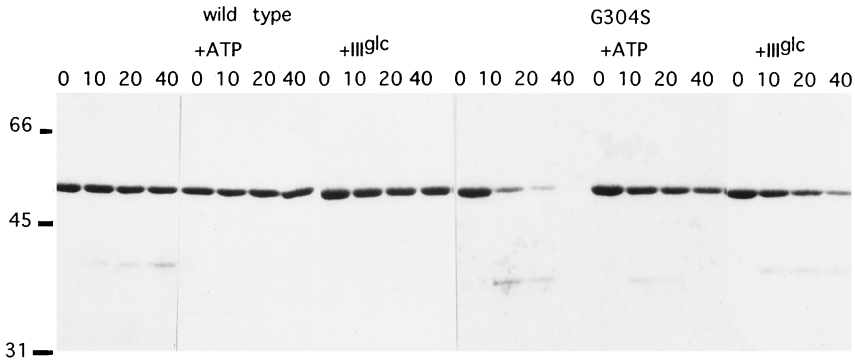

FIG. 3. Comparison of conformations of wild-type and G-304-S glycerol kinases by proteolysis. The glycerol kinases were incubated with α-chymotrypsin for the time (in minutes) indicated above each lane prior to quenching and SDS-PAGE as described in Materials and Methods. Glycerol was present at 1 mM in all digests, and ATP (2 mM) and III^{Glc} (5.5 μM for wild-type glycerol kinase and 16.5 μM for G-304-S) were added as indicated above the lanes; 0.1 mM Zn(II) was added to the digests with III^{Glc}. The positions of molecular weight markers (in thousands) are indicated on the left. The figure is a composite of four different gels.

FIG. 4. Location of G-304 in the structure of wild-type glycerol kinase. The figure shows the α-carbon backbone of the glycerol kinase subunit with the products ADP and glycerol 3-phosphate (cyan) bound to the active site. Domain I is shown in red, and domain II is shown in yellow. Residues G-304 (magenta) and F-270 (yellow) are shown in space-filling representation. The III^{Glc} binding site is a magenta helix at the extreme left side of the subunit in this view. The coordinates used to construct the figure were taken from the Zn(II) complex of glycerol kinase and III^{Glc} (1glc in the Brookhaven Protein Data Bank), and the figure was constructed by using InsightII from Biosym Technologies, Inc.

Glycerol kinase is a member of a family of proteins that shows a high degree of structural similarity even though they share little sequence similarity and includes actin, hexokinase, and the Hsp70 heat shock proteins (7). Members of this family include the ATPase domain of Hsc70 and *E. coli* DnaK, which show 51% sequence identity to one another (1). As shown in Fig. 4 for glycerol kinase, the structures of these proteins show two domains separated by a deep cleft which contains the nucleotide binding site. Actin and the heat shock proteins share the functional property of ATP-dependent conformational changes. A common mechanism for the nucleotide-dependent conformational change has been postulated in which the active site cleft is closed by rotation of the two domains relative to one another (7). The proteolysis results with glycerol kinase show that the conformations of the wild-type and G-304-S glycerol kinases are changed by binding of ATP.

This domain closure motion has been postulated as an explanation for the properties of *E. coli* DnaK mutants (9). The mutants A-174-T and D-201-N show increased Michaelis constants for ATP and changes in regulatory properties. Figure 5 shows the relationship between the sites of these substitutions in the ATPase domain of hsc70 and the site of G-304-S in glycerol kinase. Structure modeling of the A-174-T mutant of DnaK (A-179 in hsc70) showed significant steric overlaps of the substitution in the α helix in domain I (S helix) with side chains of residues 335 and 337 located in the domain II β sheet. It was postulated that the effects of the substitution on the catalytic and regulatory properties are due to interference by the steric overlaps with the domain closure motion (9). In the G-304-S glycerol kinase, the substitution could result in overlaps between the domain II β-sheet residue F-270 and residue Q-246 from the domain I α helix similar to those postulated for the DnaK A-174-T mutant. The location of residue D-201 in DnaK (D-206 in hsc70) corresponds closely to that of F-270 in glycerol kinase. The D-201-N substitution in DnaK changes the effect of peptide binding on the ATPase activity from activation to inhibition, which is analogous to the change of III^{Glc} inhibition to activation in the G-304-S glycerol kinase. Thus, the region in glycerol kinase that is affected by the G-304-S substitution is substantially the same region affected by the substitutions A-174-T and D-201-N in DnaK, and the effects of the G-304-S substitution on the catalytic and regulatory properties of glycerol kinase are similar to the effects of the corresponding substitutions in DnaK. The effects of the G-304-S substitution are consistent with a role for the domain closure motion in the catalytic and regulatory properties of glycerol kinase, and the domain closure mechanism provides an explanation for the global effects of the substitution.

The G-304-S substitution affects regulation by the allosteric effectors III^{Glc} and FBP differently. There are several possible explanations for this observation. (i) The activation by III^{Glc} may reflect its binding to a different site on the G-304-S enzyme. The existence of a site for interactions with other macromolecules which increase the activity of glycerol kinase is inferred from studies showing that the glycerol facilitator protein (GlpF) is required for rapid metabolism of glycerol even though it is not required for rapid uptake into the cell (23). The different effects of III^{Glc} on the rates of proteolysis of the wild-type and G-304-S enzymes may also reflect binding at different sites. However, the enhancement of binding obtained with Zn(II) is consistent with binding to the same site as that on the wild-type enzyme. (ii) The effect that allosteric ligand

FIG. 5. Location of G-304 relative to the sites implicated in the domain movement of DnaK. The figure shows a close-up view of the active site region of glycerol kinase. Its orientation relative to that shown in Fig. 4 can be seen by comparing the positions of the bound ADP (cyan) in each figure. The figure was constructed by superimposing the domain I α-helices (S helix) of glycerol kinase and the ATPase domain of hsc70 (3hsc in the Brookhaven Protein Data Bank). The relevant side chains from hsc70 are labeled and are shown in green. Domain I of glycerol kinase is shown in red, and domain II is shown in yellow. The relevant side chains from glycerol kinase are labeled.

binding has on the activity may depend on the oligomeric state of the enzyme. The apparent molecular weight of the G-304-S enzyme suggests that a substantial fraction of it could be present in the activity assay as the monomer. In this case, III^{Glc} could activate, rather than inhibit, the monomer. Alternatively, formation of tetramers could affect the domain movement such that the effect of FBP binding is the same as for the wild-type enzyme. The decreased apparent affinity for FBP associated with the decreased extent of tetramer formation is consistent with results that we obtained for the A-65-T and D-72-N glycerol kinases, for which the substitutions occur in a tetramer interface (10). At sufficiently high concentrations of FBP, inhibition is observed for the G-304-S enzyme and the apparent molecular weight is increased. This result suggests that the mutant enzyme is able to form tetramers, and the effect of FBP binding to the tetramers is the same as that for the wild-type enzyme. (iii) The detailed mechanisms of inhibition by FBP and III^{Glc} may be different. If this is the case, it would suggest that multiple factors are involved in glycerol kinase catalytic activity, and the activity can be inhibited by altering any of the factors. Possible roles for multiple factors are indicated by results of active site mutagenesis studies of the ATPase domain of hsc70 (6, 24). We have obtained similar results in mutagenesis studies of the conserved-active-site Asp residues of glycerol kinase, D-10 and D-245 (14a). Furthermore, FBP inhibition is dependent on tetramer formation, while III^{Glc} inhibition is not (10). The existence of other mutant glycerol kinases which have lost one but retain the other allosteric regulation (13) may also indicate different regulatory mechanisms.

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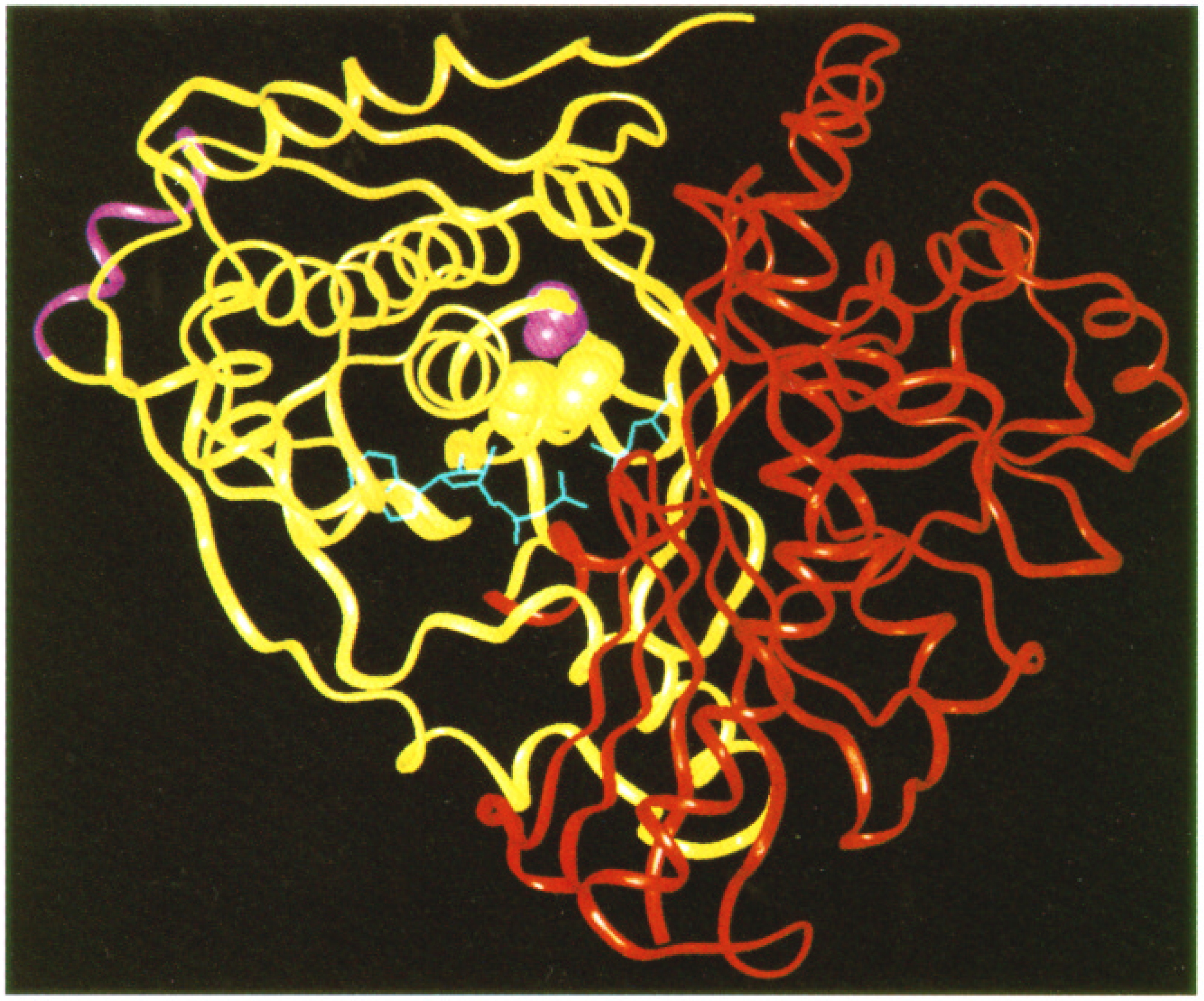
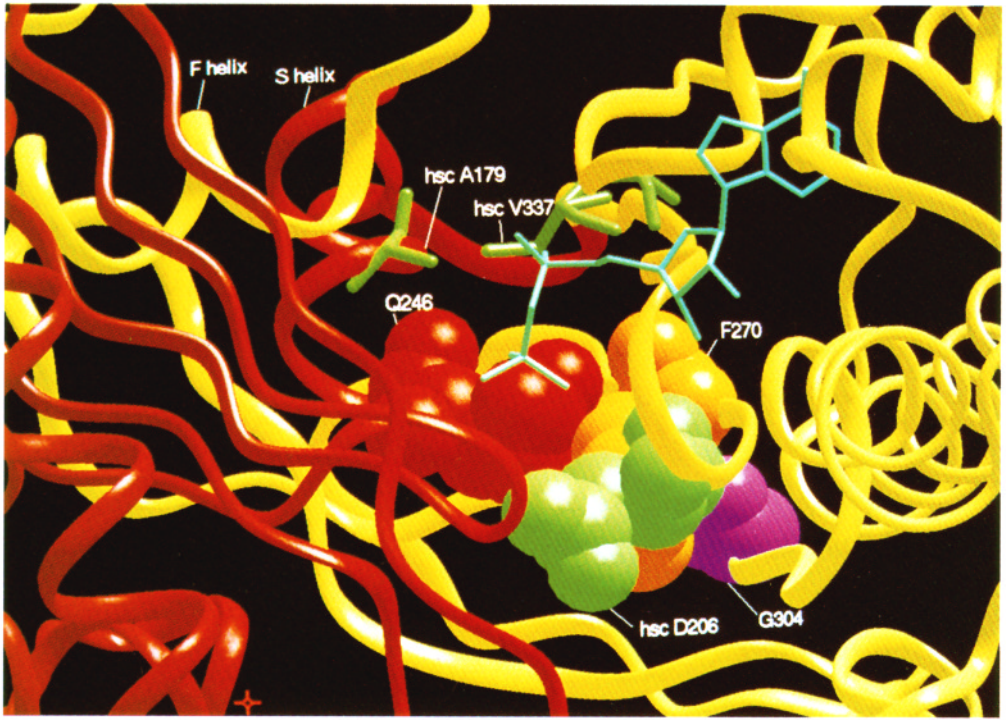
REFERENCES

1. **Buchberger, A., A. Valencia, R. McMacken, C. Sander, and B. Bukau.** 1994. The chaperone function of DnaK requires the coupling of ATPase activity with substrate binding through residue E171. EMBO J. **13:**1687–1695.
2. **Creighton, T. E.** 1983. Proteins: structure and molecular properties. W. H. Freeman and Company, New York.
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. **Faber, H. R., D. W. Pettigrew, and S. J. Remington.** 1989. Crystallization and preliminary X-ray studies of *Escherichia coli* glycerol kinase. J. Mol. Biol. **207:**637–639.
5. **Feese, M., D. W. Pettigrew, N. D. Meadow, S. Roseman, and S. J. Remington.** 1994. Cation-promoted association of a regulatory and target protein is controlled by protein phosphorylation. Proc. Natl. Acad. Sci. USA **91:**3544–3548.
6. **Flaherty, K. M., S. M. Wilbanks, C. DeLuca-Flaherty, and D. B. McKay.** 1994. Structural basis of the 70-kilodalton heat shock cognate protein ATP

hydrolytic activity. II. Structure of the active site with ADP or ATP bound to wild type and mutant ATPase fragment. J. Biol. Chem. **269:**12899–12907.
7. **Holmes, K. C., C. Sander, and A. Valencia.** 1993. A new ATP-binding fold in actin, hexokinase, and hsc70. Trends Cell Biol. **3:**53–59.
8. **Hurley, J. H., H. R. Faber, D. Worthylake, N. D. Meadow, S. Roseman, D. W. Pettigrew, and S. J. Remington.** 1993. Structure of the regulatory complex of *Escherichia coli* III^{glc} with glycerol kinase. Science **259:**673–677.
9. **Kamath-Loeb, A. S., C. Z. Lu, W.-C. Suh, M. A. Lonetto, and C. A. Gross.** 1995. Analysis of three DnaK mutant proteins suggests that progression through the ATPase cycle requires conformational changes. J. Biol. Chem. **270:**30051–30059.
10. **Liu, W. Z., R. Faber, M. Feese, S. J. Remington, and D. W. Pettigrew.** 1994. *Escherichia coli* glycerol kinase: role of a tetramer interface in regulation by fructose 1,6-bisphosphate and phosphotransferase system regulatory protein IIIglc. Biochemistry **33:**10120–10126.
11. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
12. **Meadow, N. D., D. K. Fox, and S. Roseman.** 1990. The bacterial phosphoenolpyruvate:glycose phosphotransferase system. Annu. Rev. Biochem. **59:**497–542.
13. **Novotny, M. J., W. L. Frederickson, E. B. Waygood, and M. H. Saier, Jr.** 1985. Allosteric regulation of glycerol kinase by enzyme III^{glc} of the phosphotransferase system in *Escherichia coli* and *Salmonella typhimurium*. J. Bacteriol. **162:**810–816.
14. **Pelton, J. G., D. A. Torchia, N. D. Meadow, C.-Y. Wong, and S. Roseman.** 1991. ^{1}H, ^{15}N, and ^{13}C NMR signal assignments of III^{glc}, a signal-transducing protein of *Escherichia coli*, using three-dimensional triple-resonance techniques. Biochemistry **30:**10043–10057.
14a. **Pettigrew, D. W.** Unpublished observations.
15. **Pettigrew, D. W., D.-P. Ma, C. A. Conrad, and J. R. Johnson.** 1988. *Escherichia coli* glycerol kinase: cloning and sequencing of the *glpK* gene and the primary structure of the enzyme. J. Biol. Chem. **263:**135–139.
16. **Pettigrew, D. W., G.-J. Yu, and Y. Liu.** 1990. Nucleotide regulation of *Escherichia coli* glycerol kinase: initial-velocity and substrate binding studies. Biochemistry **29:**8620–8627.
17. **Plunkett, G., III, V. Burland, D. L. Daniels, and F. R. Blattner.** 1993. Analysis of the Escherichia coli genome. III. DNA sequence of the region from 87.2 to 89.2 minutes. Nucleic Acids Res. **21:**3391–3398.
18. **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.
19. **Postma, P. W., J. W. Lengeler, and G. R. Jacobson.** 1993. Phosphoenolpyruvate:carbohydrate phosphotransferase systems of bacteria. Microbiol. Rev. **57:**543–594.
20. **Saier, M. H., Jr.** 1989. Protein phosphorylation and allosteric control of inducer exclusion and catabolite repression by the bacterial phosphoenolpyruvate:sugar phosphotransferase system. Microbiol. Rev. **53:**109–120.
21. **Silhavy, T. J., M. L. Berman, and L. W. Enquist.** 1984. Experiments with gene fusions, p. 137–138. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
22. **Stewart, G. A. B., S. Lubinsky-Mink, C. G. Jackson, A. Cassel, and J. Kuhn.** 1986. pHG165: a pBR322 copy number derivative of pUC8 for cloning and expression. Plasmid **15:**172–181.
23. **Voegele, R. T., G. D. Sweet, and W. Boos.** 1993. Glycerol kinase of *Escherichia coli* is activated by interaction with the glycerol facilitator. J. Bacteriol. **175:**1087–1094.
24. **Wilbanks, S. M., C. DeLuca-Flaherty, and D. B. McKay.** 1994. Structural basis of the 70-kilodalton heat shock cognate protein ATP hydrolytic activity. J. Biol. Chem. **269:**12893–12898.
25. **Zwaig, N., W. S. Kistler, and E. C. C. Lin.** 1970. Glycerol kinase, the pacemaker for the dissimilation of glycerol in *Escherichia coli*. J. Bacteriol. **102:**753–759.
26. **Zwaig, N., and E. C. C. Lin.** 1966. Feedback inhibition of glycerol kinase, a catabolic enzyme in *Escherichia coli*. Science **153:**755–757.




F helix
S helix
hsc A179
hsc V337
Q246
F270
hsc D206
G304