Mutational Analyses of the Metal Ion and Substrate Binding Sites of Phosphorylase Kinase γ Subunit†

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ABSTRACT: Phosphorylase kinase (PhK) and truncated γ subunit, denoted γ_{1-300}, can phosphorylate seryl and tyrosyl residues dependent on the metal ion [Yuan, C.-J., Huang, C. F., & Graves, D. J. (1993) *J. Biol. Chem. 268*, 17683–17686]. Recombinant γ_{1-300} was used to explore its dual specificity and the location of the metal ion binding sites by using site-directed mutagenesis. Two approaches were taken to generate 26 mutants. First, on the basis of the crystal structure of cAMP-dependent protein kinase (cAPK), the invariant Asn^{155} and highly conserved Asp^{168}-Phe^{169}-Gly^{170} residues were mutated. Changes included production of N155H, D168E, D168N, F169R, G170V, G170I, G170L (less than 1% of enzymatic activities were found in these mutants), F169W, and G170A mutants. Second, charge to alanine and charge reversal scanning mutations were used to probe the metal ion binding sites. Two mutants, E111K and E154R, showed very different metal ion response compared to wild-type γ and were further characterized. The mutants F169W, G170A, E111K, and E154R had 15%, 5%, 8%, and 25% specific activity relative to wild-type γ, respectively. The folding pattern of wild-type and mutated enzyme forms of γ was determined by photoacoustic infrared spectroscopy. Conformational disruptions were found in G170V, G170I, and G170L mutants, but the conformation of the rest of the mutants was similar to that of wild-type γ, suggesting that the loss of enzymatic activities of these mutants was not because of incorrect refolding. Kinetic analyses of mutants indicate that Asn^{155} and Asp^{168} residues influence maximal velocity and that Glu^{111}, Glu^{154}, Phe^{169}, and Gly^{170} residues influence binding of MgATP and phosphorylase *b*. The interactions of divalent cations, Mg^{2+} and Mn^{2+}, with E111K, E154R, N155H, F169W, and G170A mutants were different from those with the wild-type, suggesting that Glu^{111}, Glu^{154}, Asn^{155}, Phe^{169}, and Gly^{170} contribute to the character of the metal ion binding sites. Our results suggest that the metal ion binding sites reside between the D^{168}FG loop and the E^{111}–KPE^{154}N loop, similar to the metal ion binding sites in cAPK. The tyrosine kinase activity of γ_{1-300} in the presence of Mn^{2+} was increased in E154R (251%), unchanged in E111K, F169W, and G170A, and reduced in N155H (5%). Typically, the mutations had a more pronounced effect on serine kinase activity than on tyrosine kinase activity. The activity ratio (tyrosine kinase activity to serine kinase activity) increased in the mutants, suggesting that these five residues have different roles in the two activities. Our results support the view that different conformational states induced by metal ions are important for dual specificity.

Phosphorylase kinase (PhK) catalyzes the phosphorylation and activation of glycogen phosphorylase *b*. The subunit composition in white skeletal muscle is $(\alpha,\beta,\gamma,\delta)_4$ with a molecular mass of about 1300 kDa [reviewed in Pickett-Gies and Walsh (1986) and Heilmeyer (1991)]. The γ subunit is catalytically active (Kee & Graves, 1986), and its N-terminal region shares sequence homologous with the catalytic domains of other protein kinases (Hanks et al., 1988). We have expressed PhK γ subunit (full-length and truncated forms) in *Escherichia coli* (Huang et al., 1993). A truncated form, γ_{1-300}, has several advantages over full-length γ_{1-386}, such as greater purity and greater turnover number, yet has the same K_m values as full-length γ (Huang et al., 1993). Furthermore, the truncated form has the same metal-ion dependence as the full-length γ_{1-386} (Huang et al., 1993). We show in this work that the truncated γ_{1-300} form can be used to study the role of specific residues involved in catalysis and metal-ion responses of the PhK γ subunit. In this study, all the mutant enzymes are the truncated γ_{1-300} form.

Previous studies have demonstrated that free Mg^{2+} activates γ and that free Mn^{2+}, on the contrary, inhibits its serine kinase activity (Kee & Graves, 1987; Cox & Johnson, 1992; Huang et al., 1993). The data revealed the existence of a free metal ion binding site, or second metal ion binding site, in addition to the requirement for a metal-chelated ATP, the first metal ion binding site. However, these two metal ion binding sites are not well characterized in γ. Recently, several reports have shown that some protein kinases have dual specificity [reviewed in Lindberg et al. (1992)]. PhK is among them (Yuan et al., 1993). The evidence for most dual-specificity kinases is derived from autophosphorylation studies, phosphotyrosine antibody screening, and peptide substrate phosphorylation (Lindberg et al., 1992). However, PhK and γ have the unique property that the dual specificity is modulated by metal ion.

In cAPK, there are two metal ion binding sites. Unlike the γ subunit, binding of the free metal ions, such as Mg^{2+} and Mn^{2+}, at the second metal ion binding site inhibited the kinase activity of cAPK (Armstrong et al., 1979). From X-ray crystallographic studies of the cAPK, Zheng et al. (1993a,b) have shown that the invariant residue, Asp^{184} (equivalent to Asp^{168} in γ), participates in catalysis by directly chelating

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Mg^{2+} (first metal ion binding site) of the MgATP complex and is also involved in the binding of free metal ions. This invariant Asp^{184} residue is in the conserved DFG loop of the protein kinase family (Hanks et al., 1988). Knighton et al. (1991a,b) showed that the $DLKPEN^{171}$ loop is involved in catalysis, peptide substrate and MgATP binding, and free metal ion binding. Although Zheng et al. (1993a,b) showed that the invariant Asn^{171} (equivalent to Asn^{155} in γ) interacts with the metal ion in cAPK, this residue is not responsible for the binding of the metal ion in the crystal structures of the CDK2 and its MgATP complex (De Bondt et al., 1993). There is, however, a lack of experimental data to address the function of this invariant residue. Site-directed mutagenesis was performed to investigate the role of this residue. We constructed a series of mutations in γ at the highly conserved DFG region to investigate (a) the roles of specific residues, Asp^{168}-Phe^{169}-Gly^{170}, within the catalytic domain of γ subunit and (b) whether the change of the nearby residues would alter the requirement for, or response to, divalent metal ions, particularly because we reported that the enzyme has tyrosine kinase activity in the presence of Mn^{2+} (Yuan et al., 1993). To search for the other essential negatively charged residue(s) in the large lobe of the kinase domain that might participate in chelating metal ions, we used charge to alanine and charge reversal scanning mutations.[1] Seventeen mutants were generated, and the two that had significant differences on the metal ion response were further characterized.

All the mutant proteins were purified to homogeneity, and their secondary structures were analyzed by photoacoustic infrared spectroscopy, which has been utilized recently in our laboratory to evaluate the secondary structures of proteins (Luo et al., 1994). This is the first example for the use of photoacoustic infrared spectroscopy in the analyses of mutant proteins.

MATERIALS AND METHODS

Materials. Restriction enzymes were purchased from New England Biolabs or Promega. Q-Sepharose was obtained from Pharmacia, and dithiothreitol (DTT) was from Pierce. [γ-^{32}P]-ATP and [γ-^{32}P]GTP were purchased from ICN Biomedicals. The rest of reagents were purchased from Sigma. Phosphorylase *b* was prepared from rabbit skeletal muscle as described in Fischer and Krebs (1958), and residual AMP was removed by treatment with acid-washed Norit. The peptide substrate (Lys-Arg-Lys-Gln-Ile-Ser-Val-Arg-Gly-Leu) corresponding to the amino-terminal residues 9–18 of phosphorylase *b* was synthesized at the Iowa State University protein facility and purified by reverse-phase HPLC C-18 column. The cDNA of PhK γ subunit was from rabbit skeletal muscle (Huang et al., 1993).

DNA Manipulations and Oligonucleotide-Directed Site-Specific Mutagenesis. Standard DNA manipulations were carried out as described (Sambrook, 1989). Oligonucleotides used in site-directed mutagenesis were synthesized in the nucleic acid facility at Iowa State University. For construction of mutant forms of γ_{1-300}, the single-stranded DNA of pUCG300 plasmid (Huang et al., 1993), which encodes the 1–300 amino acids of PhK γ, was prepared by standard procedure (Vieira & Messing, 1987). The isolated single-stranded DNA was used as the template for site-directed mutagenesis by using an Amersham commercial kit (Sayers et al., 1988). Mutations were identified by restriction enzyme analysis and then verified by sequencing. The coding sequence for the wild-type γ and its mutant forms[2] was subcloned into pET-11a expression vectors (Studier & Moffatt 1986) and transformed into BL21(DE3) cells, which were used for expression of wild-type and mutant forms of γ (Huang et al., 1993).

Expression and Purification of Mutant γs. Wild-type and mutant forms of γ were expressed by using the T7 RNA polymerase-based expression system (Studier & Moffatt, 1986) as previously described (Huang et al., 1993). The wild-type and mutant enzymes were expressed at a high level as inclusion bodies. None of the mutants generated by oligonucleotide site-directed mutagenesis had an effect on protein synthesis or solubilization of the expressed mutant enzymes. The inclusion bodies of all γ forms (wild-type and mutants) were isolated, solubilized, renatured, and further purified by ammonium sulfate precipitation, followed by Q-Sepharose chromatography as previously described (Huang et al., 1993). All the recombinant proteins were purified to homogeneity as judged by SDS gel electrophoresis (data not shown).

Photoacoustic Infrared Spectra Analyses. The final isolated wild-type and mutant enzymes were dialyzed against 50 mM Tris-HCl (pH 7.5)/50 mM NaCl/10% glycerol/5 mM DTT and then concentrated to 1–1.5 mg/mL. Samples were prepared as a thin layer by spreading 1–2 μL (2 μg) of protein solution on a 7-mm-diameter small Teflon membrane disk made from the window of the 3M disposable IR card (Luo et al., 1994). The Perkin-Elmer Model 1800 FTIR spectrometer and MTEC photoacoustic Model 200 Photoacoustic detector were used to collect the photoacoustic infrared spectra. The spectra of each sample were obtained by scanning 2000 cycles at a 0.05 cm/s optical-path-difference velocity with 2-cm^{-1} resolution. Two mathematical manipulations were used to extract the information on protein secondary structure contents from photoacoustic infrared spectra. Secondary derivative and curve-fitting methods were utilized as described by Luo et al. (1994).

Protein Concentration Determination and SDS–Polyacrylamide Gel Electrophoresis (SDS–PAGE). Protein concentrations were determined by the Bradford assay with commercially prepared reagents from Bio-Rad (Bradford, 1976). SDS–PAGE was done in 10% acrylamide gel as described (Laemmli, 1970).

Activity Assay. The seryl kinase activity of γ was determined by incorporation of ^{32}P into phosphorylase *b* as previously described with some modification (Kee & Graves, 1986; Yuan et al., 1993). The standard assay contained the final concentrations of 50 mM Tris, 50 mM HEPES, pH 8.2, 10 mM $MgCl_2$, 10 mg/mL phosphorylase *b*, and 1 mM [γ-^{32}P]-ATP or 1 mM [γ-^{32}P]GTP. The tyrosine kinase activity assay was done using angiotensin II and MnATP as substrates and was described previously (Yuan et al., 1993). In serine kinase activity, the V_{max} and K_m values of reactions with MgATP and phosphorylase *b* at pH 8.2 were determined by standard reciprocal plots of initial-rate data. The final concentrations of wild-type and most mutant enzymes used in activity assay and kinetic analyses were 0.02–0.05 μg/mL. For mutant N155H and D168N, 50 and 200 μg/mL were used, respectively. The initial rates were determined at phosphorylase *b*

[1] The charge to alanine and charge reversal scanning mutations were also used to explore peptide specificity determinants of γ (Cox et al., manuscript in preparation).

[2] The truncated wild-type γ_{1-300} will be referred to as wild-type γ. We will use mutant enzymes to represent truncated mutated γ_{1-300} forms in this study.

Table 1: Sequence Alignment of PhK γ Subunit with Various Protein Kinases[a]

Protein kinases	Sequences
	111 154 169
PhK-γ subunit	GG***E***MF--RDLK***PE***N***I***LLDDDM--LT***DFG***FS
cAPK	KG***E***LF--RDLK***PE***N***L***LIDQQG--VT***DFG***FA
CasK II	NT***D***FK--RDVK***PH***N***V***MIDHEN--LI***DWG***LA
EGF R	FG***C***LL--RDLA***AR***N***V***LVKTPQ--IT***DFG***LA
Insulin R	HG***D***LK--RDLA***AR***N***C***MVAHDF--IG***DFG***MT
pp60$^{v\text{-}src}$	KG***S***LL--RDLR***AA***N***I***LVGENL--VA***DFG***LA

[a] Some mutated positions are indicated in boldface: E^{111}, E^{154}, N^{155}, D^{168}, F^{169}, and G^{170}. The regions that distinguish serine kinases and tyrosine kinases are underlined.

Table 2: Specific Activities of Wild-Type and Some of Its Mutant Forms

	Ser kinase act.[a] (%) (MgATP/phos *b*)	Ser kinase act. (%) (MgGTP/phos *b*)	Tyr kinase act.[b] (%) (MnATP/angiotensin II)
wild-type	100	4	100
E111K	8		78
E154R	25		251
N155H	<1		5
D168N	<1		<1
F169W	15	0.5	85
G170A	5		82

[a] The serine kinase specific activity of wild-type γ was determined in the presence of 50 mM Tris (pH 8.2), 50 mM HEPES, 5 mM DTT, 10 mM $MgCl_2$, 10 mg/mL phosphorylase *b*, and 1 mM [γ-^{32}P]ATP or 1 mM [γ-^{32}P]GTP. The wild-type specific activity [3.5 μmol of ^{32}P/(min·nmol of enzyme)] was used as 100%. [b] The tyrosine kinase activity was assayed in the presence of 50 mM Tris (pH 7.9), 50 mM PIPES, 10% glycerol, 3 mM $MnCl_2$, 1 mM [γ-^{32}P]ATP, and 2 mM angiotensin II. The wild-type specific activity [0.25 nmol of ^{32}P/(min·nmol of enzyme)] was used as 100%.

concentrations varying from 10 to 250 μM and ATP concentrations varying from 50 to 1200 μM. When GTP was used, the concentrations varied from 50 to 4000 μM. For each mutant, the kinetic parameters were obtained by varying the substrate concentrations from $^1/_2$ K_m to 5 times the K_m. In the case of F169W mutant, the GTP concentrations were from 400 to 4000 μM. All the experiments were repeated at least three times, and the samples were from at least two different preparations.

RESULTS

Properties of the Mutant Enzymes. To explore the roles of the conserved short stretch Asp^{168}-Phe^{169}-Gly^{170} (Table 1), mutations were introduced into the coding sequence of γ_{1-300}. We asked whether a larger but negatively charged glutamic acid (D168E) and a similarly sized but uncharged asparagine (D168N) could functionally substitute for Asp^{168} in γ. Incorporation of either D168E and D168N into γ nearly abolished its serine phosphotransferase activity (both mutants showed less than 1% specific activity when compared with wild-type) as measured in the standard assay.

Two mutants of γ were made in the Phe^{169} position. Phe was changed to Trp (F169W) because casein kinase II (CasK II) has this sequence, and this enzyme can utilize GTP almost as efficiently as ATP [reviewed in Pinna (1990)]. The F169W mutant had 15% the activity of the wild-type (Table 2). Incorporation of Trp did not stimulate phosphotransferase activity with GTP (Table 2). Somewhat greater activity was seen with MnGTP than with MgGTP for both the wild-type and the F169W mutant (data not shown). To alter the charge character of the region, Phe was changed to Arg (F169R). This mutant enzyme was essentially inactive.

The X-ray crystal structure of the cAPK ternary complex shows that the invariant Gly at position 170 is located at the interface between the small and large lobes, where it is surrounded by several hydrophobic residues (Knighton et al., 1991a,b). To determine whether this invariant glycine could be replaced by larger, hydrophobic residues, e.g., alanine (G170A), valine (G170V), isoleucine (G170I), and leucine (G170L), these mutants were prepared. Of the mutant proteins obtained, only the G170A mutant had appreciable kinase activity, 5% relative to wild-type γ (Table 2). The other mutants showed less than 1% specific activity.

The N155H mutant was constructed to probe the function of the invariant Asn^{155} residue. Less than 1% of serine kinase specific activity was found (Table 2). This is not surprising because of the invariance of this residue in the protein kinase family (Hanks et al., 1988). Because this residue has been implicated in free metal ion binding in cAPK but not in CDK2, the metal ion dependence of N155H was tested and characterized.

We used charge to alanine and charge reversal scanning mutations[1] to search for other negatively charged residue(s) in the large lobe of the kinase domain that might participate in chelating metal ions; 17 mutants were generated, including E111A, E111K, D114A, E118A, E124A/E126A, E136A, E154A, E154R, D159A, D160A, D161A, D186A, E189A/E193A, E197A/D202A, E210A, E253A/D255A/D256A, and D259A/D263A. Two of them, E111K and E154R, with 8% and 25%, respectively, the activity of wild-type γ (Table 2), had a different response to metal ions (see later in Figure 2). Because the substrate, phosphorylase *b*, also binds free metal ions (Graves & Wang, 1972), we wanted to exclude the possibility that this unique metal ion response might come from phosphorylase *b*. We used a peptide substrate, corresponding to residues 9–18 of phosphorylase *b*, as an alternative substrate lacking the metal ion binding property for the same assay. Similar results were obtained (data not shown), suggesting that the observed metal ion responses did not derive from phosphorylase *b*. These two enzyme mutants were further characterized.

Photoacoustic Infrared Spectroscopy Analyses. Less than 1% of enzymatic activity was found with the N155H, D168E, D168N, F169R, G170V, G170I, and G170L mutant enzymes. The loss of enzymatic activity could be due to several factors including the involvement of the mutated residues in chemical reaction, substrate binding, or conformational stability. To evaluate possible conformational changes induced by mutation, photoacoustic infrared spectroscopy was used to study the folding of recombinant γ forms (wild-type and mutants). Using this technique, we have shown previously that this method is very sensitive and that the secondary structure contents obtained by this technique are similar to those of the CD measurement (Luo et al., 1994).

Photoacoustic infrared spectra were analyzed with 2 μg of the individual mutant proteins (see Materials and Methods). There were no significant differences either in the spectra or in the calculated secondary structure content of the mutant enzymes, including D168N (Figure 1A), E111K, E154R, N155H, D168E, F169R, F169W (data not shown), and G170A (Figure 1B), when compared with the wild-type γ. The spectra of G170V, G170I, and G170L, however, were obviously different in the amide I band region when compared

Table 3: Kinetic Parameters[a] of the Wild-Type γ and Its Mutant Forms

recombinant γ forms	K_m (μM) (phos *b*)	K_m (μM) (MgATP)	K_{ia} (μM) (phos *b*)	K_{ib} (μM) (MgATP)	K_m (mM) (MgGTP)	K_{ib}' (mM) (MgGTP)	V_{max} [μmol of ^{32}P/ (min·nmol of enzyme)]
wild-type	18 ± 1	79 ± 10	24 ± 3	105 ± 21			4.1 ± 0.03
E111K	107 ± 8	305 ± 31	112 ± 12	319 ± 8			0.4 ± 0.03
E154R	113 ± 8	242 ± 9	75 ± 5	160 ± 6			6.0 ± 0.20
N155H	26 ± 3	26 ± 5	40 ± 5	39 ± 9			(4.8 ± 0.2) × 10^{-3}
D168N	23 ± 2	70 ± 4	24 ± 1	73 ± 1			(1.1 ± 0.1) × 10^{-3}
F169W	49 ● 4	290 ± 12	41 ± 4	243 ± 13			6.5 ± 0.10
G170A	178 ± 14	344 ± 9	142 ± 4	274 ± 8			0.8 ± 0.07
wild-type	76 ± 9		58 ● 6		0.1 ● 0.04	0.08 ± 0.03	0.3 ● 0.04
F169W	92 ± 10		86 ± 8		2.3 ± 0.4	2.2 ± 0.3	0.7 ± 0.06

[a] Abbreviation: phos = phosphorylase. Kinetic parameters are defined as follows: E + phos *b* = E–phos *b*, K_{ia}; E–phos *b* + ATP = E–phos *b*–ATP, K_m (ATP); E + ATP = E–ATP, K_{ib}; E–ATP + phos *b* = E–ATP–phos *b*, K_m (phos b); E + GTP = E–GTP, K_{ib}'; E–phos *b* + GTP = E–phos *b*–GTP, K_m (GTP).

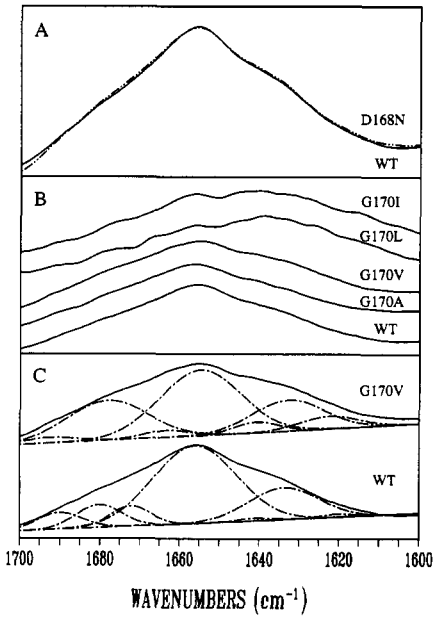

FIGURE 1: Photoacoustic infrared spectroscopy analyses of γ and its mutant forms. (A) Comparisons of photoacoustic infrared spectra of wild-type (solid line) and D168N mutant (dashed line); (B) comparisons of photoacoustic infrared spectra of wild-type and its mutant forms in the amide I region; (C) results of curve-fitting of G170V mutant (top) and wild-type (bottom).

with that of wild-type enzyme (Figure 1B). Spectral differences, especially assignments of β-sheet structure, are illustrated in Figure 1C with curve-fitting comparisons of wild-type and G170V mutant. The significant spectral differences suggest conformational disruptions in these mutants. The calculated secondary structure contents, derived from mathematical manipulations of curve-fitting as described in Luo et al. (1994), indicate the gradually increasing β-sheet structures of the mutant enzymes. The estimated β-sheet contents of wild-type γ and G170A mutant were 33% and 31%, respectively. However, it increased to 53% for G170V, 65% for G170I, and 59% for G170L. The increasing β-sheet contents of these mutants are coincident with decreasing levels of the α-helix and random coil contents. There were no significant differences in the percentage of turn structures. Clearly, the position of the invariant residue Gly^{170} cannot accommodate larger residues, suggesting that one of the possible functions of this residue is involved in the structural integrity of the protein kinase family.

Kinetic Analyses. Kinetic studies were undertaken to determine why the mutants with folding similar to that of wild-type γ had lower molar-specific activity. But the interpretation of the significance of the kinetic parameters is uncertain unless the kinetic mechanism is known. If the mechanism is random bi bi, dissociation constants can be calculated for the binding of the two substrates. Phosphorylase kinase was shown earlier to use a random bi bi kinetic mechanism (Tabatabai & Graves, 1976), but studies with γ-calmodulin complex suggest a random or ordered mechanism (Farrar & Carlson, 1991; Sanchez & Carlson, 1993). Using dead-end inhibitors, we found that the kinetic mechanism of the recombinant γ_{1-300} is a random bi bi kinetic mechanism[3] (Huang and Graves, manuscript in preparation). Therefore, studies were done with various proteins and ATP to obtain different kinetic parameters. Because K_m values may not necessarily reflect binding character, the dissociation constants for the binding of phosphorylase *b* (K_{ia}) and MgATP (K_{ib}) were calculated from the linear secondary plots. Note that similar patterns (Table 3) are seen among K_m values, K_{ia}, and K_{ib} values for most of the mutant forms, suggesting that K_m values in these instances are a measure of binding, except that E154R has a 3-fold higher K_m for MgATP but a similar K_{ib} value to the wild-type. In this case, we conclude that mutation E154 to R does not influence MgATP binding. If the comparison is based solely on K_m values, a wrong conclusion can be reached. Table 3 presents the results of the kinetic studies of wild-type and mutant γs (E111K, E154R, N155H, D168N, F169W, and G170A) at pH 8.2 for both MgATP and phosphorylase *b*.

Comparison of the kinetic parameters of N155H and D168N mutants with the wild-type γ showed similar K_m, K_{ia}, and K_{ib} values for both substrates but an 850- and 4000-fold decrease in V_{max}, suggesting that Asn^{155} and Asp^{168} residues may have an important role in catalysis like cAPK.

Interestingly, E154R and F169W mutants had higher K_m values for both substrates, a 3–6-fold increase, but their V_{max} values are essentially the same as values of the wild-type enzyme. E111K and G170A mutants showed an increase in K_m values for both substrates, a 4–10-fold increase, but a 10- and 5-fold reduction in V_{max}, respectively. These mutants seem to have a less well-formed active site region than the wild-type but are competent enzyme forms.

Kinetic analyses were also done with GTP as a phosphate donor with the wild-type and the F169W mutant. In this instance, the K_m values (for phosphorylase *b*) suggest that the wild-type binds phosphorylase *b* more poorly than when ATP is present and that mutation of F169W causes no further

[3] We have shown that peptide PhK13 ($\gamma_{302-326}$) (Dasgupta et al., 1989) is a competitive inhibitor for phosphorylase *b* and a noncompetitive inhibitor for ATP; ADP is a competitive inhibitor to ATP and a noncompetitive inhibitor to phosphorylase *b*. The results suggest that recombinant γ_{1-300} has the random bi bi mechanism (Huang and Graves, manuscript in preparation).

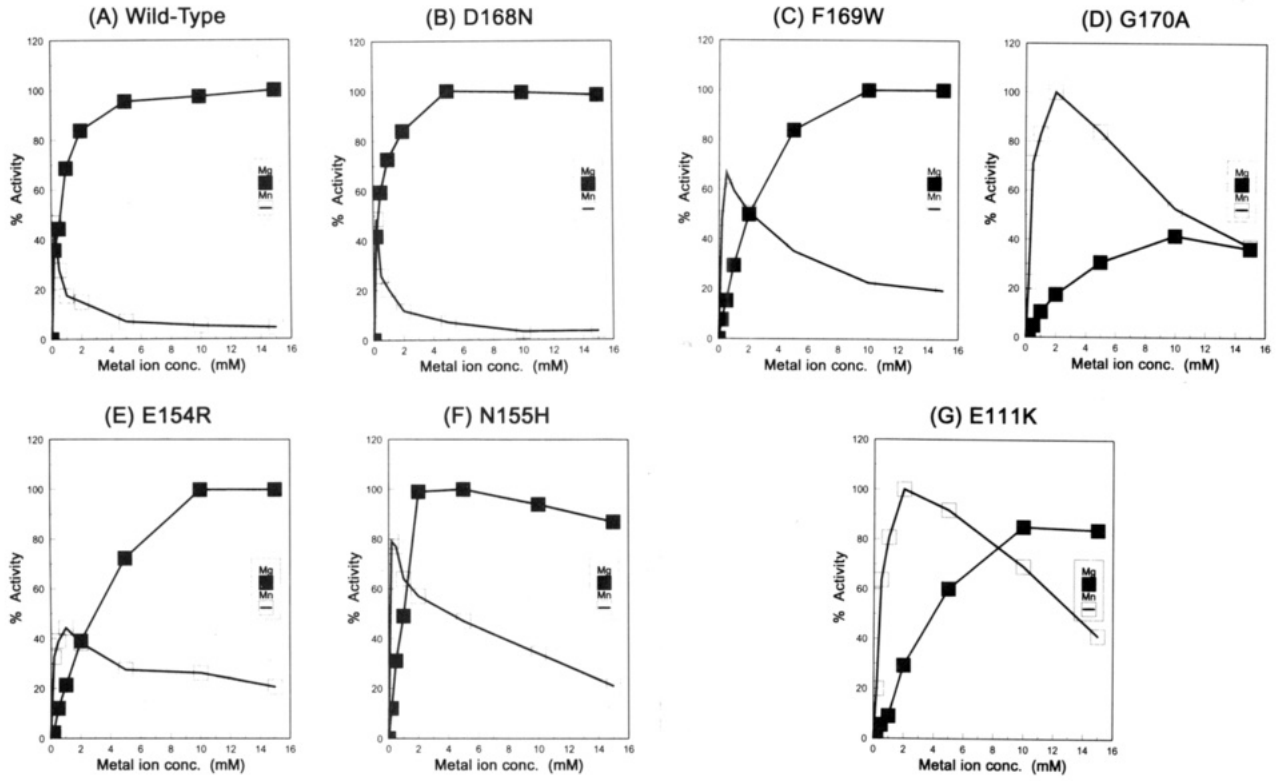

FIGURE 2: Effect of divalent metal ions on the serine kinase activity of γ and mutant forms. Serine kinase activities of (A) wild-type, (B) D168N, (C) F169W, (D) G170A, (E) E154R, (F) N155H, and (G) E111K were assayed at pH 8.2 as described in Materials and Methods, except 0.2 mM [γ-^{32}P]ATP and varied concentrations of $MgCl_2$ or $MnCl_2$ were used. The highest activity in each panel is taken as 100%, and relative specific activities are shown in Table 2.

change. The F169W mutation, however, increased the K_m for MgGTP 23-fold and caused a 2-fold increase in V_{max} relative to the wild-type enzyme.

Metal Ion Responses and the Second Metal Ion Binding Site. Earlier, it was found that free Mg^{2+} activated the serine kinase activity of γ but that free Mn^{2+} inhibited it (Kee & Graves, 1987; Cox & Johnson, 1992; Huang et al., 1993). In cAPK, Asp^{184} has been implicated as the residue involved in the chelation of Mg^{2+} in the MgATP complex (Knighton et al., 1991a,b). Therefore, it was particularly relevant to investigate whether a change in the neighboring residues of Asp^{168} (or itself) would cause different effects of divalent metal ions on γ. Figure 2A shows the effects of these metal ions on the wild-type γ when phosphorylase *b* is used as a substrate. Similar metal ion responses were found with both the D168N (Figure 2B) and D168E mutants (data not shown). Both mutants, as well as wild-type γ, reached maximal activation at 5 mM Mg^{2+}, and all exhibited the same inhibition pattern in the presence of free Mn^{2+}.

Unlike wild-type γ, the F169W, G170A, E154R, and E111K mutants showed quite different metal ion effects (Figure 2C–E,G). A shift in the concentration required for maximal activation from 5 mM Mg^{2+} to 10 mM is shown in Figure 2, indicating that all four mutants can be activated by free Mg^{2+} but not as effectively as the wild-type enzyme. More direct evidence resulted from examining the effects of free Mn^{2+}. All mutants, F169W, G170A, E154R, N155H, and E111K, displayed significantly inhibition patterns different from those of wild-type γ. Activation, instead of inhibition, was observed at low concentrations of Mn^{2+} in all instances, especially G170A and E111K, which had the highest activity in the presence of free Mn^{2+} but not free Mg^{2+}. Because of these results, the Glu^{111}, Glu^{154}, Asn^{155}, Phe^{169}, and Gly^{170} residues are possibly very close to the metal ion binding sites or the side chain of the mutants pointed to the metal ion binding sites. Mutations on these five residues might induce a subtle, spatial configuration change around the metal ion binding sites and then cause these different metal ion effects.

Metal Ion Response and Tyrosine Kinase Activity of γ Subunit and Its Mutant Enzymes. Recently, we have reported that PhK and γ are metal-ion-dependent dual-specificity kinases (Yuan et al., 1993). Seven mutants, E111K, E154R, N155H, D168E, D168N, F169W, and G170A, were assayed for their tyrosine kinase activity. With MnATP, D168N and D168E showed no tyrosine kinase activity, indicating that this residue is involved in phosphoryl transfer to tyrosine or serine. Wild-type enzyme and most mutant enzymes showed low tyrosine kinase activity in the presence of Mg^{2+} (data not shown). E154R had a slightly higher tyrosine kinase activity than wild-type in the presence of Mg^{2+}, but Mn^{2+} still favored for tyrosine kinase activity (data not shown). Tyrosine kinase activity of wild-type γ and its mutant forms has been analyzed with MnATP and is shown in Table 2. Interestingly, E154R had a 2.5-fold higher tyrosine kinase activity than wild-type enzyme. The higher tyrosine kinase activity of E154R was reflected by the increase of V_{max} (Huang and Graves, unpublished result).

DISCUSSION

Eight mutants have been constructed to elucidate the possible roles of the conserved short stretch, Asp-Phe-Gly, found in the subdomain VII of protein kinases (Hanks et al., 1988). Several investigators have reported site-directed mutagenesis of the invariant Asp residue (Moran et al., 1988; Gibbs & Zoller, 1991). The loss of enzymatic activity with the D168E and D168N mutants reported here was consistent with these other reports. Obviously, γ does not tolerate well the conservative replacement in Asp^{168} with respect to size or charge. From our kinetic analyses, we conclude that the

invariant Asp^{168} residue is involved in catalysis. This conclusion is supported by comparison of the cAPK crystal structure.

X-ray crystallographic studies of the cAPK ternary complex have shown that the invariant Asp^{184} residue (equivalent to Asp^{168} in γ) is involved in chelating Mg^{2+} in the MgATP complex and free Mg^{2+}. However, both D168N (Figure 2B) and D168E (data not shown) have metal ion responses identical to the responses of the wild-type γ. Because cAPK and γ are structurally and functionally related proteins (Reimann et al., 1984), an analysis of the structure of the cAPK ternary complex was made, with substitution of invariant Asp^{184} with Asn and Glu to probe the spatial relationship between this invariant residue and the two metal ions. The results of computer simulation showed that these replacements did not enlarge the distance (D184N) or shorten the distance (D184E) to either metal ion[4] in cAPK (Zheng, Cox, and Taylor, personal communication). Thus, we suggest that, even though the mutants have properties similar to those of the wild-type, Asp^{168} of γ is probably involved in binding of both metal ions.

Because both Phe^{169} and Gly^{170} are highly conserved in protein kinases, these two residues probably have a general function in the protein kinase family. The loss of enzymatic activity of the F169W mutant was not because of a gross conformational change, on the basis of photoacoustic infrared spectrum, and was not involved in catalysis, on the basis of kinetic analyses. Because the Phe residue is spatially close to the MgATP binding site in cAPK, the higher K_m values for MgATP and phosphorylase *b* might be indicative of the mutation causing changes in MgATP binding, which indirectly influences the phosphorylase *b* binding.

The replacement of Phe^{169} by a positively charged Arg (F169R) greatly diminishes the activity. The X-ray crystal structure of cAPK shows that Phe is located at the interface between small and large lobes and is a member of the hydrophobic pocket. Introducing a hydrophilic residue, arginine, into this position might be unfavorable to the hydrophobic interactions in the region. Substitution of Phe with hydrophobic residues showed partial phosphotransferase activity. Similar mutation results were obtained in CasK II (Jakobi & Traugh, 1992). In the recombinant CasK II α subunit, replacement of the hydrophobic Trp (equivalent to the position of Phe^{169} in γ) with Lys produced a mutant enzyme without kinase activity.

Most protein kinases cannot utilize GTP (or use it only poorly) as the phosphoryl donor when compared with ATP (Bradford, 1976; Pocinwong et al., 1981). The DFG region is thought to be associated with MgATP binding (Sambrook et al., 1989; Vieira & Messing, 1987), and the substitution in CasK II, Trp instead of Phe, might explain this unique feature of CasK II in using GTP. The F169W mutant of γ was generated to probe the possibility of utilizing GTP, as well as ATP, as the phosphoryl donor. However, the higher K_m and K_{ib} values for MgGTP in the F169W mutant suggested that (a) the precise interactions between Phe^{169} and the surrounding residues cannot be functionally replaced by another hydrophobic residue and that (b) the binding of MgGTP (or utilization of MgGTP as a phosphate donor) was not solely dependent on a single residue, Phe or Trp. The Phe residue helps to orient MgATP to facilitate phosphoryl transfer, but other residues may be responsible for the utilization of MgGTP in both CasK II and γ.

We did not observe any conformational disruption in the G170A mutant, but can clearly show a conformational change in other mutants at this position (G170V, G170I, and G170V). Increases in the size of the residue at this position might cause a perturbation of the structure of the catalytic core. Our kinetic data showed that the G170A mutant not only had a decreased V_{max} but also had increased K_m, K_{ia}, and K_{ib} values for substrates. The invariant Gly residue probably helps to maintain the flexibility of the active site region needed for substrate entrapment and product release.

In cAPK (from *Saccharomyces cerevisiae*), Gibbs and Zoller showed that Glu^{171} and Glu^{214} (equivalent to Glu^{111} and Glu^{154} in γ) were involved in peptide substrate binding (Gibbs & Zoller, 1991a,b). A mutation at those positions in cAPK, E171A and E214A, showed a 90- and 126-fold increase in K_m values for peptide but no significant change in K_m for MgATP or V_{max}. Unlike such dramatic increases in K_m values, our kinetic data showed that E111K and E154R mutants had a 5- and 3-fold increase in K_{ia} values, respectively. Our findings argue that both positions (E111 and E154) are involved in phosphorylase *b* binding but may be not the identical interactions as cAPK with kemptide. The results suggest that these changes, Glu to Lys or Arg, perhaps not only alter the phosphorylase *b* binding region but also indirectly influence the binding of metal ion and MgATP (higher K_m for MgATP). This might explain the observed metal-ion effects on E111K and E154R (Figure 2). The responses to metal ions of E111K, E154R, N155H, F169W, and G170A mutants are different from those of wild-type γ (Figure 2). The significant change in serine phosphotransferase activity with free Mn^{2+} prompts us to suggest that these five residues or the side chains of the mutants may be close to metal ion binding sites and be participating in the binding of metal ions. We argue that the metal ion binding sites are possibly between the DFG loop and the E^{111}–$KPEN^{155}$ loop, which may be similar to cAPK. In the cAPK ternary complex, Zheng et al. (1993a,b) showed that the location of the second metal ion binding site is between Asp^{184} (equivalent to Asp^{168} in γ) and Asn^{171} (equivalent to Asn^{154} in γ) and is flanked by an α- and γ-phosphate of ATP.

In E111K, E154R, N155H, F169W, and G170A mutants, mutations have a pronounced effect on serine kinase activity and seem to decrease the inhibitory effect of Mn^{2+} on serine kinase activity. In contrast, these five mutants have a higher tyrosine kinase activity than their relative serine kinase activity. The changes in activities in these mutants did not parallel each other. The higher tyrosine kinase activity supports our hypothesis that two conformations exist because of the different metal ions. Both D168E and D168N had less than 1% serine and tyrosine kinase activity, suggesting that Asp^{168} was important for both types of phosphorylation in γ. The results with the D168E and D168N mutants argue that there is one single active site.

The protein kinase subdomain VI contains specific residues characteristic of either the serine/threonine or tyrosine kinases (Hanks et al., 1988). In most serine/threonine kinases, a Lys-Pro-Glu^{154} (γ numbering) sequence is found, whereas the tyrosine kinases contain either an Arg-Ala-Ala or Ala-Ala-Arg sequence (Table 1). The E154R mutant was constructed because of this sequence alignment and has a 2.5-fold greater tyrosine kinase activity than the wild-type γ. It is unclear why this point mutation causes such dramatic change in the tyrosine kinase activity. Mutagenesis seems to alter the conformation around the metal ion binding sites and facilitate changes to favor tyrosine kinase substrates after the binding of the metal ion. The results suggest further that the configurations of the active site region between serine/threonine and tyrosine kinases are different.

[4] The results of computer simulation were provided by Drs. Jianhua Zheng and Sarah Cox.

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