Serine-53 at the Tip of the Glycine-Rich Loop of cAMP-Dependent Protein Kinase: Role in Catalysis, P-Site Specificity, and Interaction with Inhibitors†

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Received December 6, 1999; Revised Manuscript Received May 2, 2000

ABSTRACT: The glycine-rich loop, one of the most important motifs in the conserved protein kinase catalytic core, embraces the entire nucleotide, is very mobile, and is exquisitely sensitive to what occupies the active site cleft. Of the three conserved glycines [$G^{50}TG^{52}SFG^{55}$ in cAMP-dependent protein kinase (cAPK)], Gly^{52} is the most important for catalysis because it allows the backbone amide of Ser^{53} at the tip of the loop to hydrogen bond to the γ-phosphate of ATP [Grant, B. D. et al. (1998) *Biochemistry 37*, 7708]. The structural model of the catalytic subunit:ATP:$PKI_{(5-24)}$ (heat-stable protein kinase inhibitor) ternary complex in the closed conformation suggests that Ser^{53} also might be essential for stabilization of the peptide substrate−enzyme complex via a hydrogen bond between the P-site carbonyl in PKI and the Ser^{53} side-chain hydroxyl [Bossemeyer, D. et al. (1993) *EMBO J. 12*, 849]. To address the importance of the Ser^{53} side chain in catalysis, inhibition, and P-site specificity, Ser^{53} was replaced with threonine, glycine, and proline. Removal of the side chain (i.e., mutation to glycine) had no effect on the steady-state phosphorylation of a peptide substrate (LRRASLG) or on the interaction with physiological inhibitors, including the type-I and -II regulatory subunits and PKI. However, this mutation did affect the P-site specificity; the glycine mutant can more readily phosphorylate a P-site threonine in a peptide substrate (5−6-fold better than wild-type). The proline mutant is compromised catalytically with altered k_{cat} and K_m for both peptide and ATP and with altered sensitivity to both regulatory subunits and PKI. Steric constraints as well as restricted flexibility could account for these effects. These combined results demonstrate that while the backbone amide of Ser^{53} may be required for efficient catalysis, the side chain is not.

Protein kinases play a central role in a wide variety of normal and pathological processes. All members of this enzyme family have a structurally conserved catalytic core (*3*). The overall architecture of this core is bilobal with the larger carboxyl-terminal lobe possessing the determinants for substrate binding and the primary residues involved in catalysis. The smaller, amino-terminal lobe is critical for the binding of the nucleotide triphosphate, usually ATP. The glycine-rich nucleotide positioning motif [residues 47−56 in the cAMP-dependent protein kinase (cAPK) catalytic (C) subunit][1] in the small lobe is one of the most prominent features of the protein kinase core (*4*). This motif is highly conserved, with the mobile tip of the loop (the phosphate binding motif) having a characteristic sequence of G^{52}-X-G-X-X-G^{55}. Residues in this motif make contact[5] with all three parts of the ATP: the adenosine ring, the ribose ring, and the triphosphate moiety (*4*−*8*), with the tip of the loop being specifically responsible for positioning the phosphates. The α- and β-phosphates of ATP are stabilized via interactions with the backbone amides of residues 54 and 55 while the γ-phosphate interacts with the amide of residue 53.

cAPK, one of the first protein kinases to be discovered and purified (*9*), also is one of the simplest in terms of its regulation and, therefore, serves as a paradigm for the entire kinase family (*3*). The catalytic subunit (C) of cAPK has been characterized extensively by various biochemical and biophysical methods; it provided the first crystallographic model of a protein kinase (*2*, *10*, *11*). The glycine-rich loop of the cAPK C-subunit has been studied in detail by mutagenesis and kinetic analysis. The sequence in cAPK ($G^{50}TG^{52}SFG^{55}$) is indicative of most protein kinases described to date (*12*). The conserved glycines appear to be critical for the optimal positioning of the phosphates of ATP for efficient phosphoryl transfer (Figure 1). Although Gly^{52}

† This work was supported by a grant from the National Institutes of Health awarded to S.S.T. R.T.A. was supported by a Postdoctoral Fellowship from the American Heart Association, Western State Affiliates.

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[1] Abbreviations: dithiothreitol, DTT; 2-mercaptoethanol, β-ME; adenosine 3′:5′ cyclic monophosphate, cAMP; adenosine triphosphate, ATP; cAMP-dependent protein kinase, cAPK; cAPK catalytic subunit, C; isopropyl-β-D-thiogalactopyranoside, IPTG; nicotinamide adenine dinucleotide, NADH; fast performance liquid chromatography, FPLC; heat-stable protein kinase inhibitor, PKI; polyacrylamide gel electrophoresis, PAGE. Mutant proteins with amino acid substitutions are indicated in parentheses with the wild-type residue, the numbered position within the sequence, and the substituted amino acid [e.g., C(S53G) is the C-subunit with Ser^{53} replaced with glycine].

10.1021/bi992800w CCC: $19.00
Published on Web 06/21/2000

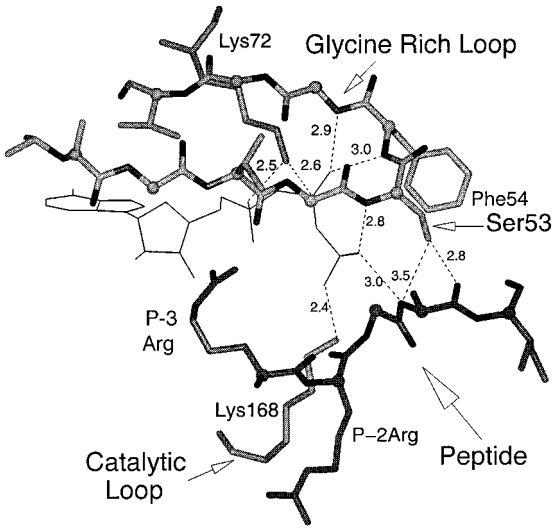


FIGURE 1: Structure of tip of the glycine-rich nucleotide-positioning loop of cAPK catalytic subunit bound to ATP and PKI. Close-up view of the cAPK C-subunit:ATP:PKI ternary complex near Ser^{53}. The γ-phosphate of ATP, the P-site (Ala^{21}) of the inhibitor peptide, Ser^{53}, and Lys^{168} are indicated with the balls (atoms) and the sticks (covalent bonds). Potential hydrogen bonds observed in the crystallographic structure model are indicated by the dashed lines. Distances are indicated in Å.

does not contact either ATP or the peptide substrate directly, mutation of this residue had more severe kinetic consequences than the replacement of either Gly^{50} or Gly^{55} (*1*). Substitution of Gly^{52} by either alanine or serine led to dramatic changes in the k_{cat}'s and the K_m's for both peptide substrate and ATP (*13*). The importance of the residues found in the nucleotide-positioning loop were examined previously by Lee et al. (*14*) and showed that mutation of residues other than the first two conserved glycines or Val^{33} (equivalent to Val^{58} in cAPK C) in phosphorylase kinase had little effect on the phosphorylation of phosphorylase by this enzyme.

Crystallographic models of the cAPK C-subunit complexed with a variety of nucleotides and substrate inhibitor peptides indicate that the tip of this loop, specifically Ser^{53} and Phe^{54}, is the most mobile part of the enzyme (*15*). The only stable conformation of the tip of the loop was observed in ternary complexes with a 20-residue fragment of the heat-stable protein kinase inhibitor (PKI), PKI_{5-24}, and either ATP (*10*, *11*) or an ATP analogue, adenylyl imidodiphosphate (AMPPNP) (*2*). These structures suggested that the specific interactions of Ser^{53} with both the γ-phosphate of the nucleotide and the substrate peptide at the tip of the loop (Figure 1) may play an important role in phosphoryl transfer. In these models, the backbone amide of Ser^{53} hydrogen bonds to an axial oxygen of the ATP γ-phosphate. A second potential hydrogen bond between the β-hydroxyl of Ser^{53} and the backbone carbonyl of the P-site residue also was observed. Another axial oxygen of the γ-phosphate of ATP hydrogen bonds to Lys^{168} in the large lobe and also may form a hydrogen bond with the backbone amide of the P-site. The third axial oxygen is bound to the activating magnesium ion. These observations have led to the prediction that the transition-state intermediate for phosphoryl transfer requires both the backbone amide of Ser^{53} and the side-chain hydroxyl (*2*, *4*, *6*). Both also could contribute to the synergistic high-affinity binding of ATP and PKI.

To test this hypothesis, Ser^{53} of the mouse cAPK C-subunit was mutated to assess which of the functional groups, if any, at this site are important for catalysis. The β-hydroxyl of Ser^{53} was removed by changing this residue to a glycine, both the β-hydroxyl and the backbone amide were blocked by mutating the serine to a proline, and the serine was replaced with a threonine to examine the effects of potential steric hindrance at the tip of the loop. These mutants were examined for effects on steady-state kinetic parameters for ATP and a synthetic peptide substrate, Kemptide. Inhibition of these mutant cAPK C-subunits by both the type-I and -II regulatory subunits and PKI also was examined. In addition, a synthetic peptide substrate with a threonine as the phosphoacceptor residue was used to evaluate the effect of Ser^{53} on P-site specificity. These experiments provide new information as to the role of Ser^{53} on the catalytic ability of cAPK as well as the binding of substrates and inhibitors.

EXPERIMENTAL PROCEDURES

Materials. ProBind Ni^+ resin and pRSETB expression vector were from Invitrogen (Carlsbad, CA). [^{32}P]-γ-ATP was from New England Nuclear−DuPonte (Boston, MA). *E. coli* strains BL21(DE3) and BL21(DE3)pLysS were from Novagen (Madison, WI). P81 filter paper was from Whatman, Inc. (Clifton, NJ). EcoLume scintillation fluid was from ICN (Costa Mesa, CA). ATP, DTT, phosphoenolpyruvate, pyruvate kinase, lactate dehydrogenase, reduced NADH, and cAMP were from Sigma (St. Louis, MO). Sodium dodecyl sulfate (SDS) and 3-(*N*-morpholino)-propanesulfonic acid (MOPS) were from Amersham−United States Biochemical (Arlington Heights, IL). Mono S HR10/10 was from Pharmacia (Piscataway, NJ). The Muta-Gene site-directed mutagenesis kit was purchased from BioRad (Hercules, CA). The heptapeptides LRRASLG (Kemptide), LRRATLG (Thr-Kemptide), and LRRAALG were synthesized at the Peptide and Oligonucleotide Facility at the University of California−San Diego on a Milligen 9050 PepSyn peptide synthesizer using standard Fmoc methodology activator and purified by high-performance liquid chromatography.

Site-Directed Mutagenesis of the cAPK Catalytic Subunit. The cDNA for the murine cAPK C-subunit in the bacterial expression vector pRSETB was used as a template for Kunkel-based site-directed mutagenesis (*16*). All mutations were made using the Muta-Gene kit as per the manufacturer's recommendations. The presence of the correct mutation was confirmed by DNA sequence analysis as described previously (*17*). All mutant cDNAs were subjected to complete sequence analysis to ensure no other mutations were present.

Expression of Murine cAPK Catalytic Subunit. Wild-type and mutant C-subunits were expressed in *E. coli* strains BL21(DE3) or BL21(DE3)pLysS (*18*). Cells were grown in YT medium containing 100 μg/mL ampicillin at 37 °C to an optical density at 600 nm of 0.6−0.8, induced with 0.5 mM isopropyl-β- D-thiogalactopyranoside, incubated for an additional 6−8 h at 24 °C, collected by centrifugation, and stored frozen. Cells were lysed with a French pressure cell (American Instruments) at pressures between 16 000 and 24 000 psi using 10−20 mL of lysis buffer/L of culture. Insoluble material was removed by centrifugation at 25000×*g* at 4 °C for 45 min.

Purification of Catalytic Subunit. Wild-type C-subunit was purified using phosphocellulose chromatography and Mono

S FPLC as described previously (*19*). Mutant proteins were purified by formation of holoenzyme with a poly-histidine-tagged type-II regulatory-subunit mutant [H_6RII(R213K)] (*20*). Cells were suspended in 20 mL/L of culture in 50 mM potassium phosphate, 150 mM KCl, 2 mM $MgCl_2$, 0.2 mM ATP, and 2 mM 2-mercaptoethanol (β-ME) (pH 8.0). Cells expressing C-subunit mutants and H_6RII(R213K) were mixed in varying ratios, depending on expression levels (typically 3/2, w/w). The cell suspension was lysed as indicated above, and the soluble fraction was adjusted to pH 8.0 and allowed to incubate with ProBind resin [1 mL bed volume/L of H_6-RII(R213K)] for 2 h at 4 °C. The resin was collected by centrifugation at low speeds, washed 3 times with 20 bed volumes of buffer, and the catalytic subunit was eluted with 3 × 1 bed volumes of buffer, including 1 mM cAMP. Fractions were pooled and dialyzed into 20 mM potassium phosphate, 50 mM KCl, and 2 mM β-ME (pH 6.5), applied to a Mono S HR10/10 column attached to a BioLogic HR system (BioRad), and developed with a linear gradient of 0−250 mM KCl over 50 mL. Phosphoisoforms of the C-subunits eluted as described previously (*19*, *21*).

Purification of RI(R209K), RII(R213K), and PKI. The type-I regulatory subunit mutant was purified as described previously (*22*). RII(R213K) was purified by Ni-NTA affinity. Briefly, cells were suspended in 20 mM Tris (pH 8.0), 0.3 M NaCl, and 5 mM β-ME and lysed as described above. Insoluble material was removed by centrifugation, and the soluble proteins were incubated for 2 h at 4 °C with 1 mL of ProBind resin/L of culture. Unbound proteins were removed, the resin was washed extensively with buffer containing 15 mM imadazole, and the H_6RII(R213K) was eluted with buffer containing 0.25 M imadazole. Protein-containing fractions were pooled, dialyzed into 20 mM MOPS (pH 7.0), 0.15 M KCl, 10% glycerol, and 1 mM β-ME, concentrated by ultrafiltration; and stored at −20 °C until used. The heat-stable protein kinase inhibitor PKI was purified as described previously (*23*).

Mass Spectrometry. Electrospray/mass spectrometry was performed using a Hewlett-Packard 59887A electrospray mass spectrometer. Protein was desalted prior to analysis by narrow bore chromatography.

Catalytic Activity Assays. Kinetic activity was measured using a coupled-enzyme spectrophotometric assay using the peptide LRRASLG (Kemptide) (*24*) or a threonine-containing derivative, LRRATLG, as described previously (*25*). C-subunit was used (25 nM) in a final volume of 0.5 mL of 50 mM MOPS (pH 7.0), 1 mM DTT, 0.2 mM NADH, 1 mM phosphoenolpyruvate, 12 units of lactate dehydrogenase, 4 units of pyruvate kinase, and varying amounts of ATP and Kemptide. The concentration of $MgCl_2$ was 10 mM in excess of ATP. Reactions were initiated by the addition of Kemptide after the catalytic subunit had equilibrated with ATP for at least 1 min at 25 °C. All measurements were taken on a Hewlett-Packard 8453 UV−visible spectrophotometer equipped with UV−visible ChemStation Software (Rev. A 02.04).

Alternatively, C-subunit activity was followed by a direct phosphorylation filter-binding assay using [^{32}P]-γ-ATP (*26*). The C-subunit (0.1−2.0 nM) was incubated in 50 mM MOPS (pH 7.0), 0.1 M KCl, 10 mM $MgCl_2$, 1 mM DTT, 100 μg/mL bovine serum albumin, 2−10 μCi of [^{32}P]-γ-ATP, unlabeled ATP, and peptide substrate. For K_m of ATP determinations, peptide substrate concentrations were held constant, and the total ATP concentration was varied from 1.0 μM to 2.0 mM. For K_m's of peptide substrates, the ATP concentration was fixed, and peptide substrate was varied [LRRASLG, 1−400 μM for wild-type C, C(S53G), and C(S53T); 20 μM−4.0 mM for C(S53P); and 10 μM−10 mM for LRRATLG]. Reactions were initiated with the addition of peptide substrate and incubated at 30 °C in a final volume of 50 μL. Reactions were terminated with 20 μL of 50% acetic acid. Aliquots were spotted onto P81 filter disks and washed together in 0.5% H_3PO_4 (4 times; 500 mL for 10 min, each wash). Filter disks were rinsed once with 2-propyl alcohol, air-dried, and counted in 5 mL of EcoLume. Background reactions containing no peptide substrate were subtracted from all data. All reactions were performed in triplicate.

Kinetic data were fitted to the equation $v = V_{max}\cdot[S]/([S] + K_m)$ where v is the reaction rate, V_{max} is the maximum rate, [S] is the concentration of the variable substrate, and K_m is the Michaelis constant. Inhibition constants were determined by assaying activity at varying concentrations of the inhibitor (regulatory subunits, 0.1 nM−10 μM; PKI, 0.1−250 nM; LRRAALG, 10 μM−10 mM) at several fixed substrate concentrations. Data were fitted to a single-site binding model using GraphPad Prizm Software, and K_i values were extrapolated from IC_{50} values using the relationship of Cheng and Prusoff: $K_i = IC_{50}/(1 + [S]/K_m)$ (*27*).

Solution Viscosity Measurements. The relative viscosity (η) of buffers containing sucrose was measured relative to 50 mM MOPS (pH 7.0) at 30 °C using an Ostwald viscometer. Sucrose concentrations of 15% and 25% (w/v) gave relative viscosities of 1.6 and 2.3, respectively. All measurements were performed in triplicate using the direct phosphorylation assay described above and as described previously (*28*, *29*). Presence of the viscosogen did not affect the binding of the peptide to P81.

RESULTS

Expression and Purification of Ser^{53} Mutants. To examine the role of Ser^{53} in catalysis, substrate specificity, and the ability of cAPK C-subunit to interact with pseudosubstrate inhibitors, this residue was subjected to site-directed mutagenesis. Ser^{53} was replaced with a glycine [C(S53G)], threonine [C(S53T)], or proline [C(S53P)] and expressed in *E. coli*, either BL21(DE3) or the pLysS variant for C(S53G), because leaky expression from the T7 promoter appeared to be toxic. Unlike wild-type C-subunit, these mutants failed to bind to phosphocellulose and, therefore, were purified via formation of holoenzyme with a polyhistidine-tagged type-II regulatory-subunit mutant [His_6RII(R213K)] (*20*). C-subunit mutants were eluted from the immobilized holoenzyme complex with cAMP and analyzed by SDS−PAGE (data not shown). While C(S53G) and C(S53T) purified similar to other mutants, C(S53P) did not bind well to His_6-RII(R213K), and a large percentage of the protein was in the unbound fraction.

Eluted fractions containing C-subunit were pooled, applied to a Mono S cation-exchange column, and eluted with a linear gradient of KCl in order to further purify and resolve the various phosphoisoforms. Figure 2 shows the Mono S FPLC elution profiles of the wild-type and the three Ser^{53}

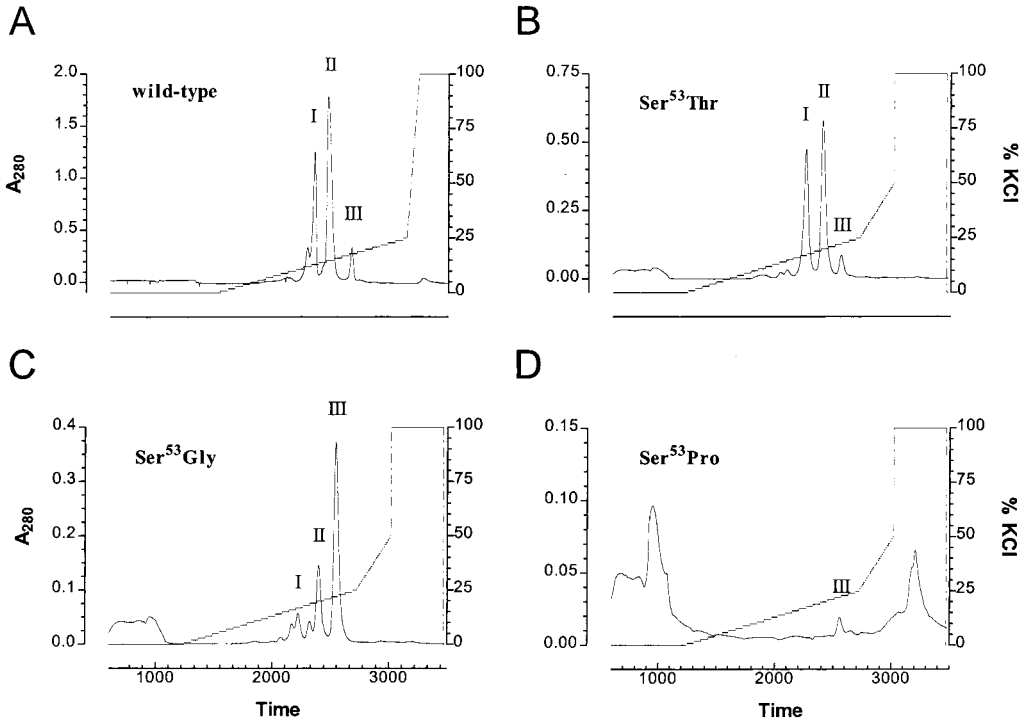


FIGURE 2: Purification profiles of wild-type C-subunit and the Ser[53] mutants from Mono S FPLC. Chromatograms from Mono S ion-exchange chromatography. Absorbance at 280 nm (solid lines) is shown on the left axis; percent of 1 M KCl elution buffer (dashed lines) is indicated on the right axis. Characteristic elution profiles for (A) wild-type C-subunit, (B) C(S53T), (C) C(S53G), and (D) C(S53P). Phosphoisoforms of peaks are indicated by roman numerals with peaks I, II, and III corresponding to the C-subunit with 4, 3, and 2 phosphates, respectively.

Table 1: Mass Determination of Mutant cAPK Catalytic Subunit Mutants

mutant (isoform)[a]	no of PO_4[b]	expected mass (Da)	experimental mass (Da)[c,d]
wild-type (I)	4	40760	40763
wild-type (II)	3	40680	40684.2 ± 6.9
wild-type (III)	2	40600	40601
Ser[53]Gly (I)	4	40730	40725.5 ± 6.1
Ser[53]Gly (II)	3	40650	40644.1 ± 6.4
Ser[53]Gly (III)	2	40570	40575.2 ± 7.0
Ser[53]Thr (I)	4	40774	40779.7 ± 6.4
Ser[53]Thr (II)	3	40694	40693.8 ± 7.2
Ser[53]Thr (III)	2	40614	40618.5 ± 6.4
Ser[53]Pro (III)	2	40610	40594.7 ± 14.9

[a] Isoforms of wild-type and mutant cAPK catalytic subunits are numbered based on the Mono S elution profile for wild-type cAPK as described previously (*19*). [b] The number of phosphates was based on elution from Mono S and native isoelectric focusing gel electrophoresis and confirmed by mass spectrometry. [c] Values shown are the mean of at least two independent measurements ± the average standard deviation. [d] Values for wild-type isoforms I and III are taken from Herberg et al. (*19*). For C(S53P), insufficient material was purified for isoforms I and II and, therefore, no mass was determined.

mutants. The threonine and glycine mutants purified similar to wild-type C-subunit; however, the proline mutant yielded markedly less protein, the majority of which eluted with higher salt. Analysis of the peak fractions by electrospray mass spectrometry (Table 1) and isoelectric focusing gel electrophoresis (data not shown) indicated that these peaks correspond to distinct phosphorylation states of the C-subunit as described earlier (*19*). Similar to the wild-type C-subunit (*30*), the specific activities of the different phosphoisoforms of a given C mutant (with at least two phosphates) were the same (data not shown). Previous studies have demonstrated that phosphoisoform III of native and recombinant wild-type C-subunit, as well as glycine-rich loop mutants of cAPK C, is phosphorylated exclusively on T197 and S338 (*13*, *19*, *30*, *31*). Therefore, all subsequent studies utilized phosphoisoform III (two phosphates) of wild-type and mutant C-subunit.

Determination of the Steady-State Kinetic Parameters for the Peptide Substrate LRRASLG. cAPK preferentially phosphorylates serine residues which lie in the consensus sequence RRX(S/T)Hyd (*32*, *33*). To assess whether Ser[53] is important in phosphorylation of a canonical peptide substrate, the steady-state kinetic parameters of the Ser[53] mutants with the heptapeptide, LRRASLG (Kemptide), were examined. Both C(S53G) and C(S53T) behaved similarly to wild-type, with K_m (for both peptide and ATP) and k_{cat} values within 2-fold of the wild-type C values (Table 2). These results demonstrate unambiguously that the β-hydroxyl of Ser[53] is not required for the phosphorylation of a peptide substrate. The proline mutant, however, showed dramatic elevation in its K_m for both Kemptide (~20-fold) and ATP (~10-fold) and a modest decrease in k_{cat} (2.5−5-fold). The mutations did not have substantial effects on the K_i of ADP, suggesting that affinity for ADP was not altered. In addition, the K_m values for peptide substrate were independent of ATP concentration (and vice versa), suggesting that the Ser[53] mutants maintain an ordered kinetic mechanism (*34*, *35*).

Ser[53] Influences the P-Site Specificity of cAPK C-Subunit. To further characterize the role of Ser[53] in the ability of the C-subunit to phosphorylate peptide substrates, the steady-state kinetic parameters of wild-type C-subunit and the Ser[53] mutants with a threonine-containing peptide substrate were determined. Wild-type C phosphorylates the threonine residue in the heptapeptide LRRATLG approximately 100-fold less efficiently (k_{cat}/K_m) than the serine residue of Kemptide

Table 2: Steady-State Kinetic Parameters of Wild-Type and Mutant cAPK Catalytic Subunits[a]

parameter	ligand	wild-type	C(S53T)	C(S53G)	C(S53P)
k_{cat} (s^{-1})	LRRASLG	21.7 ± 0.7	20.8 ± 0.3	31.9 ± 1.5	10.4 ± 0.2
K_m (μM)	LRRASLG	21.9 ± 2.4	14.5 ± 0.9	22.2 ± 3.5	489.5 ± 20.7
k_{cat}/K_m (μM^{-1} s^{-1})	LRRASLG	1.0	1.4	1.4	0.021
k_{cat} (s^{-1})	LRRATLG	7.9 ± 0.5	9.1 ± 0.5	24.5 ± 1.0	nd
K_m (μM)	LRRATLG	654.0 ± 108.3	745.6 ± 102.0	365.7 ± 48.4	nd
k_{cat}/K_m (μM^{-1} s^{-1})	LRRATLG	0.012	0.012	0.067	nd
K_m (μM)	ATP	12.7 ± 0.5	12.5 ± 0.4	25.1 ± 0.9	108 ± 18.1
K_i (μM)	ADP	14.6 ± 0.3	12.5 ± 0.4	30.7 ± 0.9	43.5 ± 1.1

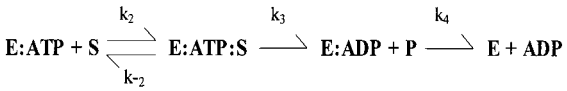
[a] All values were determined using the [^{32}P] incorporation assay as described in Experimental Procedures, with the exception of K_m for ATP. All values represent the mean of three independent experiments ± standard error of the mean.

Table 3: Estimates of the Microscopic Rate Constants Derived from Solution Viscosity Analysis and Mechanistic Scheme 1[a]

	LRRASLG			LRRATLG	
	wild-type	C(S53G)	C(S53P)	wild-type	C(S53G)
$(k_{cat})^{\eta}$	0.99 ± 0.06	1.00 ± 0.03	0.09 ± 0.01	0.19 ± 0.03	0.39 ± 0.05
$(k_{cat}/K_m)^{\eta}$	0.05 ± 0.02	0.09 ± 0.03	0.00 ± 0.06	0.11 ± 0.03	0.03 ± 0.05
k_3 (s^{-1})	500 ± 60[b]	fast[c]	*11.4*[d]	9.8	40.2
k_4 (s^{-1})	21.7	31.9	*111.6*	41.6	62.8

[a] Values were derived as follows: $k_3 = k_{cat}/(1-k_{cat}^{\eta})$; $k_4 = k_{cat}/k_{cat}^{\eta}$. Values for $(k_{cat})^{\eta}$ and $(k_{cat}/K_m)^{\eta}$ are the slopes of the lines of the ratio of these parameters in the absence and presence of viscosogen plotted against relative viscosity as described previously (*28*). The microscopic rate constants were determined using the values of k_{cat} and K_m reported in Table 2. Due to the near-zero values of $(k_{cat}/K_m)^{\eta}$, determination of microscopic rate constants k_2 and k_{-2} according to Scheme 1 was not possible (*36*). [b] Parameters determined by pre-steady-state kinetic quench-flow analysis (*38*). [c] Value for k_3 cannot be accurately determined due to the low value of k_{cat}^{η}. [d] Values of k_3 and k_4 are italicized to indicate that they cannot be accurately determined due to the low value of $(k_{cat})^{\eta}$.

Scheme 1



(Table 2). However, C(S53G) is actually a better threonine kinase than the wild-type C-subunit. The k_{cat} is 3-fold greater, and the K_m is one-half of that for the wild-type C-subunit.

Viscosity Effects on Steady-State Kinetic Parameters. The mutations at Ser[53] could influence the steady-state kinetic parameters in several ways: the binding of peptide substrate could be affected, the chemical transfer step could be altered, and/or the release of ADP following catalysis could be changed. Changes in K_m for the peptide do not necessarily reflect changes in substrate affinity. To examine which step(s) is(are) altered by these mutations, the effect of solvent viscosity was examined as described previously (*1*, *28*, *36*, *37*). Wild-type, C(S53G), and C(S53P) were examined using Kemptide as a substrate, and the microscopic rate constants were determined (Scheme 1).

The ratios of k_{cat} and k_{cat}/K_m in both the absence and the presence of viscosogen were plotted versus the relative viscosity (η). The slopes of the lines are designated $(k_{cat})^{\eta}$ and $(k_{cat}/K_m)^{\eta}$, respectively, and are reported in Table 3. The wild-type C-subunit and C(S53G) appear to have similar kinetic mechanisms, with k_{cat} largely dependent on k_4 (ADP-release), thus confirming that the side chain of Ser[53] is not an important feature that contributes to phosphoryl transfer. In contrast, the k_{cat} for C(S53P) appears to be limited by the chemical transfer of the phosphate to the peptide (k_3). The observed changes in the rate constants could be due to changes in affinity of the peptide substrate. However, the low values of $(k_{cat}/K_m)^{\eta}$ prevent an accurate determination of K_s, the peptide binding constant. To circumvent this problem, the K_i for an inhibitory version of Kemptide, LRRAALG, where the P-site serine is replaced with an alanine, was examined with wild-type C-subunit and the C(S53G). While this is not a direct measure of the K_d for the substrate peptide, it does measure the affinity of a pseudosubstrate peptide with similar sequence. The experimental values for wild-type C-subunit (200 ± 20 μM) and the serine-to-glycine mutant (209 ± 39.1 μM) are in close agreement with the published K_i [190 μM (*38*)], suggesting that mutation of Ser[53] to glycine does not alter the affinity of the peptide substrate. Therefore, the majority of the effect on the mechanism produced by the serine-to-proline change appears to result from a decrease in k_3, the rate of phosphoryl transfer.

When phosphorylation of LRRATLG was examined, the changes in the kinetic parameters k_{cat} and K_m, when compared to phosphorylation of Kemptide, again appear to be due mainly to effects on k_3. The K_d for this substrate peptide was not affected compared to Kemptide, suggesting that affinity is not responsible for the 80-fold drop in k_{cat}/K_m for the phosphorylation of LRRATLG.

Role of Ser[53] in the Inhibition of cAPK by PKI. Analysis of the crystallographic model of the cAPK catalytic subunit in a ternary complex with ATP and PKI_{5-24} suggested that the high-affinity binding of PKI ($K_i = 10^{-10}$ M) might be due, in part, to a potential hydrogen bond between the β-hydroxyl of Ser[53] and the P-site carbonyl of PKI (*4*, *5*). This hypothesis was tested using this series of mutants and full-length PKI. The results are summarized in Table 4. In contrast to what was predicted, phosphorylation of Kemptide by C(S53G) was inhibited by PKI as effectively as the wild-type C-subunit was inhibited. The C(S53T) mutant also was just as sensitive to PKI inhibition as the wild-type C-subunit. In contrast, however, C(S53P) is nearly 3 orders of magnitude less sensitive to PKI. These data indicate that a hydrogen bond between the side chain of Ser[53] and PKI is not required for the tight and synergistic binding of PKI to C:ATP and

Table 4: Inhibition Constants for RI(R209K), RII(R213K), and Wild-Type PKI with Wild-Type and Mutant cAPK Catalytic Subunits[a]

inhibitor C-subunit	wild-type	C(S53T)	C(S53G)	C(S53P)
RI(R209K) (10^{-9} M)	0.15 ± 0.03	0.20 ± 0.05	0.12 ± 0.04	5.98 ± 1.09
RII(R213K) (10^{-9} M)	0.23 ± 0.04	0.19 ± 0.08	0.30 ± 0.05	1.19 ± 0.27
PKI (10^{-9} M)	0.40 ± 0.06	0.27 ± 0.06	0.12 ± 0.07	103 ± 16.0

[a] All values were determined using varying inhibitor concentrations and at least three fixed Kemptide concentrations above and below their respective K_m values. K_i values were calculated from IC_{50} values and substrate concentrations using the relationship: $K_i = IC_{50}/(1 + [S]/K_m)$ (*27*). Constants are given as the mean ± the standard error of the mean.

suggest the hydrogen bonds between the γ-phosphate of ATP and both Ser[53] and Lys[168] (*5*, *10*, *11*) in the catalytic loop are the more important interactions for efficient phosphoryl transfer.

Effect of Mutations on the Interaction of the C-Subunit with the Regulatory Subunits. To determine if Ser[53] has a role in the interaction of the C-subunit with either the type-I or -II regulatory subunits, the effect of the three mutations on the sensitivity of C to these physiological inhibitors was examined. Two regulatory subunit mutants, RI(R209K) and RII(R213K), which are defective in cAMP binding (*20*, *22*) were exploited in order to avoid having to urea-strip the bound cAMP from the inhibitors. The results in Table 4 show that C(S53G) and C(S53T) have no change in sensitivity to these inhibitors. Indeed, the inhibitor potency of PKI for C(S53G) may be marginally enhanced compared to that of the wild-type C-subunit. Thus, even though ATP is required for high-affinity binding of PKI and the type-I regulatory subunit, hydrogen bonding of the β-hydroxyl of Ser[53] to the inhibitor protein is not. The proline mutant, however, is much less sensitive to both inhibitors. Of the two regulatory subunit types, type-I regulatory subunit binding, with an increased IC_{50} of 40-fold, is affected most dramatically. The effect on PKI inhibition is even greater, with the K_i increased by 250-fold. Therefore, while the potential hydrogen bond between the β-hydroxyl of Ser[53] and the P-site is not important, the affinity for ATP, PKI, and RI for the C-subunit would appear to depend critically on the hydrogen bond between the tip of the loop and the γ-phosphate of ATP.

DISCUSSION

The glycine-rich nucleotide-positioning motif plays a critical role in protein kinase function. Not only do residues in this sequence position and secure the ATP in the active site cleft, several residues potentially interact with the phosphoacceptor−peptide sequence. Mutations of the conserved glycines of this motif in phosphorylase kinase (*14*) and the cAPK C-subunit (*1*) indicate that these residues play a critical role in catalysis. In the cAPK C-subunit, Ser[53] lies at the distal tip of the loop very near the site of phosphotransfer. Structural models indicated that this residue can form hydrogen bonds to both the γ-phosphate of ATP and the P-site carbonyl of the substrate peptide (Figure 1). This led to the hypothesis that these interactions might influence the binding of substrate peptides and thereby effect catalysis. In addition, these potential hydrogen bonds were thought to be important for the synergism observed with ATP and PKI when binding to the C-subunit (*31*, *39*).

In this report, Ser[53] of the cAPK C-subunit was subjected to site-directed mutagenesis in an attempt to test the above hypotheses. While there is a risk of altering the structure and the overall kinetic mechanism of the enzyme, thereby producing effects that distort the importance of the specific residue targeted, this approach, nonetheless, can be a useful tool to answer basic functional questions. Removal of the Ser[53] side chain [i.e., C(S53G)] resulted in a functional kinase with steady-state enzymatic properties similar to the wild-type C-subunit. Phosphorylation of a canonical peptide substrate, LRRASLG (Kemptide), and sensitivity to the physiological inhibitors, PKI and type-I and -II regulatory subunits, were unaffected. These results clearly negate the importance of the potential hydrogen bond observed in the crystallographic models. Similarly, C(S53T) was identical kinetically to C(S53G) and wild-type C-subunit, further supporting the conclusion that the serine side chain per se does not play a critical role in catalysis or interactions with physiological inhibitors. Because Ser[53] was not replaced with a bulkier amino acid (e.g., leucine) the potential importance of steric factors cannot be evaluated but should not be ignored. Previously, the glycine loop of phosphorylase kinase was subjected to site-directed mutagenesis and the effects on catalysis were examined (*14*). When Val[29], which is equivalent to Ser[53] of cAPK C, was changed to a serine, only small effects on catalysis were observed. These data support the conclusions drawn from this study. One caveat to these conclusions is that the precise details of the kinetic mechanisms for the cAPK C−Ser[53] mutants were not examined rigorously. While these studies are important to fully understand the role of the glycine-rich loop in catalysis, they are beyond the scope of this report.

Results obtained with the C(S53P) mutant were rather different. This mutant has compromised ability to phosphorylate Kemptide (k_{cat}/K_m nearly 100-fold lower than that of wild-type C-subunit). These effects were manifested as a lower k_{cat} and reduced K_m's for both ATP and Kemptide. The K_i for ADP suggests that the binding of the nucleotide is reduced, but that at least part of the change in affinity for ATP may be due to lost interactions with the γ-phosphate. This mutant also has a reduced sensitivity to PKI and the type-I regulatory subunit, but binding to the type-II regulatory subunit is largely unaffected (Table 4). This is consistent with the requirement of ATP for the high-affinity binding of both PKI and the type-I regulatory subunit and further supports our model that these two inhibitors bind to the closed conformation of the kinase. The type-II regulatory subunit, which actually is a substrate for cAPK, binds in an ATP-independent manner. Therefore, that the binding of the type-II regulatory subunit is not affected is consistent with the role of the backbone amide of Ser[53] in ATP binding. This finding also suggests that the C(S53P) mutant does not alter the binding site for this macromolecular physiological inhibitor of the C-subunit.

On the basis of protein-footprinting experiments and in correlation with different structural models, it appears that binding of ATP is sufficient for the C-subunit to adopt the "closed" catalytically poised conformation (*40*). Binding of the type-I regulatory subunit and PKI is synergistically dependent on high-affinity ATP binding to the active site and, therefore, probably requires a closed conformation of the C-subunit. The glycine loop is the most mobile motif in

the cAPK C-subunit because it displays the highest crystallographic temperature factors (*6*, *10*). Indeed, the glycine loop moves a considerable distance during the transition from the open to the closed conformation (*5*, *41*). This mobility is thought to be critical for the function of the kinase. Introduction of a proline at the tip of the glycine loop could alter the inherent flexibility of this structure. This, in turn, could slow conformational changes required for catalysis and thereby affect the catalytic efficiency. Changes in the phosphotransfer rate and/or the release of products would not be unexpected.

Alternatively, reduced flexibility of the glycine loop may destabilize the closed conformation or make it less favorable energetically. Because this loop must move for the release of ADP and the binding of ATP to complete the catalytic cycle, changes in the loop's flexibility could alter enzyme function. While changing Ser^{53} to a proline did result in a 5-fold increase in k_4 (i.e., the ADP release rate) and a 3-fold decrease in ability of ADP to inhibit the C-subunit, the importance of the interaction of the γ-phosphate with the backbone amide of Ser^{53} cannot be ruled out. Additionally, this mutation leads to a measurable decrease in the chemical transfer of phosphate from ATP to peptide.

Finally, this mutation could adversely affect the overall fold of the C-subunit, leading to an enzyme with an altered structure, potentially affecting binding and/or changing the exact catalytic mechanism of the enzyme.

Ser^{53} also lies very close to the P-site of the peptide substrate in the cAPK C:ATP:PKI and C:AMPPNP:PKS ternary complexes (*2*, *11*). Therefore, it is not unlikely that this residue may influence the P-site specificity of cAPK. Interestingly, while cAPK appears to prefer to phosphorylate serine residues, two of the best protein substrates for cAPK, protein phosphatase inhibitor-1 (PPI-1) and dopamine and cAMP-regulate phosphoprotein (DARPP-32), are both phosphorylated on threonines (*42*−*44*). Therefore, it is enigmatic why threonine is phosphorylated much less efficiently when presented in the context of a short peptide substrate. One possible reason is that the side chain of Ser^{53} may conflict with the side chain of a threonine at the P-site. When C(S53G) was tested for its ability to phosphorylate a threonine-containing derivative of Kemptide (LRRATLG), this mutant was found to be 5 times more efficient at phosphorylating threonine than the wild-type C-subunit. The use of viscosogens to determine the individual microscopic rate constants revealed that the actual chemical transfer of the γ-phosphate from ATP to the threonine is 4 times higher with C(S53G) than with the wild-type. This accounts almost entirely for the observed elevation in k_{cat} of C(S53G) toward LRRATLG. Estimation of k_2 and k_{-2} from the solution viscosity experiments suggests that the K_m for this reaction with both the wild-type and C(S53G) is closer to the K_d of the peptide, although the K_d for LRRATLG is not much different from that of Kemptide (*38*). These findings indicate the importance of using more physiological substrates to examine the role on catalysis of these C-subunit point mutations. These studies currently are in progress.

In summary, the Ser^{53} side chain does not appear to play an essential role either in phosphorylation of Kemptide or in the interaction with physiological inhibitors such as PKI or either type of regulatory subunit. This study negates the hypothesis that the potential hydrogen bond between the β-hydroxyl of Ser^{53} and the carbonyl of the P-site backbone is critical for the synergistic binding of ATP and PKI to the C-subunit. However, with the caveat that changing this residue at the tip of the flexible glycine-rich loop to a proline may reduce its inherent mobility, eliminating the interaction of this residue with the γ-phosphate of ATP reduces the efficiency of the kinase. Therefore, the role of Ser^{53} seems to be to interact with the γ-phosphate of ATP, possibly positioning it for chemical transfer and potentially keeping the C-subunit in a "closed" and catalytically poised conformation.

The side chain of Ser^{53} may influence the phosphoacceptor preference of the cAPK C-subunit. It remains unclear whether the Ser^{53} side chain interferes when there is a threonine at the P-site in the substrate peptide. Interestingly, C(S53G) is more efficient than wild-type C-subunit at phosphorylating DARPP-32, a good threonine-containing substrate for cAPK (W. Hemmer and S. Taylor, unpublished observation). These results imply that, while Ser^{53} may influence P-site specificity, additional interactions between the C-subunit and the target substrate peptide−protein are important for influencing P-site specificity.

ACKNOWLEDGMENT

The authors thank Siv Garrod and Larry Gross for mass spectrometry, Cindy Gribskov for providing wild-type C-subunit, Elzbieta Radzio-Andzelm for computer graphics, and Joe Adams for assistance with the viscosity experiments and critical discussion.

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BI992800W