# Cation-Promoted Association of *Escherichia coli* Phosphocarrier Protein IIA$^{Glc}$ with Regulatory Target Protein Glycerol Kinase: Substitutions of a Zinc(II) Ligand and Implications for Inducer Exclusion†

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ABSTRACT: In *Escherichia coli*, inducer exclusion is one mechanism by which glucose prevents unnecessary expression of genes needed for metabolism of other sugars. The basis for this mechanism is binding of the unphosphorylated form of the glucose-specific phosphocarrier protein of the phospho*enol*pyruvate: glycose phosphotransferase system, IIA$^{Glc}$ (also known as III$^{Glc}$), to a variety of target proteins to prevent uptake or synthesis of the inducer. One of these target proteins is glycerol kinase (EC 2.1.7.30, ATP: glycerol 3-phosphotransferase), which is inhibited by IIA$^{Glc}$. Glycerol kinase is the only IIA$^{Glc}$ target protein for which the structure of the complex is known. Association of these two proteins forms an intermolecular binding site for Zn(II) with metal ligands contributed by each protein, and Zn(II) enhances IIA$^{Glc}$ inhibition [Feese, M., Pettigrew, D. W., Meadow, N. D., Roseman, S., and Remington, S. J. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 3544−3548]. Here, we show that the Zn(II) enhancement can be described quantitatively by a model with binding of Zn(II) to the complex with an apparent dissociation constant of less than 1 $\mu$M at pH 7.0 and 25 °C. Initial velocity studies show that IIA$^{Glc}$ is an uncompetitive inhibitor with respect to both substrates, and the mechanism of inhibition is not altered by Zn(II). The Zn(II)-liganding residue contributed by glycerol kinase (Glu478) is substituted by using site-directed mutagenesis to construct the enzymes E478C, E478D, E478H, and E478Q. The substitutions have only small effects on the inhibition by IIA$^{Glc}$ in the absence of Zn(II), the catalytic properties, or other allosteric regulation. However, all of the substitutions abolish the Zn(II) enhancement of IIA$^{Glc}$ inhibition, and the X-ray crystallographic structures of the complexes of IIA$^{Glc}$ with the E478C and E478H mutants show these substitutions abolish binding of Zn(II) to the intermolecular site. These results support the hypothesis that Zn(II) enhances the affinity for complex formation by binding at the intermolecular site, i.e., cation promoted association. The high affinity for Zn(II) binding to the complex and the ability of the other four amino acid residues to efficiently substitute for Glu478 in all functions except binding of Zn(II) suggest that cation promoted association of these two proteins may have a role in inducer exclusion in vivo.

In *Escherichia coli*, the glucose-specific phosphocarrier protein of the PTS, IIA$^{Glc}$, participates in at least four different physiological functions via its interactions with at least 10 other proteins (*1*):[1] (a) it serves as a phosphoryl carrier in the uptake and phosphorylation of glucose, (b) phospho-IIA$^{Glc}$ is thought to be an activator of adenylate cyclase, (c) IIA$^{Glc}$, but not phospho-IIA$^{Glc}$, inhibits certain non-PTS permeases (lactose, melibiose, and maltose), a phenomenon called inducer exclusion, which has been reviewed recently (*2*−*4*), and (d) IIA$^{Glc}$, but not phospho-IIA$^{Glc}$, inhibits glycerol kinase (EC 2.1.7.30, ATP:glycerol 3-phosphotransferase), the subject of this paper (*5*, *6*). Glycerol is a unique substrate for *E. coli* in that it is taken up by facilitated diffusion and is then trapped in the cell as glycerol 3-phosphate after MgATP-dependent phosphorylation catalyzed by glycerol kinase (*7*). Glycerol 3-phosphate induces the glycerol phosphate regulon (*7*), and thus, IIA$^{Glc}$ inhibition of glycerol kinase is, in effect, inducer exclusion. Surprisingly, these multiple interactions of IIA$^{Glc}$ with other proteins are effected without any consensus sequence in the target proteins (*8*).

The structure of only one complex between IIA$^{Glc}$ and a target protein has been established, the IIA$^{Glc}$·glycerol kinase

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[1] Abbreviations: IIA$^{Glc}$ (also known as III$^{Glc}$), the phosphocarrier protein of the phosphotransferase system specific for glucose and methyl α-D-glucopyranoside; FBP, fructose 1,6-bisphosphate; GK, glycerol kinase; gol, glycerol; kb, kilobase pairs; MES, 2-(*N*-morpholino)-ethanesulfonic acid; PTS, phospho*enol*pyruvate:glycose phosphotransferase system; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis. Mutant proteins are denoted by using the single-letter abbreviation for the amino acid residues, e.g., E478Q means that the glutamate residue at position 478 is replaced with glutamine.

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complex (*9*). Glycerol kinase binds to $IIA^{Glc}$ primarily via a hydrophobic patch on the surface surrounding the active-site histidine residues of $IIA^{Glc}$. The interaction between the two proteins involves remarkably few amino acid residues, seven from glycerol kinase and 10 from $IIA^{Glc}$. Subsequent crystallographic experiments showed that addition of Zn(II) to the crystalline complex resulted in its binding at an intermolecular site with ligands and distorted tetrahedral geometry identical to the active site of thermolysin (*10*). Two Zn(II) ligands are provided by the active-site histidine residues of $IIA^{Glc}$ (H75 and H90), one ligand is provided by a glutamate residue of glycerol kinase (E478), and the fourth coordination position is occupied by a molecule from the solvent. Addition of 0.1 mM Zn(II), but not other divalent cations, to enzyme activity assays appeared to increase the apparent affinity for complex formation (*10*). These results suggested that Zn(II) binding at the intermolecular site promotes the association of the two proteins, and this phenomenon was termed cation-promoted association. If correct, this hypothesis could have important physiological consequences.

This hypothesis was partially tested by substituting one of the $IIA^{Glc}$ Zn(II) ligands, H75, with glutamine, an amino acid residue that does not usually coordinate Zn(II) with high affinity (*10*). The H75Q mutant of $IIA^{Glc}$ behaved normally insofar as protein−protein interactions were concerned, i.e., glycerol kinase activity was inhibited. However, as predicted, Zn(II) did not affect the inhibition. In this report, the Zn(II) concentration dependence of $IIA^{Glc}$ inhibition of the wild-type glycerol kinase and site-directed mutagenesis of the Zn(II)-liganding residue of glycerol kinase are used to test the cation-promoted association hypothesis more extensively.

## EXPERIMENTAL PROCEDURES

*Materials.* All materials were purchased from Sigma Chemical Co. of St. Louis, MO, unless indicated otherwise. $IIA^{Glc}$ was purified as described (*11*).

*Construction of Site-Directed Mutants.* The molecular biology procedures used for cloning the DNA fragments and DNA sequencing were described previously (*12*). Mutations of the *glpK* gene were constructed by using the Kunkel method (*13*) for site-directed mutagenesis using the Bio-Rad MutaGene kit with *E. coli* strain CJ236 (*dut ung*) and the entire *glpK* gene (1.5 kb) carried on a 2.8 kb *Hin*dIII insert (*14*) in M13mp19. Mutagenic primers (20 mers) were synthesized by the Gene Technologies Laboratory of the Department of Biology of Texas A&M University. Mutated genes were identified by DNA sequencing, using Sequenase 2.0 (U.S. Biochemicals) with α-[$^{35}$S]dATP (DuPont NEN) and a primer previously used for sequencing the *glpK* gene. Inserts bearing the mutated genes were transferred to the *Hin*dIII site of the expression vector pHG165 (*15*). The altered enzymes were expressed in *E. coli* DG1 (*ara* Δ(*lac-proAB*) (*Ø80 lacZ*Δ*M*15) *rpsL glpK*202 *hsdR4*) (*12*) and purified to near homogeneity using the same protocol as for the wild-type enzyme, i.e., ion-exchange chromatography on DEAE-cellulose and DEAE-Sephadex. No obvious differences were noted for the expression or purification of any of the altered enzymes relative to the wild-type enzyme, indicating that the amino acid changes do not produce gross alterations in enzyme structure. SDS−PAGE of the purified proteins showed them to have the same extent of purity as the wild-type enzyme (>95%) and gave the same apparent subunit molecular weight. The enzymes were stored as microcrystals in ammonium sulfate at 4 °C. At least two different preparations of each enzyme were used in these studies, and no significant differences in their properties were found.

Because the site-directed mutagenesis was performed using the entire cloned *glpK* gene, the occurrence of spurious second site mutations was evaluated. Two different procedures were used, as described below, and the results showed no second site mutations in any of the genes. For all of the mutations, multiple independent isolates were identified by DNA sequencing. For the substitutions E478D and E478Q, glycerol kinase was purified from each of two independent isolates and found to have identical properties. If second site mutations occurred, they would either have to be the same mutation or confer the same properties on the mutant enzyme; the probability of either of these cases is very small. For the substitutions E478C and E478H, the absence of second site mutations was evaluated by DNA sequencing. The entire mutated *glpK* gene was sequenced using the Applied Biosystems, Inc., model 373A DNA sequencer in the Laboratory for Crop Genome Analysis of the Crop Biotechnology Center of Texas A&M University. Double-stranded plasmid DNA was prepared for sequencing by using the Qiagen tip 10 mini kit. The primers used for the sequencing were the same as used previously for sequencing the wild-type gene. No mutations other than those intended were found in these two *glpK* genes.

*Determinations of Protein Concentration.* The protein concentration of $IIA^{Glc}$ was determined after dialysis versus 0.1 M triethanolamine-HCl, pH 7.0, by using the Bio-Rad protein assay with bovine serum albumin as the standard. The concentration of glycerol kinase was determined from absorbance at 280 nm using the extinction coefficient 1.73 $(mg/mL)^{-1}$ $cm^{-1}$. This extinction coefficient differs from that previously used [1.4 $(mg/mL)^{-1}$ $cm^{-1}$], which was determined by a dry weight method (*16*). The difference was noted when the extinction coefficient was calculated on the basis of the amino acid composition (*17*) deduced from the DNA sequence and was confirmed by determining the extinction coefficient by using the method of Edelhoch (*18*). The molar concentrations of the proteins were calculated using molecular weights of 56 100 for glycerol kinase (*14*) and 18 100 for $IIA^{Glc}$ (*19*).

*Enzyme Assays.* Initial velocities were measured by using an ADP-coupled continuous spectrophotometric assay at 25 °C (*12*). The assay contained 0.05 M triethanolamine-HCl, pH 7.0 (adjusted at room temperature with KOH), 5 mM $MgCl_2$, 20 mM KCl, 1 mM $\beta$-mercaptoethanol, varied concentrations of ATP and glycerol, and other additions as indicated in the tables and figure legends. Zn(II) was added as $ZnCl_2$ (Aldrich). Reactions were initiated by addition of enzyme (final concentration 0.2−0.5 $\mu$g/mL) to the equilibrated cuvette. All of the enzymes were prepared for assay by removing glycerol and ammonium sulfate used in crystallizing the enzymes for storage by either dialysis or Sephadex G-25 chromatography using 0.1 M triethanolamine-HCl, pH 7.0, 1 mM dithiothreitol.

Initial velocity data were analyzed as described for the wild-type enzyme (*20*); enzyme kinetics parameters were

estimated from fits of the initial velocity data at ATP concentrations ≤0.1 mM to the following equation for a sequential bisubstrate enzyme mechanism:

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

Data from steady-state kinetics studies of IIA$^{Glc}$ inhibition were fitted to the following equation for uncompetitive inhibition:

$$v = VA/K + A(1+ [I]/K_{ii}) \quad (2)$$

*Analysis of IIA$^{Glc}$ Inhibition Data.* IIA$^{Glc}$ inhibition was characterized by measuring the specific activity of the wild-type or mutant glycerol kinases by using the assay described above with different concentrations of IIA$^{Glc}$ with or without added Zn(II). The IIA$^{Glc}$ inhibition of glycerol kinase was analyzed in terms of Scheme 1:

Scheme 1

$$\begin{array}{ccc} GK + IIA^{Glc} & \overset{K_d}{\rightleftharpoons} & GK{\cdot}IIA^{Glc} \\ +Zn & & +Zn \\ K_1 \downdownarrows\uparrow & & \downdownarrows\uparrow K_{Zn} \\ GK + Zn{\cdot}IIA^{Glc} & \overset{K_2}{\rightleftharpoons} & GK{\cdot}Zn(II){\cdot}IIA^{Glc} \end{array}$$

The terms $K_d$ and $K_{Zn}$ are defined as apparent dissociation constants for formation of the GK·IIA$^{Glc}$ complex and for binding of Zn(II) to the GK·IIA$^{Glc}$ complex, respectively. The terms $K_1$ and $K_2$ are defined as apparent dissociation constants for formation of the Zn(II)·IIA$^{Glc}$ complex and for binding of glycerol kinase to the Zn(II)·IIA$^{Glc}$ complex, respectively. This scheme yields the following equation for the effect of Zn(II) on the fractional saturation of glycerol kinase with IIA$^{Glc}$:

$$\nu = \frac{[IIA^{Glc}]_{tot}}{K_{app} + [IIA^{Glc}]_{tot}} \quad (3)$$

where

$$K_{app} = K_d \frac{1 + [Zn(II)]_f/K_1}{1 + [Zn(II)]_f/K_{Zn}}$$

The data for the mutant glycerol kinases were analyzed by fitting to eq 4 to obtain estimates for $K_{app}$ and maximum inhibition:

$$SA, \% = 100\% - (\text{maximum inhibition}\% \times \nu) \quad (4)$$

For analysis of the data obtained for the wild-type enzyme at different concentrations of Zn(II), the full expression for $K_{app}$ was substituted into eq 4, and the fits yield estimates of $K_d$, $K_{Zn}$, $K_1$, and maximum inhibition. The specific activities were normalized to the specific activity at 0 IIA$^{Glc}$ for each data set. This was done for data presentation because the specific activity is decreased by addition of Zn(II). Control experiments show this decrease is not due to effects on the coupling enzymes of the assay.

Analysis of the data obtained for the wild-type enzyme at different concentrations of Zn(II) by using eq 4 requires estimates of $[Zn(II)]_f$, which must take the binding of Zn(II) to ATP into account. In addition, the competition of Mg(II) for ATP must be considered. $[Zn(II)]_f$ was estimated by using Microsoft Excel to perform successive approximations to evaluate the simultaneous multiple equilibria following the principles outlined by Storer and Cornish-Bowden (*21*). Association constants for formation of MgATP and ZnATP ($M^{-1}$) of $2 \times 10^4$ and $1.6 \times 10^5$, respectively (*22*), were used for the calculations of $[Zn(II)]_f$ by this method. The binding of Zn(II) to IIA$^{Glc}$ alone was not included in the successive approximations to estimate $[Zn(II)]_f$. Additional calculations which include binding of Zn(II) to IIA$^{Glc}$ alone using the value $K_1$ determined from the fits to the inhibition data show that the free Zn(II) concentration changes only from 11 $\mu$M at 0 IIA$^{Glc}$ to 9.5 $\mu$M at 25 $\mu$M total IIA$^{Glc}$. Thus, the free concentration of Zn(II) is effectively buffered by the ATP, and ignoring binding of Zn(II) to IIA$^{Glc}$ alone in calculating $[Zn(II)]_f$ does not introduce significant errors in the data analysis.

Nonlinear least-squares fitting of data to equations was done by using NonLin for Macintosh obtained from R. J. Brenstein, Robelko Software, Carbondale, IL. For all the data analyses, the best fit values of the parameters are shown with 65% confidence limits corresponding to one standard deviation in the fit given in parentheses.

*Atomic Absorption Spectroscopy.* Atomic absorption spectroscopy was used to determine the Zn(II) content of wild-type glycerol kinase and IIA$^{Glc}$ after dialysis versus 0.1 M triethanolamine buffer at pH 7.0. Zn(II) determinations were made at a wavelength of 214.2 nm by using a Perkin-Elmer 2380 atomic absorption spectrophotometer operated in the flame mode with an air-acetylene flame. Zn(II) reference standard solution (1000 ppm) was from Fisher Scientific. The standard curve was linear over the range 0.5−6 $\mu$M Zn(II).

*X-ray Crystallography.* The E478C and E478H mutant enzymes were crystallized by hanging drop vapor diffusion against sodium acetate as the regulatory complex with intact IIA$^{Glc}$ (IIA$^{Glc}$ slow). The crystals were isomorphous to wild-type cocrystals described previously (*9*). Crystal setups contained IIA$^{Glc}$-glycerol kinase in the molar ratio of 3.2:1, total protein 29 mg/mL diluted 50% with well solution, with 1 mL 0.7−0.9 M sodium acetate and 0.1 M MES, pH 6.0, as the well solution. Addition of 2 mM zinc acetate to the mother liquor did not affect the crystals of the E478C complex. However, crystals of the E478H complex diffracted poorly and became very sensitive to radiation damage after addition of Zn(II), so complete data sets could not be collected from these crystals. Diffraction data to the maximum resolution obtainable were collected using a Xuong-Hamlin area detector and reduced with the supplied software (*23*). Data collection statistics are given in Table 1. The atomic and electron density maps were inspected using FRODO (*24*), and the models were refined using conjugate gradient least-squares using the TNT package (*25*). Statistics for the refined models are presented in Table 1.

## RESULTS

*Effect of Zn(II) on IIA$^{Glc}$ Inhibition of Wild-Type Glycerol Kinase.* Figure 1 shows titrations of the catalytic activity

Table 1: X-ray Crystallographic Data Collection and Model Statistics

| | | |
|---|---|---|
| mutant enzyme | E478C | E478H |
| space group | *I*222 | *I*222 |
| cell dimensions (Å) | | |
| *a* | 123.0 | 123.0 |
| *b* | 125.1 | 125.0 |
| *c* | 134.6 | 135.0 |
| resolution (Å) | 20−2.9 | 20−2.6 |
| reflections (total) | 46 571 | 78 087 |
| reflections (unique) | 17 783 | 28 020 |
| $R_{merge}$[a] | 0.065 | 0.070 |
| completeness (%) | 81 | 85 |
| atomic model statistics | | |
| atoms | 4982 | 4926 |
| solvent atoms | 0 | 0 |
| *R*-factor[b] | 0.179 | 0.180 |
| bond length deviations (Å) | 0.013 | 0.025 |
| bond angle deviations (deg) | 2.3 | 3.6 |
| *B*-factor restraints (Å$^2$) | 8.7 | 11.2 |

[a] $R_{merge} = \sum|I - \langle I\rangle|/\sum\langle I\rangle$, where $\langle I\rangle$ is the mean value of individual measurements of intensities *I*. [b] *R*-factor is the standard crystallographic residual $\sum||F_o| - |F_c||/\sum|F_o|$.

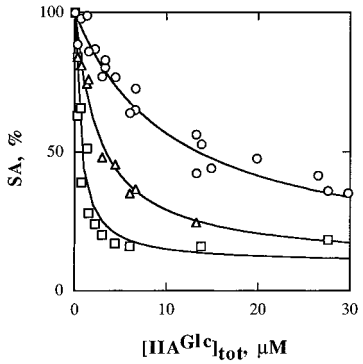


FIGURE 1: $IIA^{Glc}$ inhibition of wild-type glycerol kinase at different concentrations of Zn(II). The specific activity of wild-type glycerol kinase was determined by using the assay described in the Experimental Procedures with 2.5 mM ATP, 2 mM glycerol, and additions of $ZnCl_2$ and $IIA^{Glc}$ as indicated. The solid lines drawn through the data points show the best simultaneous fit of all the data to eq 4, with the parameters given in the text. Legend: ○, 0 $\mu$M $ZnCl_2$; △, 10 $\mu$M $ZnCl_2$; □, 100 $\mu$M $ZnCl_2$.

of wild-type glycerol kinase with $IIA^{Glc}$ at different total concentrations of Zn(II). The points show results of several individual titrations performed using several different preparations of glycerol kinase and $IIA^{Glc}$. As the total concentration of Zn(II) is increased, the shift in the titration curve is consistent with increased apparent affinity for binding of $IIA^{Glc}$ to glycerol kinase, as shown previously (*10*). The effect of Zn(II) on $IIA^{Glc}$ inhibition of the wild-type glycerol kinase can be quantitatively described by the model shown in Scheme 1 in which Zn(II) binding to the complex increases the apparent affinity for complex formation. The lines show the best fit of all the data simultaneously to eq 4 (square root of the variance = 6.6), which provides a good description of the data and yields the following parameter values, with the 65% confidence intervals in parentheses: maximum inhibition, 90 (85, 97)%; $K_d$, 11 (8, 13) $\mu$M; $K_{Zn}$, 0.3 (0.2, 0.5) $\mu$M; $K_1$, 11 (5, 24) $\mu$M. Comparison of the values of $K_1$ and $K_{Zn}$ shows that the apparent affinity of $IIA^{Glc}$ alone for binding Zn(II) is about 30-fold less than that of the glycerol kinase·$IIA^{Glc}$ complex. The apparent affinity for binding of Zn(II) to $IIA^{Glc}$ alone is consistent with the affinity for Zn(II) binding observed for other bidentate sites containing nitrogen ligands (*26*, *27*). The value of $K_1$ can also be estimated from the Zn(II) inhibition of the phosphorylation of $IIA^{Glc}$ by HPr, the kinetics of which have been characterized (*28*, *29*). Preliminary results of these inhibition experiments indicate that the dissociation constant for Zn(II) binding to $IIA^{Glc}$ is about 10 $\mu$M, which is also the value obtained from preliminary results of equilibrium binding studies (Meadow, N. D., and Pettigrew, D. W., unpublished experiments). The dissociation constants in Scheme 1 are related by the expression, $K_1K_2 = K_dK_{Zn}$. The value of $K_2$ that is calculated from this relation (0.3 ± 0.3 $\mu$M) is in good agreement with the value of $K_{app}$ at 100 $\mu$M total Zn(II) [0.6 (0.4, 0.9) $\mu$M], which is consistent with saturation of the glycerol kinase·$IIA^{Glc}$ complex with Zn(II) that is expected at the free concentration of Zn(II) under these conditions (~10 $\mu$M).

The kinetic mechanism of $IIA^{Glc}$ inhibition was investigated by using steady-state kinetics studies at 0 and 100 $\mu$M total Zn(II), which were performed as described previously (*20*). Double-reciprocal plots of the data for inhibition versus ATP or glycerol are shown in Figure 2. The plots are parallel for both substrates without and with Zn(II), showing uncompetitive inhibition and that Zn(II) does not alter the mechanism of inhibition. The observation of uncompetitive inhibition shows that $IIA^{Glc}$ binds only to the enzyme·substrate complexes and not to the free enzyme. Inhibition parameters that were obtained by fitting the data to eq 2 are summarized in Table 2. The values of $K_{ii}$ obtained for inhibition with respect to each substrate are the same. With 100 $\mu$M total Zn(II), neither the mechanism of inhibition nor the apparent value of $K_{ii}$ are altered by changing the concentration of the fixed substrate ATP. Observation of uncompetitive inhibition with the same value of $K_{ii}$ for both substrates, which is not dependent on the concentration of the fixed substrate, is consistent with binding of $IIA^{Glc}$ to the ternary enzyme·substrate/product complexes and/or to one or both of the binary enzyme·product complexes. This result is consistent with earlier in vivo studies in the closely related organism *Salmonella typhimurium*, which showed that glycerol is required for $IIA^{Glc}$ inhibition of glycerol kinase (*30*). Importantly, these results show that the titration curves in Figure 1 are not dependent on substrate concentration and provide an accurate estimate of the fraction of glycerol kinase that is bound by $IIA^{Glc}$ in the absence or presence of Zn(II). The value of $K_{ii}$ obtained at 0 Zn(II) corresponds to $K_d$ and the value of $K_{ii}$ obtained at 100 $\mu$M total Zn(II) corresponds to $K_2$. In each case, the value obtained from the steady-state inhibition studies is in good agreement with the value obtained from the data shown in Figure 1.

Inhibition by $IIA^{Glc}$ was previously reported to be noncompetitive with respect to both substrates (*5*). While the reasons for the difference in the results reported here and the previous results are not known, differences in experimental conditions should be noted. In particular, the earlier study was performed using pH 6.5 and 37 °C, whereas pH 7.0 and 25 °C is used here. In the earlier study, higher concentrations of the fixed substrate (5 mM ATP, 0.5 mM glycerol) and a single concentration of $IIA^{Glc}$ (22 $\mu$M) were used. As shown in Table 2, however, neither the type of inhibition nor $K_{ii}$ is changed by increasing the concentration of the fixed substrate ATP.

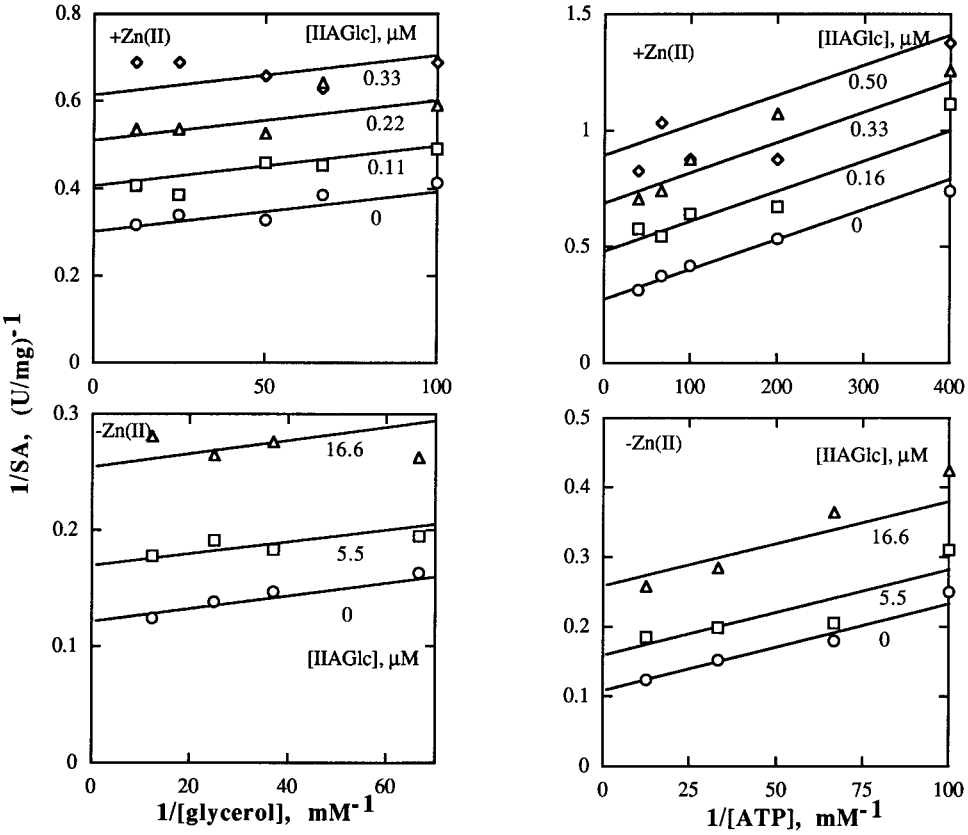


FIGURE 2: Double-reciprocal plots of steady-state kinetics studies of IIA$^{Glc}$ inhibition of wild-type glycerol kinase. Initial velocities were determined at pH 7.0 and 25 °C by using the coupled assay described in the Experimental Procedures with additions of II$^{AGlc}$ and Zn(II) as indicated. The points show the experimental data and the lines show the best fit of the data to eq 2, yielding the parameter values for $K_{ii,app}$ shown in Table 2. The concentration of glycerol kinase was 0.4 $\mu$g/mL. The concentrations of the fixed substrate are given in Table 2.

Table 2: Steady-State Kinetics Parameters for IIA$^{Glc}$ Inhibition of Glycerol Kinase[a]

| variable substrate | fixed substrate [concn ($\mu$M)] | $K_{ii}$ ($\mu$M) |
|---|---|---|
| | no added Zn(II) | |
| glycerol | ATP [50] | 15 (14, 17) |
| ATP | glycerol [50] | 13 (11, 15) |
| | 100 $\mu$M Zn(II) | |
| glycerol | ATP [20] | 0.32 (0.17, 0.55) |
| glycerol | ATP [500] | 0.2 (0.18, 0.23) |
| ATP | glycerol [20] | 0.22 (0.18, 0.25) |

[a] The steady-state parameters for IIA$^{Glc}$ inhibition of glycerol kinase were determined by fitting kinetics data shown in Figure 2 to eq 2. The parameters $K_{ii}$ are shown as the best-fit value with the 65% confidence limits shown in parenthesis.

In the crystal structure of the glycerol kinase·IIA$^{Glc}$ complex, glycerol kinase is a tetramer and there is one IIA$^{Glc}$ binding site per subunit of glycerol kinase (*9*). In solution, glycerol kinase undergoes a reversible equilibrium between dimer and tetramer, and at the enzyme concentrations in these assays (0.2 $\mu$g/mL), the wild-type glycerol kinase is present as the dimer (*31*, *32*). There are two binding sites for IIA$^{Glc}$ per dimer of glycerol kinase, hence two binding sites for Zn(II) per molecule of IIA$^{Glc}$·glycerol kinase complex. If the binding sites for each of the ligands were not identical and independent, the complete description of the binding equilibria would require a model more complex than that shown in Scheme 1, with second-order terms and cross terms in the concentrations of each of the ligands. However, IIA$^{Glc}$ inhibition of glycerol kinase and the effect of Zn(II) on the inhibition are well described in terms of Scheme 1, which assumes that the binding sites are identical and independent. For the IIA$^{Glc}$ binding sites, this result is consistent with the crystal structure of the complex with glycerol kinase, which shows there are no direct interactions between the IIA$^{Glc}$ molecules that are bound to each subunit of glycerol kinase.

The Zn(II) contents of one preparation each of purified wild-type glycerol kinase and IIA$^{Glc}$ were determined as described in the Experimental Procedures. The concentrations of the proteins after dialysis were determined to be glycerol kinase, 2.3 mg/mL (41 $\mu$M subunits); IIA$^{Glc}$, 3.4 mg/mL (190 $\mu$M). The ratio of Zn(II) to protein (mol/mol) was 0.04 for glycerol kinase and 0.001 for IIA$^{Glc}$. Thus, neither protein alone binds Zn(II) tightly, and only added Zn(II) contributes to the cation effects reported here.

*Consequences of Structure Changes in the Zn(II)-Liganding Residue of Glycerol Kinase for the Effect of Zn(II) on IIA$^{Glc}$ Inhibition.* Site-directed mutagenesis was used to verify that the effects of Zn(II) on IIA$^{Glc}$ inhibition are due to its binding at the intermolecular site seen in the crystal structure and to determine whether amino acid side chains that are known to be Zn(II) ligands in other proteins might substitute for glutamate 478. The mutant glycerol kinases E478C, E478D, E478H, and E478Q were constructed and purified as described in the Experimental Procedures. The effects of the substitutions on cation-promoted association of the glycerol kinase·IIA$^{Glc}$ complex were evaluated by

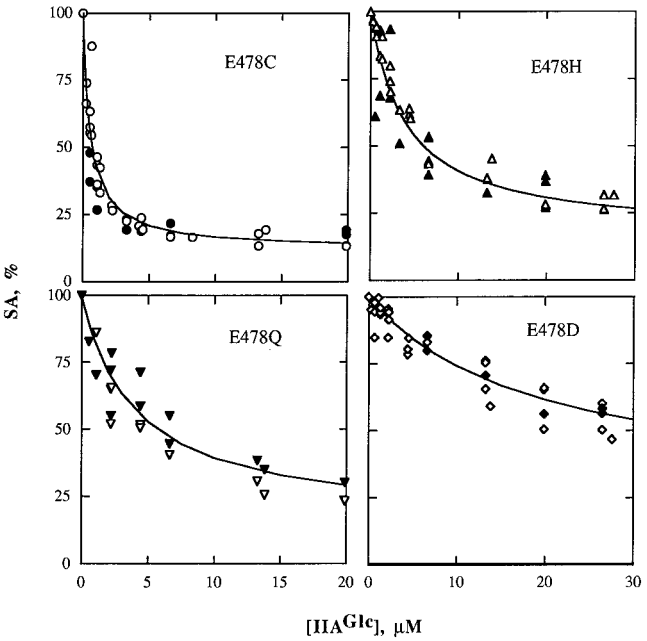


FIGURE 3: Effect of Zn(II) on $IIA^{Glc}$ inhibition of glycerol kinase E478 mutants. The specific activities of the mutant glycerol kinases were determined as described in the legend to Figure 1. Open symbols indicate assays performed without added Zn(II) while filled symbols indicate assays performed with 100 $\mu$M $ZnCl_2$ added. The points show the specific activity expressed as a percentage relative to the specific activity at 0 $IIA^{Glc}$, which is decreased by the addition of Zn(II). The lines drawn through the data points were calculated using the parameters obtained by fitting data obtained both in the presence and absence of Zn(II) to eq 4 simultaneously.

determining the effect of Zn(II) on the $IIA^{Glc}$ inhibition. Figure 3 shows titrations of the activity of the E478 mutant glycerol kinases with $IIA^{Glc}$ in the absence of added Zn(II) (open symbols) and with 100 $\mu$M Zn(II) added (filled symbols). Examination of the results appears to indicate that the titration curves are not altered by the addition of 100 $\mu$M Zn(II). This is confirmed by analysis of the titration curves, the results of which are summarized in Table 3 and show that, in contrast to the wild-type enzyme, $K_{app}$ is not changed significantly by 100 $\mu$M Zn(II) for any of the mutant enzymes. This is consistent with greatly reduced apparent affinity for Zn(II) binding to the complex and with binding of Zn(II) at the intermolecular site as the basis for its effect on $IIA^{Glc}$ inhibition, i.e., cation-promoted association. Comparison of the values for the apparent dissociation constant in the absence of Zn(II) relative to the wild-type glycerol kinase shows that the E478C substitution increases the affinity 10−15-fold while the other substitutions do not significantly alter it. None of the substitutions has a significant effect on the maximum extent of inhibition.

*Zn(II) Binding to Complexes of $IIA^{Glc}$ with the E478C and E478H Mutant Glycerol Kinases.* The structures of the glycerol kinase·$IIA^{Glc}$ complex were determined by X-ray diffraction methods for the E478C and E478H mutant enzymes as described under Experimental Procedures. Figure 4a shows the final refined model for the E478C complex superimposed on an ($F_o - F_c$) difference electron density map in the vicinity of the intermolecular Zn(II) site. The sulfur atom of cysteine 478 was removed for the purpose of the phase calculation, and the contour level set at $+3\sigma$. The contour cage shows the electron density expected for a fully occupied sulfur atom at the position of the substitution, and, for comparison, a comparable or higher level density would be expected for a bound Zn(II) ion. No trace for bound Zn(II) is observable at the binding site (*10*), and the side chain of cysteine 478 appears to be too far from the two histidines of $IIA^{Glc}$ to form a ligand for bound metal. The minor density peaks at the glycerol kinase-$IIA^{Glc}$ interface may be due to small coordinate errors. The sulfur atom of cysteine 478 does not appear to be oxidized even though reducing agent was not added to the crystal setups. This experiment clearly shows that the substitution E478C has not altered the structure of glycerol kinase or the ability of the regulatory protein $IIA^{Glc}$ to bind to glycerol kinase; however, the complex no longer binds Zn(II) even at a concentration of 2 mM Zn(II) added to the crystallization buffer.

Table 3: Parameters for Effect of Zn(II) on Binding of $IIA^{Glc}$ To E478 Mutants of Glycerol Kinase[a]

| enzyme | −Zn(II) | +100 $\mu$M Zn(II) | combined |
|---|---|---|---|
| E478C | | | |
| $K_{app}$ ($\mu$M) | 0.7 (0.5, 0.9) | 0.4 (0.3, 0.5) | 0.6 (0.4, 0.7) |
| maximum inhibition | 89 (85, 95) | 85 (91, 90) | 88 (84, 92) |
| E478D | | | |
| $K_{app}$ ($\mu$M) | 17 (8, 45) | 30 (15, 85) | 19 (9, 47) |
| maximum inhibition | 71 (52, 134) | 94 (64, 197) | 75 (55, 172) |
| E478H | | | |
| $K_{app}$ ($\mu$M) | 5 (4,7) | 3 (2,5) | 4 (3,6) |
| maximum inhibition | 86 (80, 93) | 79 (71, 90) | 83 (76, 95) |
| E478Q | | | |
| $K_{app}$ ($\mu$M) | 3 (2, 4) | 4 (2,6) | 3 (2, 5) |
| maximum inhibition | 89 (83, 96) | 81 (69, 97) | 85 (76, 95) |
| wt | | | |
| $K_{app}$ ($\mu$M) | 9 (6, 14) | 0.6 (0.4, 0.9) | |
| maximum inhibition | 83 (71, 103) | 91 (83, 101) | |

[a] The parameters for the mutant glycerol kinases were estimated by fitting the data in Figure 3 to eq 4. The columns that are labeled −Zn(II) and +100 $\mu$M Zn(II) were obtained from separate fits of the data in the absence or presence of Zn(II), respectively, while the column labeled combined was obtained by fitting the data in the absence and presence of Zn(II) simultaneously. For the wild-type (wt) enzyme, the parameters are from fits to the 0 Zn(II) and 100 $\mu$M Zn(II) data in Figure 1. The parameters are shown as the best-fit value with the 65% confidence limits shown in parenthesis.

Figure 4b is similar to Figure 4a and shows the vicinity of the intermolecular Zn(II) site for the complex with the E478H mutant glycerol kinase. The histidine 478 side chain was removed for phase calculation and the contour level for the ($F_o - F_c$) map set to $+3\sigma$. Zn(II) was not present in this experiment, so difference features at the metal site are not expected, but the map does reveal that, as with the E478C substitution, the structure of neither glycerol kinase nor the complex is perturbed by the substitution. Difference electron density maps calculated as ($F_{o,Zn} - F_o$) with partial data sets (typically 50% complete) obtained in the presence of 2 mM Zn(II) were noisy, but no large features could be seen at the position of the Zn(II) (data not shown). It was not determined why these crystals became radiation sensitive. Thus, despite its presence at a concentration of 2 mM, Zn(II) does not occupy the intermolecular cation binding site in either structure, showing that the E478C and E478H substitutions of the glycerol kinase Zn(II)-liganding residue greatly diminish the binding of Zn(II) to the complex.

*Consequences of Changes in the Zn(II)-Liganding Residue of Glycerol Kinase for Other Properties of the Enzyme.* The catalytic properties of the mutant glycerol kinases were

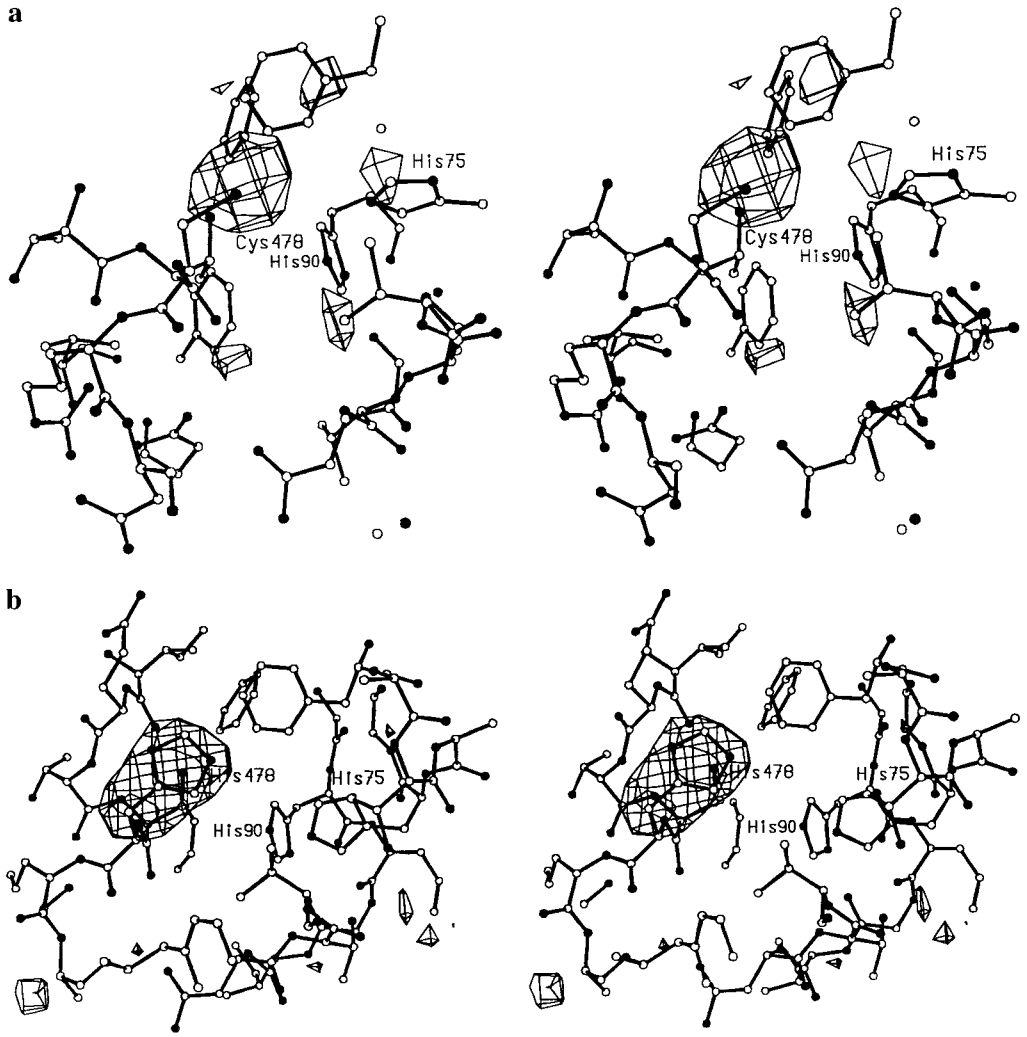


FIGURE 4: Structures in the vicinity of the Zn(II) binding site in the complex of E478C and E478H mutant glycerol kinases with IIA$^{Glc}$. (a) Stereoview of a portion of the final refined model of the glycerol kinase-IIA$^{Glc}$ complex for the E478C glycerol kinase superimposed on an ($F_o - F_c$) difference electron density map contoured at $+3\sigma$. Zn(II) is present at a concentration of 2 mM in these crystals. The diagram is centered on the approximate location of the intermolecular Zn(II) binding site. The side chain of cysteine 478 was modeled as alanine in the phase calculation. The level of difference electron density shows what would be expected (and is seen) for a fully occupied sulfur, and comparable or higher density would be expected for bound Zn(II). (b) As in panel a, but showing the result for the E478H mutant glycerol kinase. The side chain of histidine 478 was modeled as glycine for the phase calculation to show the expected level of density for bound Zn(II).

determined to assess the effects of the substitutions. The dependence of the initial velocity of each of the mutant enzymes on ATP concentration gives the biphasic double-reciprocal plot seen for the wild-type enzyme (*20*) (not shown). Thus, none of these substitutions alters qualititatively the apparent ATP activation of the enzyme. The initial velocity data were analyzed as described in the Experimental Procedures, and the initial velocity parameters obtained for the mutant enzymes are compared to those of the wild-type enzyme in Table 4. The results shown for the wild-type enzyme are in good agreement with an earlier report (*20*). Each of the substitutions decreases the $V_{max}$. The smallest effect on $V_{max}$ is seen for the E478D substitution, which conserves the charge of the amino acid side chain. The substitutions have small effects on substrate binding, as shown by the decreased values for $K_{iatp}$. The activity of glycerol kinase is also regulated by FBP (*12*, *32*). The effects of the substitutions on FBP regulation were determined to assess possible global effects on the regulatory properties. Titrations of the activity of the wild-type and mutant glycerol kinases with FBP performed at the same enzyme concentrations (not shown) show no significant differences. This is consistent with the absence of global regulatory effects of the substitutions. These results show that, except for binding of Zn(II) at the intermolecular site, aspartate, cysteine, glutamine, or histidine can be substituted for the glutamate residue at position 478 in glycerol kinase with little or no effect on its functional properties.

Table 4: Initial Velocity Parameters for Glycerol Kinase Mutants[a]

| enzyme | $V_{max}$ (units/mg) | $K_{atp}$ ($\mu$M) | $K_{gol}$ ($\mu$M) | $K_{iatp}$ ($\mu$M) |
|---|---|---|---|---|
| E478C | 5 (4.7, 5.4) | 3 (2, 4) | 5 (4, 6) | 32 (17, 46) |
| E478D | 11 (8, 13) | 7 (4, 11) | 11 (6, 16) | 12 (4, 20) |
| E478H | 6 (5, 7) | 4 (2, 5) | 5 (3, 7) | 12 (4, 18) |
| E478Q | 8 (7, 9) | 5 (2, 7) | 5 (2, 9) | 25 (6, 43) |
| wt | 15.3 (14.7, 16) | 9 (8, 11) | 5 (3, 7) | 54 (31, 76) |

[a] Kinetic parameters were estimated from fitting initial velocity data as described in the Experimental Procedures. The parameters are shown as the best-fit value with the 65% confidence limits shown in parenthesis.

## DISCUSSION

The results of this study strongly support the hypothesis that the action of Zn(II) on $IIA^{Glc}$ inhibition of glycerol kinase is due to cation-promoted association. The effect of Zn(II) on $IIA^{Glc}$ inhibition of the wild-type glycerol kinase can be described quantitatively by a model in which Zn(II) binds to the protein−protein complex. The effect of Zn(II) on $IIA^{Glc}$ inhibition of glycerol kinase and binding of Zn(II) to the $IIA^{Glc}$•glycerol kinase complex are greatly decreased by substitutions of the glycerol kinase Zn(II)-liganding amino acid, E478. Thus, substitutions of the Zn(II)-liganding amino acid residues in either glycerol kinase or $IIA^{Glc}$ greatly reduce the effect of Zn(II) on $IIA^{Glc}$ inhibition of glycerol kinase. This result is consistent with the crystal structure of the ternary complex of the two proteins with Zn(II) (*10*).

In the construction of the E478 substitutions, the cysteine, aspartate, and histidine amino acid residues were chosen because they are known to be Zn(II) ligands in other proteins. Glutamine was chosen to provide an isosteric substitution with an amino acid residue that does not generally function as a Zn(II) ligand. The loss of Zn(II) enhancement of $IIA^{Glc}$ inhibition of the E478Q mutant glycerol kinase is consistent with the results obtained for the H75Q mutant of $IIA^{Glc}$ and is the result expected for decreased affinity for Zn(II) binding due to loss of a good Zn(II) ligand at the intermolecular site. The substitutions E478C, E478D, and E478H result in the same loss of Zn(II) enhancement of $IIA^{Glc}$ inhibition as the E478Q substitution, suggesting that they also decrease the affinity for Zn(II) binding to the intermolecular site. The decreased affinity for Zn(II) binding to the complexes with the E478C and E478H mutant glycerol kinases is verified by the crystallographic experiments which indicate that the dissociation constant for Zn(II) binding to the intermolecular site is much greater than 1 mM since no bound Zn(II) is detectable with 2 mM Zn(II) added to the crystals. These substitutions thus decrease the affinity for Zn(II) by more than 3 orders of magnitude, even though aspartate, cysteine, and histidine are good Zn(II) ligands in other proteins. Studies of an artificial Zn(II) site in carbonic anhydrase II have identified several factors that are important for high-affinity binding of Zn(II) (*33*). The primary factors are distance between the liganding atoms and the Zn(II) ion, the coordination geometry, and favored rotamer conformation of the amino acid side chain. The peptide backbone must be sufficiently flexible to allow the movement required for establishing the optimal metal−ligand distance, and there must be sufficient space to allow the preferred rotamer conformation. The inability of aspartate, cysteine, or histidine to substitute for glutamate in the intermolecular Zn(II) site in the $IIA^{Glc}$•glycerol kinase complex indicates the lack of the required flexibility. This suggests that the ability of the residue at position 478 to function as a Zn(II) ligand is dependent on the other amino acids in the binding site. In $IIA^{Glc}$ binding sites on other regulatory targets, a different Zn(II)-liganding residue could be associated with a different primary structure for the remainder of the site to optimize Zn(II) coordination. Cation-promoted association may thus provide an explanation for the apparent lack of a consensus $IIA^{Glc}$ binding sequence among the target proteins.

Although none of the four amino acid residues that were examined can replace glutamate as a Zn(II) ligand, each effectively replaces glutamate in other functions of glycerol kinase. That is, the catalytic properties and other regulatory properties, including $IIA^{Glc}$ inhibition in the absence of Zn(II), differ little from those of the wild-type enzyme. In the absence of Zn(II), the structures of the $IIA^{Glc}$ complexes of the E478C and E478H glycerol kinases differ from the wild-type only at the site of the substitution. Thus, the only apparent advantage to having glutamate at position 478 relative to the other amino acids is binding of Zn(II), unless the glutamate residue is essential for other, unknown, interactions or functions of glycerol kinase. The E478C substitution does increase the affinity for $IIA^{Glc}$, such that the affinity for $IIA^{Glc}$ binding to this mutant enzyme is the same as for the wild-type enzyme with Zn(II). The fact that glutamate, rather than cysteine, has been selected by evolution for position 478 in glycerol kinase suggests that the ability to bind Zn(II) rather than the affinity for $IIA^{Glc}$ binding in the absence of Zn(II) is important and infers that cation-promoted association may be an important aspect of this signal transduction pathway in vivo.

The apparent high affinity for Zn(II) binding to the wild-type glycerol kinase•$IIA^{Glc}$ complex is also consistent with a possible physiological role for cation-promoted association. The total and free intracellular concentrations of Zn(II) are important factors for regulation via cation-promoted association. Possible scenarios in which changes in Zn(II) concentration in response to cellular signals control the activities of subclasses of transcription factors have been considered (*34*). However, there is surprisingly little known about these concentrations or mechanisms of uptake and Zn(II) homeostasis in living organisms, including *E. coli* (*35*, *36*). The high affinity of the intermolecular site for Zn(II) binding to the complex, indicated by the dissociation constant of less than 1 $\mu$M, shows that low concentrations of free Zn(II) are sufficient to enhance complex formation, thus decreasing the concentration of the dephospho form of $IIA^{Glc}$ that is required for efficient inducer exclusion and freeing $IIA^{Glc}$ to serve in its other functions. The affinity for Zn(II) binding to $IIA^{Glc}$ is low, as shown by the absence of Zn(II) in atomic absorption spectroscopic measurements on the dialyzed preparation of $IIA^{Glc}$ and the 30-fold lower affinity for Zn(II) binding to $IIA^{Glc}$ relative to the $IIA^{Glc}$•glycerol kinase complex. The difference in affinity ensures that concentrations of free Zn(II) sufficient to enhance complex formation will not interfere with the PTS function of $IIA^{Glc}$. The ability of Zn(II) to enhance effective allosteric control without interfering with other roles may be an important aspect of cation-promoted association and may reflect one solution to the evolutionary problem of multiple physiological functions for the same site on a protein.

The difference in Zn(II) affinities of the complex and the individual components raises interesting questions about the mechanisms of Zn(II) binding and complex formation. In crystallographic experiments, Zn(II) is added to the cocrystal of the complex and is able to bind at the intermolecular site. This binding apparently occurs without dissociation of the complex since it is observed in the crystal and, for the wild-type enzyme, does not disrupt the crystal. The Zn(II) at the intermolecular site is not accessible to the solvent (*10*), which may be one reason that its affinity for binding is $10^6$-fold less than that for binding of Zn(II) at the structurally identical site in thermolysin which is solvent accessible (*37*). Ad-

ditional studies using the wild-type and specifically substituted mutant proteins should provide information about mechanisms of Zn(II) binding.

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