IIA^{Glc} Allosteric Control of *Escherichia coli* Glycerol Kinase: Binding Site Cooperative Transitions and Cation-Promoted Association by Zinc(II)[†]

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ABSTRACT: The catalytic activity of glycerol kinase (EC 2.7.1.30, ATP:glycerol 3-phosphotransferase) from *Escherichia coli* is inhibited allosterically by IIA^{Glc} (previously known as III^{Glc}), the glucose-specific phosphocarrier protein of the phosphoenolpyruvate:glycose phosphotransferase system. A sequentially contiguous portion of glycerol kinase undergoes an induced fit conformational change involving coil, α-helix, and 3_{10}-helix upon IIA^{Glc} binding. A second induced fit occurs upon binding of Zn(II) to a novel intermolecular site, which increases complex stability by cation-promoted association. Eight of the ten sequentially contiguous amino acids are substituted with alanine to evaluate the roles of these positions in complex formation. Effects of the substitutions reveal both favorable and antagonistic contributions of the normal amino acids to complex formation, and Zn(II) reverses these contributions for two of the amino acids. The consequences of some of the substitutions for IIA^{Glc} inhibition are consistent with changes in the intermolecular interactions seen in the crystal structures. However, for the amino acids that are located in the region that is α-helical in the absence of IIA^{Glc}, the effects of the substitutions are not consistent with changes in intermolecular interactions but with increased stability of the α-helical region due to the higher α-helix propensity of alanine. The reduced affinity for IIA^{Glc} binding seen for these variants is consistent with predictions of Freire and co-workers [Luque, I., and Freire, E. (2000) *Proteins: Struct., Funct., Genet. 4*, 63−71]. These variants show also increased cation-promoted association by Zn(II) so that the energetic contribution of Zn(II) to complex formation is doubled. The similarity of effects of the alanine substitutions of the amino acids in the α-helical region for IIA^{Glc} binding affinity and cation-promoted association by Zn(II) indicates that they function as a cooperative unit.

Glycerol kinase from *Escherichia coli* (EC 2.7.1.30, ATP: glycerol 3-phosphotransferase) catalyzes the MgATP-dependent phosphorylation of glycerol to yield *sn*-glycerol 3-phosphate. In addition to its roles in carbon and phospholipid metabolism, glycerol 3-phosphate is the inducer for expression of the genes of the *glp* regulon (*1*). Regulation of expression of this regulon, of which the *glpK* element encodes glycerol kinase, is necessary for efficient metabolism of glycerol. As part of a signal transduction mechanism to modulate inducer levels, the catalytic activity of glycerol kinase from *E. coli* is regulated posttranslationally by at least two different allosteric effectors: the glycolytic intermediate fructose 1,6-bisphosphate (FBP) and the glucose-specific phosphocarrier protein of the phosphoenolpyruvate:glycose phosphotransferase system, IIA^{Glc} (previously known as III^{Glc}) (*2*). These allosteric effectors mediate glucose control of the expression of the elements of the *glp* regulon. Here, we report investigation of allosteric regulation by IIA^{Glc}.

The crystal structures of *E. coli* glycerol kinase without (*3*) and with (*4*) bound IIA^{Glc} have been determined to 2.62 and 2.6 Å resolution, respectively, and reveal that it is a member of the sugar kinase/actin/heat shock 70 superfamily of enzymes (*5*). Glycerol kinase is a tetramer in the crystal structures, and there is a single binding site for IIA^{Glc} per glycerol kinase monomer, located ∼30 Å from the active site. A small number of amino acids on glycerol kinase were identified crystallographically to interact with IIA^{Glc}: R402 and the sequentially contiguous amino acids P472-G473-I474-E475-T476-T477-E478-R479-N480-Y481. Comparison of the structure of IIA^{Glc} alone (*6*) with its structure in the complex reveals that its conformation is not changed upon binding to glycerol kinase. However, a conformational change is induced in glycerol kinase at positions 472−479. The nature of the induced fit conformational change has been described (*3*) and is shown in Figure 1. In the absence of IIA^{Glc}, the backbone conformation of this region consists of a coil from positions 472 to 475 and an α-helix from positions 476 to 481. This α-helix extends to position 499, and interactions between these α-helices in adjacent subunits constitute a large portion of the dimer interface. Upon binding of IIA^{Glc}, the first amino-terminal turn of the α-helix is unwound to a 3_{10}-helix, and the conformation at positions 472−475 changes from coil to 3_{10}-helix. This conformational change is restricted to positions 472−479; no other amino acid positions in glycerol kinase show significant movement.

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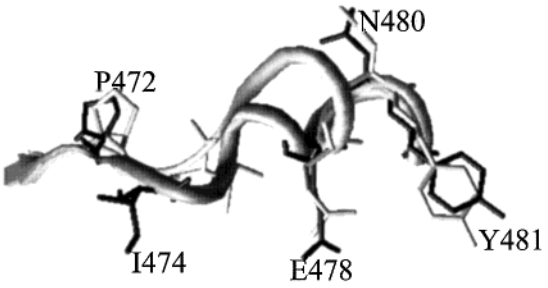

Position	I474	E475	T476	T477	E478	R479	N480	Y481
- IIA^{Glc}	coil	coil	α	α	α	α	α	α
+IIA^{Glc}	3_{10}	3_{10}	3_{10}	3_{10}	3_{10}	α	α	α

FIGURE 1: Conformational change of the sequentially contiguous amino acids upon binding of IIA^{Glc} to *E. coli* glycerol kinase. The crystal structures of normal glycerol kinase without (PDB ID: 1GLF) and with (PDB ID: 1GLB) bound IIA^{Glc} were superimposed by using the Swiss-PdbViewer v3.1 (Swiss Institute of Bioinformatics, Geneva). The overall backbone rmsd for the entire superimposed structures is 0.49 Å. The figure shows the peptide backbone structure from positions 471 to 481 and selected amino acid side chains in each of the structures. The backbone conformation at each position in each of the structures is shown in the lower portion of the figure. Legend: light gray, structure without IIA^{Glc}; dark gray/black, structure with bound IIA^{Glc}. The figure was constructed with the Swiss-PdbViewer.

The crystal structures of glycerol kinase−IIA^{Glc} complexes also revealed the presence of a novel intermolecular Zn(II) site that is formed upon association of the two proteins (*7*). The metal ligands consist in three amino acid side chains, H75 and H90 from IIA^{Glc} and E478 from glycerol kinase, and solvent at the fourth coordination position. Binding of Zn(II) at this site stabilizes the complex by cation-promoted association. Comparison of the structures of the complexes without and with Zn(II) reveals that the only amino acid in the complex that shows a conformational change upon binding of Zn(II) is E478 from glycerol kinase, providing a second induced fit.

To evaluate the roles of the amino acid side chains in complex formation and the induced fit conformational changes, we substituted alanine individually for each of the sequentially contiguous amino acids at positions 474−481. The amino acids at positions 472 and 473 were not substituted by alanine in these studies. For position 472, this decision was based on the likely role of the proline in the sharp turn made by the peptide backbone at this point (Figure 1) and the conservation of this residue in members of the bacterial sugar kinase family. These considerations suggest that P472 may have a key role in the stability and folding of glycerol kinase. For position 473, the glycine was not substituted with alanine because of concerns about the possible deleterious effect of the larger amino acid with respect to the induced fit conformational change. Effects of the alanine substitutions reveal that the amino acids at positions 476−481 behave as a cooperative unit with respect to IIA^{Glc} inhibition and cation-promoted association by Zn(II).

MATERIALS AND METHODS

Materials. Chemicals and enzymes were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated. IIA^{Glc} was purified as described (*8*).

Construction of Site-Directed Variants. Mutations in the *glpK* gene were constructed by using the Kunkel method for site-directed mutagenesis (*9*) as described for earlier variants (*10*) with primers synthesized by the Gene Technologies Laboratory of Texas A&M University. The DNA sequence of both strands of each of the altered genes was determined in all cases by using the ABI prism dye terminator cycle sequencing core kit with Big Dye premix from Perkin-Elmer, and the thermocycling conditions were as recommended by ABI. Sequence products were purified by using spin columns of P-30 polyacrylamide gel from Bio-Rad. The purified product was loaded onto an ABI 373 or 377 DNA sequencing system in the Gene Technologies Laboratory. Sequence analysis was performed by using Sequencher software. The sequences showed that the variant genes contain no mutations other than those designed. Variant enzymes were expressed and purified as described (*10*). SDS−PAGE was used to assess the homogeneity, which was >95% in all cases. At least two independent purifications of each variant enzyme were performed, and no significant differences were seen in the properties of the preparations. The purified enzymes were stored as crystalline suspensions in ammonium sulfate and desalted before use by either dialysis or Sephadex G-25 chromatography.

Far-UV circular dichroism spectra at 25 °C were measured over the range of 200−260 nm by using a Jasco J-600 spectrophotometer. The concentration of glycerol kinase was 0.4 mg/mL in 10 mM triethanolamine hydrochloride, pH 7.0, and 2 mM glycerol in a cuvette with a path length of 0.2 mm.

Protein Determinations and Enzyme Assays. The concentration of glycerol kinase was determined from the A_{280} using an extinction coefficient of 1.73 $(mg/mL)^{-1}$ cm^{-1} (*10*). The concentration of IIA^{Glc} was determined with the Bio-Rad assay with bovine serum albumin as standard, and its molar concentration was calculated on the basis of the molecular mass of 18.1 kDa (*11*).

Glycerol kinase enzyme activity was measured by using the continuous ADP-coupled spectrophotometric assay at pH 7.0 and 25 °C, with 0.05 M triethanolamine hydrochloride (adjusted to pH 7.0 with NaOH), 5 mM $MgCl_2$, 20 mM KCl, and 0.1 mM β-mercaptoethanol (*10*), with other additions as indicated. Initial velocities were measured by using a Beckman DU-640 spectrophotometer with thermoelectric temperature control and a kinetics software package. One unit of glycerol kinase is the amount of enzyme that catalyzes the formation of 1 μmol of ADP/min in this assay.

To evaluate regulatory kinetic behavior with respect to ATP, the concentration of ATP was varied from 0.01 to 2.5 mM with 2 mM glycerol, as described for the normal glycerol kinase (*12*). For all of the variants, double-reciprocal plots of the ATP concentration dependence of the initial velocity display the biphasic, concave downward pattern consisting of two linear regions, one for concentrations below 0.1 mM and one for concentrations above 0.1 mM, that is seen for the normal enzyme (not shown). The ATP dependence of the initial velocity can be analyzed in two separate concentration regimes to yield kinetic parameters (*12*). However, because of the uncertainties in kinetic parameters obtained from the higher concentration ranges of ATP (*12*), evaluation of effects of amino acid substitutions on the catalytic properties of glycerol kinase is based on the lower ATP

concentration range as reported previously for other variant glycerol kinases (*10*, *13*−*15*). For these initial velocity studies, the ATP concentrations were (mM) 0.01, 0.015, 0.025, 0.037, and 0.08 with glycerol concentrations of (mM) 0.01, 0.02, 0.04, and 0.1. Glycerol kinase was added to a final concentration of 0.16−0.2 μg/mL to initiate the reaction. Initial velocity kinetic parameters were estimated from fits of the initial velocity, expressed as units per milligram, to the following equation for a sequential bisubstrate enzyme kinetic mechanism:[1]

$$v = \frac{V_{max}[ATP][gol]}{[ATP][gol] + K_{ATP}[gol] + K_{gol}[ATP] + K_{iATP}K_{gol}} \quad (1)$$

where K_{ATP} is the Michaelis constant for ATP, K_{gol} is the Michaelis constant for glycerol, and K_{iATP} is the dissociation constant for ATP binding. Values of k_{cat} were calculated from V_{max} by using the subunit molecular mass for glycerol kinase of 56 kDa (*16*) ([k_{cat} (s^{-1}) = V_{max} (units/mg)][0.93 (min·mg)/(μmol·s)]).

For inhibition studies, the assay concentrations of ATP and glycerol were 2.5 and 2 mM, respectively, and glycerol kinase was added to the assays to a final concentration of 0.5 μg/mL to initiate the reaction. FBP was added to the assay at concentrations of (mM) 0.01, 0.025, 0.1, 0.2, 0.4, 0.8, 1, 5, and 10, while IIA^{Glc} was added to the assay at concentrations ranging from 0 to 40 μM (typically nine different concentrations per inhibition curve). For assays of IIA^{Glc} inhibition with Zn(II), $ZnCl_2$ (Aldrich) was added to a final concentration of 0.1 mM. The apparent dissociation constant for binding of Zn(II) to the normal glycerol kinase−IIA^{Glc} complex is about 1 μM (*10*), so trace amounts of Zn(II) do not have significant effects and Zn(II) must be added to obtain the cation-promoted association. The free concentration of Zn(II) in these experiments is about 10 μM, which practically saturates the Zn(II) binding to the complex for the normal glycerol kinase (*10*). Cation-promoted association of IIA^{Glc} inhibition of the normal glycerol kinase is specific for Zn(II); addition of other divalent cations [Mn(II), Co(II), Cu(II), Cd(II)] does not increase the inhibition (*7*).

Allosteric inhibition by FBP or IIA^{Glc} was determined as the ratio of specific activity with inhibitor to the specific activity without inhibitor, expressed as a percentage. Parameters for FBP inhibition were obtained from fits of the dependence of the normalized specific activity (SA, %) on FBP concentration to the following equation for cooperative inhibition:

$$SA\ (\%) = 100 - [100(I_{max}[FBP]^{n_H})/((K_{0.5})^{n_H} + [FBP]^{n_H})] \quad (2)$$

where I_{max} is the maximum extent of inhibition, $K_{0.5}$ is the apparent dissociation constant for FBP binding, and n_H is the Hill coefficient. Parameters for IIA^{Glc} inhibition were obtained from fits of the dependence of the normalized specific activity on IIA^{Glc} concentration to the following equation for noncooperative inhibition:

$$SA\ (\%) = 100 - [100(I_{max}[IIA^{Glc}])/(K_i + [IIA^{Glc}])] \quad (3)$$

where I_{max} is the maximum extent of inhibition and K_i is the apparent dissociation constant for IIA^{Glc} binding. The inhibition of normal glycerol kinase by IIA^{Glc} was shown to display uncompetitive behavior with respect to both substrates, and the K_i obtained from inhibition at a single concentration of substrates is the same as the K_{ii} estimated from fits of steady-state inhibition data (*10*). The standard free energy change for formation of the glycerol kinase−IIA^{Glc} complex is given by $\Delta G° = RT \ln K_i$.

Nonlinear least-squares fitting of initial velocity data to eq 1 was performed by using the computer program NonLin for Macintosh obtained from R. J. Brenstein, Robelko Software, Carbondale, IL. The reported parameters are the average ± propagated maximum uncertainties from independent measurements of the kinetic properties of two different preparations of the enzymes; i.e., standard deviation = [(|maximum uncertainty for preparation 1|^2 + |maximum uncertainty for preparation 2|^2)/2]$^{0.5}$. The uncertainties for the parameters for each preparation were determined as the 65% confidence intervals. These intervals are generally asymmetric, and the larger of the values for each interval was used in propagating the uncertainties. Nonlinear least-squares fitting of inhibition data to eqs 2 and 3 was performed by using the computer program Kaleidagraph by Synergy Software. The uncertainties that are shown for the inhibition parameters are the propagated values of the standard errors of the parameters obtained from the fits of at least two independent experiments. For the variant glycerol kinases, uncertainties in the standard free energy changes for IIA^{Glc} binding were determined by using the estimated K_i ± the standard error as reported by the fitting program to calculate a confidence interval for $\Delta G°$. For the normal glycerol kinase, the uncertainties in the standard free energy change for IIA^{Glc} binding are given as the sample standard deviation for the indicated number of multiple independent determinations. Uncertainties in the values of $\Delta\Delta G°$ were obtained by propagating the uncertainties of each of the values of $\Delta G°$.

RESULTS

Variants of glycerol kinase with substitutions of the sequentially contiguous amino acids in the IIA^{Glc} binding site were constructed and purified as described under Materials and Methods. For alanine substitutions at positions 474−481, the variant enzymes show levels of protein expression and behavior during purification that are similar to those of the normal enzyme, suggesting that there are not large structure perturbations due to the substitutions. The effects of the substitutions on the structures of the variant enzymes were examined by using far-UV circular dichroism spectroscopy. The circular dichroism spectrum of glycerol kinase is not altered significantly by any of the substitutions (not shown), indicating also the absence of large structure changes. This is consistent with results of X-ray structure determinations for the IIA^{Glc} complexes of other glycerol kinase variants with substitutions in this region—I474D (*3*) and E478C and E478H (*10*).

The substitutions do affect the structure of glycerol kinase, as shown by changes in the catalytic parameters. For evaluation of the effects of the substitutions on the catalytic

[1] Abbreviations: gol, glycerol; n_H, Hill coefficient. Variant proteins are denoted by using the single-letter abbreviations for the amino acids; e.g., I474A means that the isoleucine residue at position 474 has been replaced with alanine.

Table 1: Functional Properties of Glycerol Kinases with Alanine Substitutions of the Sequentially Contiguous IIA^{Glc} Binding Amino Acids[a]

enzyme	wt	I474A	E475A	T476A	T477A	E478A	R479A	N480A	Y481A
V_{max}, units/mg	20.9 ± 1.6	6.5 ± 0.5	22.1 ± 1.4	22.1 ± 1.3	17.8 ± 1.4	14.2 ± 0.7	10.8 ± 0.5	32.6 ± 2.5	27.4 ± 2.2
k_{cat}/K_m, 10^{-6} M^{-1} s^{-1}	2.4 ± 0.05	1.1 ± 0.03	2.7 ± 0.08	1.2 ± 0.02	2.0 ± 0.12	1.8 ± 0.04	3.2 ± 0.36	2.2 ± 0.04	2.2 ± 0.03
$K_{0.5}$, mM	0.58 ± 0.03	0.29 ± 0.03	0.62 ± 0.04	0.56 ± 0.03	0.47 ± 0.03	0.43 ± 0.04	0.33 ± 0.02	0.62 ± 0.1	0.42 ± 0.06
$\Delta\Delta G^\circ_{-Zn(II)}$, kcal/mol	0 ± 0.1	0.3 ± 0.2	0.2 ± 0.2	1.0 ± 0.2	0.9 ± 0.3	−1.0 ± 0.2	1.8 ± 0.2	1.8 ± 0.2	1.7 ± 0.2
$\Delta\Delta G^\circ_{+Zn(II)}$, kcal/mol	0 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	0.6 ± 0.1	−0.9 ± 0.1	0.9 ± 0.13	0.5 ± 0.14	0.8 ± 0.1	0.3 ± 0.1
$\Delta\Delta G^\circ_c$, kcal/mol	−1.6 ± 0.4	−1.3 ± 0.4	−1.1 ± 0.4	−2.0 ± 0.4	−3.4 ± 0.5	0.3 ± 0.4	−2.9 ± 0.5	−2.6 ± 0.4	−2.9 ± 0.4

[a] The catalytic and regulatory properties of the purified glycerol kinases at pH 7.0, 25 °C, were determined from fits of kinetic data to the equations that are described under Materials and Methods. $\Delta\Delta G^\circ_{\pm Zn(II)}$ is given by $\Delta G^\circ_{variant} - \Delta G^\circ_{normal}$, so that values >0 indicate decreased affinity for complex formation, where ΔG° is calculated as $RT \ln K_i$. $\Delta\Delta G^\circ_c$ is given by $\Delta G^\circ_{+Zn(II)} - \Delta G^\circ_{-Zn(II)}$. wt = normal glycerol kinase.

properties of glycerol kinase, initial velocity kinetic parameters were determined as described under Materials and Methods. Kinetic parameters that are obtained from fits of the data to eq 1 are listed in Table 1 for the normal and variant glycerol kinases. The parameters were determined for two different preparations of each glycerol kinase, and the uncertainties for the parameters indicate the absence of significant differences between the preparations. For the normal enzyme, the parameters agree well with those reported previously (*10*). For the I474A variant, V_{max} is reduced more than 2-fold. A decrease in catalytic activity of about 10-fold was observed for the I474D variant glycerol kinase (*3*). For the E475A variant, V_{max} increases to a value larger than that of the normal enzyme. V_{max} decreases for each substitution thereafter until the N480A and Y481A variants, for which it is greater than that of the normal enzyme. The lower specific activity seen for the R479A variant is consistent with the 15-fold lower specific activity reported for the R479D variant (*3*). At each position, the substitution results also in change in K_{ATP}, the Michaelis constant for ATP, such that the ratio k_{cat}/K_{ATP} changes little. Similar effects are obtained for k_{cat}/K_{gol} (not shown).

Some of the substitutions also alter the apparent affinity for FBP, and the effect appears to be related to V_{max} for catalysis. Each of the variant enzymes shows positively cooperative inhibition by FBP, and the inhibition curves were fitted to eq 2 to obtain the parameters I_{max}, n_H, and $K_{0.5}$. None of the substitutions alters I_{max} or n_H (not shown). However, as shown in Table 1, $K_{0.5}$ is changed for some of the enzymes. For the positions 474−480, $K_{0.5}$ shows a position-dependent effect of the substitutions that is similar to the effect seen for V_{max}. As V_{max} decreases, the apparent affinity for FBP increases ($K_{0.5}$ decreases). The correlation coefficient for these two parameters for the substitutions at positions 474−480 is 0.93. The strong correlation between these parameters from independent experiments suggests that the small differences in V_{max} are significant.

Substitution of each of the sequentially contiguous amino acids affects IIA^{Glc} binding and inhibition of catalysis but to different extents and directions. Effects of the alanine substitutions on IIA^{Glc} inhibition of glycerol kinase were determined from fitting the IIA^{Glc} concentration dependence of the inhibition to eq 3 to obtain the inhibition parameters I_{max} and K_i. All of the variant enzymes display inhibition by IIA^{Glc}, but the substitutions alter the inhibition parameters. The primary effect of the substitutions is on the apparent affinity for formation of the glycerol kinase−IIA^{Glc} complex. A measure of the affinity is the apparent standard free energy change for complex formation, which is calculated from the K_i that is determined from the inhibition data ($\Delta G^\circ = RT \ln K_i$). The apparent standard free energy change for formation of the normal glycerol kinase−IIA^{Glc} complex is −6.8 ± 0.2 kcal/mol ($n = 10$) in the absence of added Zn(II), in good agreement with earlier reports (*7*, *10*). For the variant glycerol kinases, the effect of the substitution on the apparent affinity for IIA^{Glc} binding is given by $\Delta\Delta G^\circ_{-Zn(II)} = \Delta G^\circ_{variant} - \Delta G^\circ_{wt}$, the values of which are shown in Table 1. The alanine substitution of each amino acid except E478 decreases the apparent affinity for complex formation ($\Delta\Delta G^\circ_{-Zn(II)} > 0$), showing that the normal amino acid in these cases contributes favorably to complex formation. The substitution E478A results in increased affinity for IIA^{Glc}, indicating that the glutamate residue at position 478 in the normal enzyme makes an antagonistic contribution to complex formation. A similar increase of affinity was observed previously for the substitution E478C (*10*). Thus, the glycerol kinase amino acid side chains in the IIA^{Glc} binding site make both favorable and antagonistic contributions to complex formation.

Addition of Zn(II) produces large changes in the contributions of the sequentially contiguous amino acids to the energetics of complex formation by cation-promoted association. The cation-promoted association can be quantitated as the coupling free energy between Zn(II) binding and formation of the glycerol kinase−IIA^{Glc} complex, which is given by the difference in free energy change for IIA^{Glc} binding in the presence and absence of Zn(II), $\Delta\Delta G^\circ_c = \Delta G^\circ_{+Zn(II)} - \Delta G^\circ_{-Zn(II)}$. The effects of Zn(II) can be seen in the values for $\Delta\Delta G^\circ_{+Zn(II)}$ and $\Delta\Delta G^\circ_c$ that are shown in Table 1. For the normal enzyme, $\Delta G^\circ_{+Zn(II)} = -8.4 \pm 0.3$ kcal/mol ($n = 12$), in good agreement with earlier reports (*7*, *10*). Hence, $\Delta\Delta G^\circ_c$ for the normal enzyme is −1.6 ± 0.4 kcal/mol, showing cooperativity between binding of IIA^{Glc} and Zn(II). The magnitude of the coupling free energy is in the range expected for protein-mediated interactions (*17*). For the variant enzymes, the largest change in apparent affinity for IIA^{Glc} binding is seen for position T477, which makes an antagonistic contribution to complex formation, and E478, which makes a favorable contribution. Thus, amino acid side chains in the IIA^{Glc} binding site of glycerol kinase make both favorable and antagonistic contributions to complex stability in the presence of Zn(II) as well as in its absence, but their identities are different. The magnitudes of $\Delta\Delta G^\circ_{+Zn(II)}$ for the other variants are changed somewhat so that they show less variation than $\Delta\Delta G^\circ_{-Zn(II)}$. The cooperative contribution of I474 or E475 is increased and that of T476, R479, N480, or Y481 is decreased. The value of $\Delta\Delta G^\circ_c$ is altered little by alanine substitutions at position 474 or 475. With the exception of position 478, alanine substitutions at positions 476−481 result in increased

coupling between Zn(II) binding and IIA^{Glc} binding and inhibition. Position 476 marks a transition between little effect and an effect that approximately doubles the coupling free energy. For E478, the alanine substitution abolishes Zn(II) binding and cation-promoted association, as shown by the value of $\Delta\Delta G°_c$ in Table 1.

Two of the alanine substitutions reduce I_{max} for inhibition by IIA^{Glc}. Normal glycerol kinase shows partial allosteric inhibition by IIA^{Glc}; the value of I_{max} is 90−95% in the absence or presence of Zn(II) under the enzyme assay conditions used here. For each of the variant enzymes except I474A and R479A, the value of I_{max} also is 90−95%. For I474A and R479A, I_{max} is 73 ± 1% and 51 ± 3%, respectively, without or with added Zn(II), indicating that these alanine substitutions decrease the coupling between IIA^{Glc} binding and inhibition of catalysis such that IIA^{Glc} is unable to provide the extent of inhibition obtained for the normal enzyme and the other variant enzymes.

DISCUSSION

The effects of the alanine substitutions of the sequentially contiguous amino acids in the IIA^{Glc} binding site of glycerol kinase on binding and inhibition by IIA^{Glc} are considered in terms of the crystal structures of glycerol kinase without (*3*) and with (*4*) bound IIA^{Glc} and of the glycerol kinase-IIA^{Glc} complex without and with Zn(II) (*7*). A primary conclusion of this study is that, at some positions, consequences of the alanine substitutions for IIA^{Glc} binding and inhibition appear to be consistent with changes in intra- and intermolecular side chain interactions seen in the structures, while, at other positions, effects of substitutions and effects on cation-promoted association by Zn(II) are quite unexpected. The effects of the substitutions at the latter positions indicate that they behave as a cooperative unit in IIA^{Glc} binding and inhibition. In addition, the effects of the substitutions on the catalytic properties and FBP inhibition are coupled to the effects on IIA^{Glc} binding and inhibition, even though the sites of the substitutions are ∼30 and ∼65 Å from the other functional sites, respectively.

For I474, the effects of the alanine substitution on IIA^{Glc} inhibition appear to be consistent with the changes in interactions seen in the structures. Upon binding of IIA^{Glc}, the α-carbon of I474 moves ∼4 Å as the side chain moves from a cleft in glycerol kinase to form van der Waals interactions with IIA^{Glc}. The nature of this movement for the entire region can be seen in Figure 1. The decreased affinity for complex formation and increased partial character of IIA^{Glc} inhibition are consistent with loss of these interactions with IIA^{Glc}. The decreases in the catalytic activities of the I474A and I474D variants are consistent with increasing degrees of loss of the intramolecular interactions of I474 with the cleft on glycerol kinase and indicate important roles for these interactions in the properties of the enzyme in the absence of IIA^{Glc}. These substitutions increase the apparent affinity for FBP, further suggesting that they alter the normal catalytic states of the enzyme. The I474A substitution does not alter the cation-promoted association by Zn(II), which is consistent with the observation that the conformation at this position does not change upon Zn(II) binding.

Effects of the R479A substitution on the catalytic properties, FBP inhibition, and IIA^{Glc} inhibition in the absence of Zn(II) also appear to be consistent with the changes in interactions seen in the structures. Upon binding of IIA^{Glc} to glycerol kinase, intramolecular hydrogen bonds of the guanidinium group of R479 with the backbone carbonyls of positions 424 and 474 are disrupted, and the guanidinium group of R479 forms an intermolecular salt bridge with D38 on IIA^{Glc}. In the absence of IIA^{Glc}, the decreases in catalytic activity seen for the R479A and R479D variants indicate that the intramolecular interactions are important for normal function. Loss of these intramolecular interactions results also in increased apparent affinity for FBP for the R479A variant but not for the R479D variant (*3*). The effects of the alanine substitution on IIA^{Glc} affinity in the absence of Zn(II) are consistent with the loss of the intermolecular salt bridge with D43 of IIA^{Glc} that is seen in the crystal structures and show that this salt bridge makes a substantial contribution to the apparent affinity.

The salt bridge of R479 and the intermolecular interactions of I474 are important for IIA^{Glc} inhibition to attain its maximum value. The loss of full inhibition associated with loss of each of these interactions, seen as reduced values of I_{max}, indicates that they have important roles in stabilizing the inhibited forms of the enzyme−substrate complexes. Importance of the van der Waals interactions is consistent with results from protein design studies showing that either side chain packing (*18*−*20*) or polar interactions (*21*, *22*) can confer uniqueness to protein structures. In this case, both are necessary to stabilize the inhibited states of glycerol kinase.

The alanine substitution of E475 has only small effects on catalytic and regulatory properties of glycerol kinase. Examination of the structures suggests that the substitution could have larger effects. The α-carbon of E475 moves ∼5 Å upon binding of IIA^{Glc}, and the carboxylate group of E475 forms a hydrogen bond with the amide hydrogen of I474. The crystal structures show that the side chain of E475 may interact with the helix macrodipole in both the absence and presence of IIA^{Glc}. The small effects of the alanine substitution indicate that these interactions contribute little to the energetics of glycerol kinase functions or that their loss is fortuitously compensated by other effects of the substitution. The E475A substitution does not affect cation-promoted association by Zn(II), which is consistent with the structures showing no change at this position upon Zn(II) binding to the normal glycerol kinase−IIA^{Glc} complex.

In the absence of IIA^{Glc}, the carboxylate group of E478 forms an intramolecular salt bridge with the guanidinium group of R482. In the absence of Zn(II), the side chain of E478 may form a distant intermolecular contact with F71 of IIA^{Glc}; yet, the effect of the alanine substitutions shows that the side chain makes an antagonistic contribution to complex formation. A similar increase in affinity was seen for the E478C substitution (*10*). The unfavorable contribution likely is related to loss of the intramolecular salt bridge and burial of the charged carboxylate group in the mostly hydrophobic interface between the two proteins. The substitutions E478D, E478H, and E478Q, which retain a charged or polar amino acid at this position, do not result in the increased affinity seen with the nonpolar amino acids (*10*). The E478A substitution abolishes cation-promoted association by Zn(II). Loss of Zn(II) binding for the E478A variant is consistent with loss of a Zn(II)-liganding amino

acid side chain shown by the crystal structures and agrees with earlier results for other substitutions of E478 that abolish Zn(II) binding also (*10*).

For the amino acids at positions 476, 477, 480, and 481, the effects of the alanine substitutions on IIA^{Glc} inhibition and cation-promoted association do not appear to be consistent with changes in side chain interactions observed in the structures. The effects of the alanine substitution of T476 or T477 on the catalytic properties, FBP inhibition, and IIA^{Glc} inhibition in the absence of Zn(II) are very similar. This suggests a common feature of these positions that is affected by the alanine substitution. In the absence of IIA^{Glc}, the side chains of T476 and T477 show no intramolecular interactions with glycerol kinase. Upon binding of IIA^{Glc}, the amino acid side chain in each of these positions interacts with IIA^{Glc}. The side chain of T476 forms rather distant intermolecular contacts with V40 and V46, while the side chain of T477 makes an extensive network of intermolecular contacts with V40, F41, F88, and H90, as well as an intermolecular hydrogen bond between its hydroxyl group and D94. Thus, the similar effects of the alanine substitution are not due to similar participation of each of these amino acids in intermolecular interactions. On the basis of the crystal structures, the effects of the alanine substitution at position 480 or 481 on the catalytic properties and IIA^{Glc} inhibition in the absence of Zn(II) are completely unexpected. As seen in Figure 1, the conformation of glycerol kinase at these two positions does not appear to change significantly upon IIA^{Glc} binding. These amino acids have little or no interaction with IIA^{Glc}. N480 makes no contacts closer than 4.0 Å with IIA^{Glc}. The Cϵ of Y481 is 3.5 Å from the Cα of E97 and the Cβ of Y481 is 3.7 Å from the Cγ of V96 of IIA^{Glc}. The Cβ interaction should not be disrupted in the alanine variant. Yet, in the absence of Zn(II), the alanine substitutions at these two positions decrease the affinity for IIA^{Glc} more than do the substitutions at positions that undergo backbone conformational changes and form more extensive interactions with IIA^{Glc}. The conformation at positions 476, 477, 480, and 481 does not change upon Zn(II) binding to the normal glycerol kinase, so the large increase in the coupling free energy $\Delta\Delta G^\circ_c$ seen for the alanine variants at these positions is unexpected.

The unexpected effects of the alanine substitutions may be explained in terms of cooperativity among the IIA^{Glc} binding interactions and changes in the stability of the binding site helix. The sum of the $\Delta\Delta G^\circ_{-Zn(II)}$ values for the alanine substitutions at positions 474−481 is 6.7 ± 0.6 kcal/mol, which accounts for $\Delta G^\circ_{-Zn(II)}$ for the normal glycerol kinase to within 0.1 ± 0.6 kcal/mol. As shown by the crystal structures, the sequentially contiguous amino acids on glycerol kinase do not constitute the entire IIA^{Glc} binding site; R402 on glycerol kinase forms a salt bridge with E43 on IIA^{Glc}, and other contributions have been identified by Freire and co-workers (*23*). The observation that the sum of the $\Delta\Delta G^\circ_{-Zn(II)}$ values for these alanine substitution variants differs little from $\Delta G^\circ_{-Zn(II)}$ for the normal enzyme, without accounting for other interactions, indicates positive cooperativity among the IIA^{Glc} binding interactions. This cooperativity is increased markedly by Zn(II). The sum of the $\Delta\Delta G^\circ_{+Zn(II)}$ values for the alanine substitutions is 3.5 ± 0.3 kcal/mol, which leaves 4.9 ± 0.4 kcal/mol of $\Delta G^\circ_{+Zn(II)}$ for the normal glycerol kinase unaccounted for.

Many of the amino acids in the IIA^{Glc} binding site appear to function as a cooperative unit with respect to IIA^{Glc} inhibition and cation-promoted association by Zn(II). Effects of the alanine substitutions on the apparent affinity for IIA^{Glc} in the absence of Zn(II) increase from position 474 to position 481, except for the E478 position. The substitution at I474 or E475 has a small effect ($\Delta\Delta G^\circ_{-Zn(II)} \cong 0.2$ kcal/mol), while the substitution at T476, T477, or 479−481 has a larger effect ($\Delta\Delta G^\circ_{-Zn(II)} \cong 1-1.7$ kcal/mol). Cation-promoted association by Zn(II) is increased about 2-fold by the alanine substitutions at all of these positions, except 476, which shows a smaller increase. All of these amino acids are in an α-helical conformation in the absence of IIA^{Glc}. The similarity of the effect of substituting each of these positions with alanine with respect to IIA^{Glc} affinity in the absence of Zn(II) and increased cation-promoted association by Zn(II) strongly suggests that this region of the binding site functions as a cooperative unit with respect to IIA^{Glc} binding and inhibition.

The action of the α-helical residues as a cooperative unit is consistent with the conformational change that is observed in the crystal structures and with the behavior of α-helices during protein folding. The smaller effect of the T476A substitution on both the free energy of IIA^{Glc} binding and $\Delta\Delta G^\circ_c$ is consistent with its location as the N-terminal residue in the α-helix. The decreased apparent affinity for IIA^{Glc} seen for the alanine substitutions of residues in this cooperative unit is consistent with increased stabilization of the α-helix due to the higher helix propensity of alanine relative to the normal amino acids. For positions 476, 477, 480, and 481, the helix propensity is increased 0.57, 0.57, 0.60, and 0.47 kcal/mol, respectively, by the alanine substitution (*24*). These α-helical propensities represent the difference in stability relative to the unfolded state(s), and the difference in propensities of amino acids for formation of α-helix versus 3_{10}-helix is unknown. These results suggest that there is, in fact, a significant difference between the propensities for the two types of helix. Freire and co-workers (*23*) predicted recently that increased stability of the IIA^{Glc} binding site would decrease the affinity for IIA^{Glc} binding, and the effects of the alanine substitutions are consistent with this prediction. It appears that the alanine substitution at position 480 or 481 may stabilize the helix also at the distal positions 476−478.

Cation-promoted association by Zn(II) may be an important component of recognition of the many regulatory targets of IIA^{Glc} (*25*). The ability of Zn(II) to contribute to complex stability to overcome significant losses of affinity in the protein−protein interactions could have important implications for protein evolution. For the variants at the α-helical positions, cation-promoted association by Zn(II) offsets a portion of the loss in binding affinity that results from the substitution. Results of this study suggest that the apparent affinity for metal ion binding can be utilized to complement relatively weak protein−protein interactions and allow complex formation. Additional substitutions through evolution could enhance the affinity for the protein−protein interactions while retaining metal binding with lowered affinity so that the net affinity for complex formation remains relatively constant, retaining biological function. In such a scenario, the observed affinity for metal binding may be reduced to the point that its current functional relevance may

be overlooked; however, its true function may have been obviated by subsequent evolutionary changes to reach the current point.

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