Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 3544–3548, April 1994
Biophysics

Cation-promoted association of a regulatory and target protein is controlled by protein phosphorylation

(protein crystal structure/x-ray crystallography/signal transduction/protein–protein interaction)

MICHAEL FEESE*†, DONALD W. PETTIGREW†, NORMAN D. MEADOW§, SAUL ROSEMAN§, AND S. JAMES REMINGTON*†¶

*Institute of Molecular Biology and †Departments of Physics and Chemistry, University of Oregon, Eugene, OR 97403; ‡Department of Biochemistry and Biophysics, Texas A&M University, College Station, TX 77843; and §Department of Biology and McCollum–Pratt Institute, The Johns Hopkins University, Baltimore, MD 21218

Contributed by Saul Roseman, November 12, 1993

ABSTRACT **A central question in molecular biology concerns the means by which a regulatory protein recognizes different targets. III^{Glc}, the glucose-specific phosphocarrier protein of the bacterial phosphotransferase system, is also the central regulatory element of the PTS. Binding of unphosphorylated III^{Glc} inhibits several non-PTS proteins, but there is little or no sequence similarity between III^{Glc} binding sites on different target proteins. The crystal structure of *Escherichia coli* III^{Glc} bound to one of its regulatory targets, glycerol kinase, has been refined at 2.6-Å resolution in the presence of products, adenosine diphosphate and glycerol 3-phosphate. Structural and kinetic analyses show that the complex of III^{Glc} with glycerol kinase creates an intermolecular Zn(II) binding site with ligation identical to that of the zinc peptidase thermolysin. The zinc is coordinated by the two active-site histidines of III^{Glc}, a glutamate of glycerol kinase, and a water molecule. Zn(II) at 0.01 and 0.1 mM decreases the K_i of III^{Glc} for glycerol kinase by factors of about 15 and 60, respectively. The phosphorylation of one of the histidines of III^{Glc}, in its alternative role as phosphocarrier, provides an elegant means of controlling the cation-enhanced protein–protein regulatory interaction. The need for the target protein to supply only one metal ligand may account for the lack of sequence similarity among the regulatory targets of III^{Glc}.**

In enteric bacteria, the phospho*enol*pyruvate:glycose phosphotransferase system (PTS) catalyzes the uptake and phosphorylation of its sugar substrates. In addition, the PTS regulates adenylate cyclase, glycerol kinase (GK), and a number of non-PTS permeases (for reviews, see refs. 1–4). The central regulatory protein of the PTS is III^{Glc}, the product of the *crr* gene. There is now substantial evidence that III^{Glc} and/or phospho-III^{Glc} interacts with at least 11 target proteins. Some of these interactions are covalent, involving phosphate transfer to other PTS proteins, whereas interactions with non-PTS target proteins appear to be via protein–protein binding. It has been proposed that III^{Glc} (18.1 kDa) binds to and inhibits its non-PTS target proteins, whereas phospho-III^{Glc} does not or may even be a positive effector.

Maltose uptake is inhibited by binding of III^{Glc} to the product of the *malK* gene (MalK) and mutants of MalK insensitive to III^{Glc} regulation have been isolated and sequenced, potentially identifying a III^{Glc} binding site (5). Similar mutants of the melibiose carrier (MelB) have been identified, and the III^{Glc} binding region was postulated to be an α-helix (6). In those studies, two different regions of sequence similarity between MalK and MelB were found and postulated to be the III^{Glc} binding site. In another report, a region of GK (residues 370–385) was aligned with marginally similar regions of MalK, MelB, and LacY (7).

We have described the crystal structure of the complex of III^{Glc} with glycerol kinase (monomer size, 56 kDa) at 2.6-Å resolution (8). The III^{Glc} binding site is on one face of a 3_{10}-helix formed by residues 472–481 in GK, located >30 Å from the catalytic site; inhibition of the enzyme results from long-range conformational changes in GK (8). There is no amino acid sequence similarity between the III^{Glc} binding site on GK and any of the postulated III^{Glc} binding sites on other target proteins or, indeed, any region of the other target proteins. Thus, the question arises as to whether there is a primary structural motif by which III^{Glc} recognizes its diverse targets.

In the present report, we show that the binding of III^{Glc} to GK generates a putative intermolecular zinc coordination site. Zn(II) not only specifically binds at this site but substantially increases the apparent affinity of the two proteins and the inhibitory potency of III^{Glc} for GK. These results have significant implications for the mechanisms of regulation of multiple proteins by III^{Glc}.

MATERIALS AND METHODS

Crystals of the III^{Glc}/GK complex were prepared by hanging-drop vapor diffusion as described (8) and transferred to storage buffer containing 20 mM cation chloride, 20 mM ADP, 50 mM glycerol 3-phosphate (G3P), and 0.6 M sodium acetate in 100 mM Mes buffer, pH 6.1. Normally, all solutions were prepared in ultrafiltered water (Nanopure II; Barnstead), but the $MgCl_2$ solution described below was inadvertently prepared with reverse-osmosis water (15-fold higher conductivity than the ultrafiltered water).

Diffraction data using graphite-monochromated CuK_α radiation were collected on a San Diego Multiwire Systems area detector and reduced by the supplied software. Crystallographic refinement was performed with the TNT package of programs (9), and electron density maps were inspected with FRODO (10).

A site-directed mutant of III^{Glc}, H75Q ($His^{75} \rightarrow Gln$), was prepared as reported (11). GK assays were conducted (12) with the homogeneous enzyme in the presence or absence of III^{Glc} and Zn(II) as indicated. The effect of Zn(II) on the coupling enzyme system was investigated. ZnADP is a substrate for pyruvate kinase, but the efficacy of the coupling system is not affected by the Zn(II) concentrations used in these experiments. The specific activity of GK is constant over the range of enzyme concentrations (0.5–2.5 μg/ml) used with 0.1 mM $ZnCl_2$ added to the assay mixture.

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Abbreviations: GK, glycerol kinase; PTS, phosphotransferase system; G3P, glycerol 3-phosphate.

¶To whom reprint requests should be sent at the * address.

RESULTS

Formation of Zn(II)/III^{Glc}/GK Complex. To more fully characterize the active site of GK, we investigated the binding of Mn(II) and Mg(II) to the enzyme by soaking crystals in 20 mM solutions of the metal chloride and comparing electron density maps in the presence and absence of the metals. Crystallographic data collection statistics are summarized in Table 1. The space group is *I*222 with a = 124.0 Å, b = 125.1 Å, and c = 133.3 Å. After crystallographic refinement of the models (Table 1), the crystallographic R factors and geometric parameters were satisfactory. The density maps revealed positive features between the β-phosphate of ADP and the 3-phosphate of G3P that could be attributed to the added metal. These results and their relevance to the enzymatic mechanism will be discussed elsewhere.

During refinement of the atomic model against the Mg(II) data set, a large (11σ) positive peak was apparent in an $F_o - F_c$ difference electron density map at the primary interface between III^{Glc} and GK (Fig. 1 *Upper*). Local structural changes involving a slight collapse of the two proteins about this site and the size of the density feature suggested that the two proteins had bound a contaminating metal ion considerably more electron dense than Mg(II). No such feature was observed in the Mn(II) difference map, indicating that Mn(II) does not bind at this site at 20 mM concentration. The ligands that contact the ion are His^{75} and His^{90} of III^{Glc} and Glu^{478} of GK, which rearranges upon binding Zn(II) (Fig. 1 *Lower*). In addition, a water molecule, which accepts three bonds (two hydrogen bonds from the backbone amides of residues 95 and 96 of III^{Glc}) and thus probably a hydroxide, associates with the ion such that the coordination is distorted tetrahedral. This is precisely the coordination seen for the catalytic Zn(II) in the metalloproteinase thermolysin (13, 14).

In a second experiment, data were collected after adding 5 mM Zn(II) as the only divalent cation to the crystals (Table 1), and a 22σ peak in a difference electron density map confirmed that Zn(II) binds to the intermolecular site. A 7σ peak appeared at the location where Mg(II) and Mn(II) bind, indicating that Zn(II) binds at the GK catalytic site with lower occupancy as well. In the initial experiment described above, the Zn(II) at the intermolecular site appears to have been present at about half occupancy and was apparently a contaminant in the reverse-osmosis water used to prepare the $MgCl_2$ solution.

Comparison of Zn(II) Binding Sites: Thermolysin and the III^{Glc}/GK Complex. The intermolecular regulatory Zn(II) site in the III^{Glc}/GK complex is compared with the Zn(II) binding site of thermolysin in Fig. 2. In each case, three protein atoms coordinate the zinc: the imidazole N-3 atoms of two histidine residues and a glutamate carboxyl oxygen atom. The three atoms of the III^{Glc}/GK complex superimpose over the corresponding thermolysin atoms to rms error of 0.19 Å, within the estimated coordinate errors of the models (≈0.2 Å). In addition, the presumed hydroxide ion is near the position of the presumed catalytic water of thermolysin (14), although the zinc atom is not otherwise solvent-accessible.

Table 1. Diffraction data and atomic-model statistics for $MgCl_2$, $ZnCl_2$, and $MnCl_2$ data sets

	$MgCl_2$	$ZnCl_2$	$MnCl_2$
No. of crystals	1	1	1
Resolution, Å	2.7	2.8	2.7
No. of reflections	54,181	54,323	54,366
Unique observations	26,670	18,829	14,984
Completeness*	0.88	0.73	0.65
R_{merge}†	0.036	0.054	0.042
R factor‡	0.177	0.166	0.154
rms deviations			
Bonds, Å	0.020	0.022	0.022
Angles, degrees	2.77	2.78	2.58
Planarity, Å	0.034	0.035	0.032
Restrained B factors, Å^2	2.9	3.0	2.9

*Ratio of observed to theoretically possible reflections.
†$R_{merge} = \Sigma|I_{obs} - I_{ave}|/\Sigma|I_{obs}|$, where I_{ave} is the average value of multiple measurements I_{obs}.
‡Standard crystallographic R factor = $\Sigma||F_o| - |F_c||/\Sigma|F_o|$.

Vallee and Galdes (15) grouped zinc binding sites in proteins into three general classes—structural, regulatory, and catalytic—with catalytic sites having distorted coordination. They, and Vallee and Williams (16, 17), have suggested that this distorted coordination is a key feature of catalysis by zinc-dependent enzymes via transition-state stabilization. The present finding of a regulatory Zn(II) site with a ligand configuration essentially identical to that of a zinc peptidase may require reevaluation of this distinction.

Effect of Zn(II) on Inhibition of GK by III^{Glc}. Although the III^{Glc}/GK complex forms a Zn(II) binding site that is identical to that in thermolysin, does zinc have any physiological effect? As a first approach in answering this question, the activity of GK was studied in the presence and absence of various concentrations of III^{Glc} and/or Zn(II) (Figs. 3 and 4). Purified GK, III^{Glc}, and coupling enzymes were first dialyzed exhaustively versus 0.1 M triethanolamine/HCl (pH 7.0). It is apparent that Zn(II) amplifies the inhibitory effect of III^{Glc} on the enzyme. The decrease of activity at 0 mM III^{Glc} in the presence of Zn(II) results from the fact that ZnATP is a substrate of GK; it binds to GK more tightly than MgATP but supports a lower rate of catalysis (data not shown). Quantitative analysis of the kinetic data is given in Table 2.

The inhibition of GK by III^{Glc} in the absence of Zn(II) agrees well with earlier reports (18). In the presence of 0.01 mM Zn(II), the K_i for III^{Glc} decreases by a factor of 15, and in the presence of 0.1 mM Zn(II), the K_i decreases by a factor of about 59. Thus, Zn(II) greatly enhances the inhibitory potency of III^{Glc}, and we attribute this to a large increase in the affinity of the two proteins for each other in the presence of Zn(II).‖ The binding constants of the three components in the ternary complex remain to be determined. From the available data, we estimate the dissociation constant of Zn(II) from the GK/III^{Glc} complex to be about 10^{-5} M.

The crystal structure suggests that both imidazole N-3 atoms are required coordination sites for Zn(II). This is demonstrated by the site-directed mutant III^{Glc} H75Q. This mutant III^{Glc} is as effective as the wild-type protein in inhibiting GK, but Zn(II) has no effect on this inhibition (Fig. 4*B*).

Specificity of the Zn(II) Effect. Under the same assay conditions used for Zn(II), none of the metals Mn(II), Co(II), Cu(II), or Cd(II), added as the chloride to 0.1 mM concentration, significantly increases inhibition of GK by III^{Glc} at 0.027 mg/ml. Thus, the affinity of the regulatory site for these metals is much less than that for Zn(II).

Induced Fit. It is important to emphasize that formation of the ternary complex Zn(II)/III^{Glc}/GK is associated with conformational changes or "induced fit." In the absence of Zn(II), III^{Glc} induces the formation of its binding site in GK. Residues 472–481 are disordered in GK alone (H. R. Faber and S.J.R., unpublished results) but form a 3_{10}-helix when GK binds to

‖Preliminary fluorescence equilibrium measurements (V. Ladokhina and N.D.M., unpublished results) also suggest that Zn(II) increases the affinity of the two proteins. A preparation of the F3Y mutant of III^{Glc} (10) in which about 30% of the protein contained 5-hydroxytryptophan at residue 3, was prepared as described by Ross *et al.* (19), who showed that the fluorescence of 5-hydroxytryptophan can be quantitated without interference by a large excess of tryptophan. The fluorescence anisotropy of the 5-hydroxytryptophanyl III^{Glc} was found to increase on titration with a molar equivalent of GK. The presence of 0.1 mM Zn(II) resulted in a much larger increase of anisotropy upon titration, whereas an excess of EDTA abolished the effect of Zn(II). Mn(II) had no effect on the anisotropy of 5-hydroxytryptophanyl III^{Glc} when used in parallel experiments.

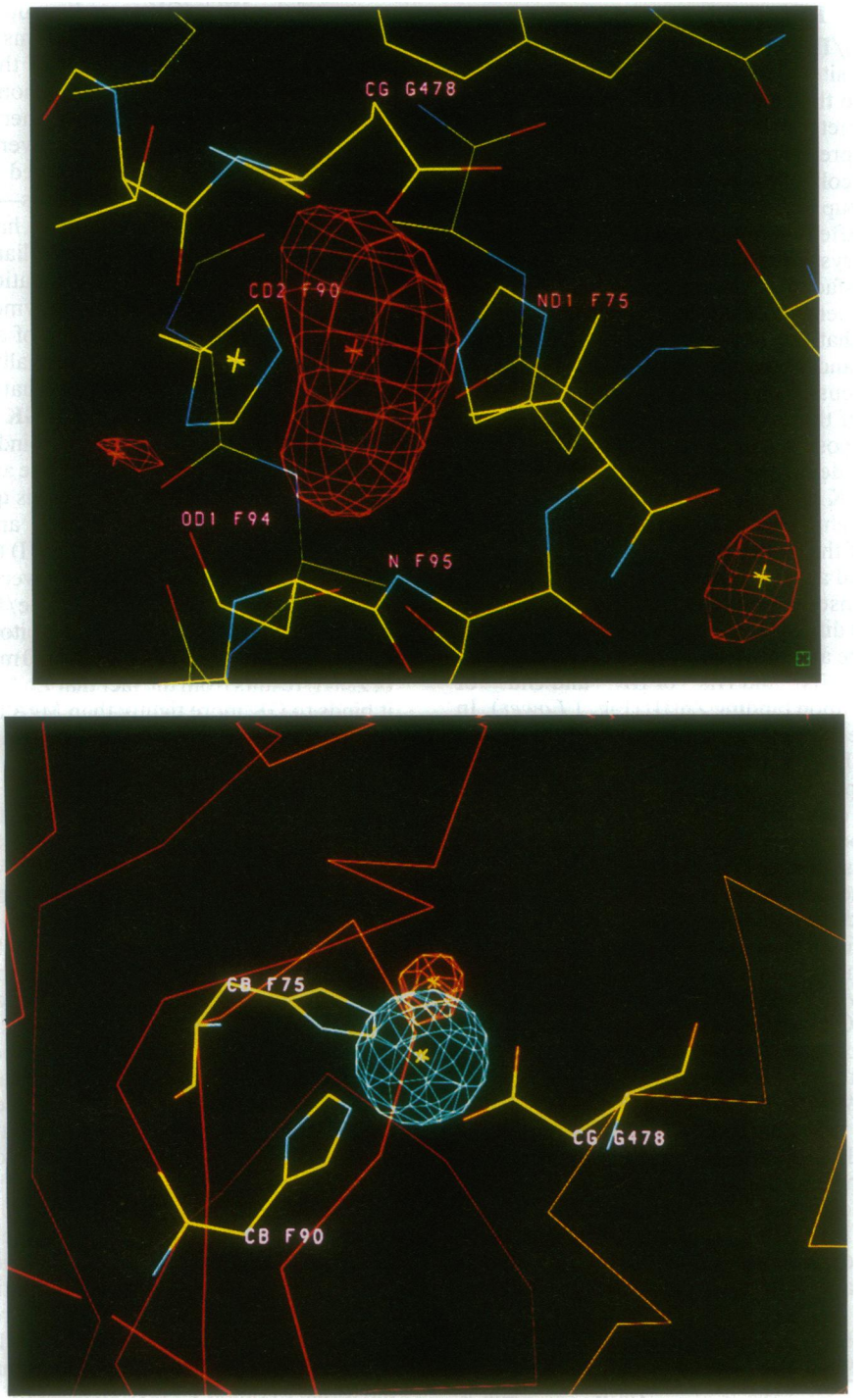

FIG. 1. (*Upper*) The 2.7-Å $F_{o(Mg)} - F_c$ difference electron density map, contoured at $+4\sigma$ calculated with phases derived from the initial III^{Glc}/GK model with no included metal ions. The elongated feature is a partially occupied Zn(II) plus solvent bound to side chains 75 and 90 of III^{Glc} and Glu^{478} of GK. (*Lower*) Final 2.7-Å $F_{o(Zn)} - F_{calc}$ difference electron density map contoured at $+10\sigma$ after removal of Zn(II) (blue) or H_2O (red) from the refined model before calculation of phases.

III^{Glc}. There is virtually no change in the structure of III^{Glc} upon binding. In a second induced fit, the carboxyl of Glu^{478} of GK changes orientation to serve as a ligand for Zn(II). It seems possible that the entropic cost associated with the (re)ordering of these structures upon binding is an important feature of the regulatory interaction, serving to tune the binding affinity and modulate regulatory levels.

DISCUSSION

The decrease in the K_i for III^{Glc} inhibition of GK that results from intermolecular Zn(II) binding has important implications for the regulatory behavior of III^{Glc} *in vivo*. According to our measurements, the *E. coli in vivo* concentrations of III^{Glc} plus phospho-III^{Glc} range from 0.08 mg/ml in exponential-phase cells grown on glycerol to 0.25 mg/ml when the cells are grown on glucose. At these concentrations, especially at 0.08 mg/ml, Fig. 4*A* shows that inhibition of GK *in vitro* is only about 50%. However, *in vivo* there is nearly complete inhibition of the enzyme when methyl α-D-glucoside is added to whole cells growing on glycerol (20). Fig. 4*A* provides an explanation for the apparent discrepancy. In the presence of Zn(II), the inhibition of GK *in vitro*

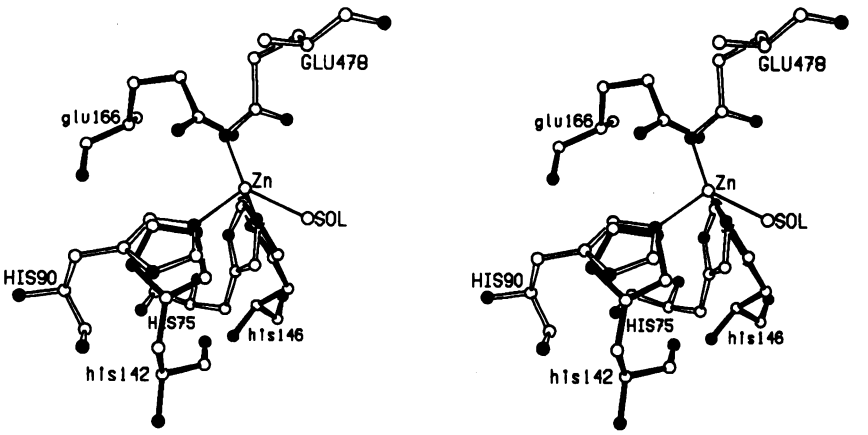

FIG. 2. Overlay of Zn(II) binding sites in thermolysin and the III^{Glc}/GK complex. Shown is a stereo drawing of a least-squares superposition of the active-site Zn(II) ligands of thermolysin (filled bonds) with those of the III^{Glc}/GK complex (open bonds). The zinc atom of only the complex is shown. Thin lines indicate bonds to ligating atoms, including a presumed hydroxide molecule labeled SOL.

increases to about 90% with III^{Glc} at 0.08 mg/ml. Similar discrepancies between *in vivo* and *in vitro* regulation have been noted for LacY and MelB (6, 21, 22) as well. Thus, the large effect of intermolecular Zn(II) on III^{Glc} regulation of GK suggests a physiological role for Zn(II) in the regulatory interactions of III^{Glc} with other target proteins.

We have previously proposed that phosphorylation of His^{90} directly prevents the binding of III^{Glc} to GK by steric interference and unfavorable electrostatic interactions of a charged phosphate in a predominately hydrophobic region (8). This mode of regulation of protein–protein interaction also applies to the results reported here. That is, phosphorylation of III^{Glc} at His^{90} destroys the intermolecular Zn(II) binding site, thereby providing another potentially important means of controlling the interaction between a regulatory protein and its target.

The generality of this type of protein–protein regulatory interaction remains to be established. Metal ions are not uncommon as bridging ligands between protein molecules in crystals (e.g., ref. 23), but at present there is only one other proven structural example of a biologically relevant intermolecular cation binding site. This is the recently determined structure of the complex of actin with the filament-severing and -capping protein gelsolin, which together form a Ca(II) binding site (24). There are intriguing similarities with the example discussed here. Actin and GK have very similar tertiary structures (8), but gelsolin does not bind to the same region of actin as does III^{Glc} to GK. Actin also contributes only one side chain (Glu^{167}) to the calcium binding site. Ca(II) enhances the affinity of the two proteins (25), although whether Ca(II) binding *per se* is regulated in some fashion to control the interaction of the two proteins is not clear.

We suggest that intermolecular cation binding will be common, and that the term "cation-promoted association" be used to describe the phenomenon. The advantage of metal ligation in protein–protein interactions is that a highly specific and very tight complex can be formed by a small number of

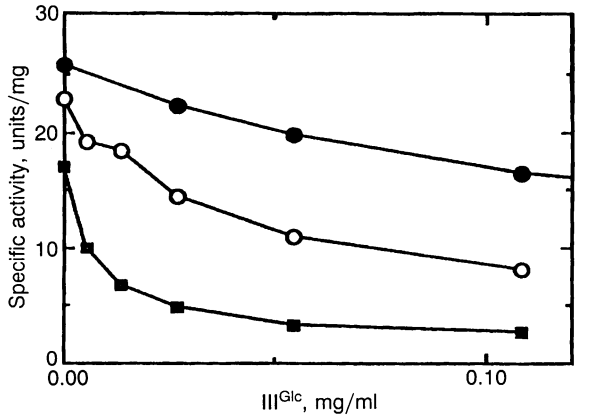

FIG. 3. Effect of Zn(II) on the inhibition of GK by III^{Glc}. The specific activity of GK at 1 μg/ml was measured by an ADP-coupled spectrophotometric assay at pH 7.0 and 25°C with 2 mM glycerol, 2.5 mM ATP, 5 mM $MgCl_2$ and additions of $ZnCl_2$ (Aldrich Chem, Metuchen, NJ) (●, 0 mM; ○, 0.01 mM; ■, 0.1 mM) and III^{Glc} as shown (12).

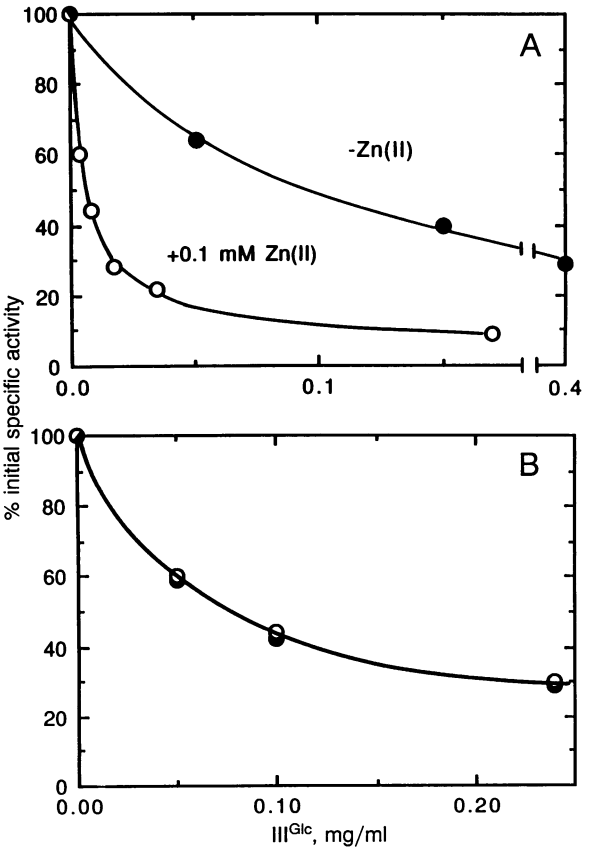

FIG. 4. Effect of Zn(II) on the inhibition of GK by wild-type III^{Glc} (*A*) and the mutant H75Q (*B*). Data are expressed as a percentage of the specific activity relative to that in the absence of III^{Glc}. Zn(II) concentrations are shown in *A*. In *B*, the symbols are reversed: ●, in the presence of 0.1 mM Zn(II); ○, absence of Zn(II).

Table 2. Effect of Zn(II) on inhibition of GK by III^{Glc}

Zn(II), mM	K_i (III^{Glc})	
	μg/ml	μM
0	300	16.6
0.01	20	1.1
0.1	5	0.28

K_i values were estimated from steady-state kinetic studies of III^{Glc} inhibition versus ATP concentration which will be described elsewhere.

appropriately aligned side chains. These in turn can be contributed by different combinations of secondary structures. Thus, a regulatory protein can specifically recognize targets that lack sequence similarity.

We thank K. Kallio for expert technical assistance and Dr. L. Brand for expert advice on fluorescence spectroscopy. This work was supported by National Institutes of Health Grants GM49992 (D.W.P.), GM42618-01A1 (S.J.R.), and 5-R37 GM38759 (S.R.), the Texas Agricultural Experiment Station Research Enhancement Program (D.W.P.), the American Cancer Society ACS 1296 (S.J.R.), and the Lucille P. Markey Charitable Trust.

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