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 (G50TG52SFG55 in cAMP-dependent protein kinase (cAPK)), Gly52 is the most important for catalysis because it allows the backbone amide of Ser53 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 Ser53 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 Ser53 side-chain hydroxyl [Bossemeyer, D. et al. (1993) EMBO J. 12, 849]. To address the importance of the Ser53 side chain in catalysis, inhibition, and P-site specificity, Ser53 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 Ser53 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 optimal positioning of the phosphates of ATP; cAMP-dependent protein kinase, cAPK; cAPK catalytic subunit, C

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, PPLC; 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 Ser53 replaced with glycine].
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 a potential hydrogen bond between the $\gamma$-hydroxyl of Ser53 and the backbone amide of the P-site. Conversely, the backbone amide of Ser53 hydrogen bonds to Lys168 in the large lobe and also may be involved in ternary complexes with a 20-residue fragment of the heat-stable protein kinase inhibitor (PKI), PKI$_{C-24}$, and either ATP or 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 Ser53 on P-site specificity. These experiments provide new information as to the role of Ser53 on the catalytic activity 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). $[^{33}P] ATP$ was from New England Nuclear—DuPont (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-Genie site-directed mutagenesis kit was purchased from BioRad (Hercules, CA). The heptapeptides LRRASLG (Kemptide), LRRATLG (Thr-Kemptide), and LRRALG were synthesized at the Peptide Synthesis Kit was purchased from BioRad (Hercules, CA).

**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 mg/mL ampicillin at 37 °C to an optical density at 600 nm of 0.6–0.8, induced with 0.5 mM isopropyl-$\beta$-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
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S FPLC as described previously (19). Mutant proteins were purified by formation of holoenzyme with a poly-histidine-tagged type-II regulatory-subunit mutant [His6RII(R213K)] (20). Cells were suspended in 20 mL/L of culture in 50 mM potassium phosphate, 150 mM KC1, 2 mM MgCl2, 0.2 mM ATP, and 2 mM 2-mercaptoethanol (pH 8.0). Cells expressing C-subunit mutants and His6RII(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 His6RII(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 x 1 bed volumes of buffer, including 1 mM cAMP. Fractions were pooled and dialyzed into 20 mM potassium phosphate, 50 mM KC1, 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 KC1 over 50 mL. Phosphoisoforms of the C-subunits eluted as described previously (19, 21).

Purification of RII(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 imidazole, and the His6RII(R213K) was eluted with buffer containing 0.25 M imidazole. Protein-containing fractions were pooled, dialyzed into 20 mM MOPS (pH 7.0), 0.15 M KC1, 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 MgCl2 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 [32P]-γ-ATP (26). The C-subunit (0.1–2.0 nM) was incubated in 50 mM MOPS (pH 7.0), 0.1 M KC1, 10 mM MgCl2, 1 mM DTT, 100 μg/mL bovine serum albumin, 2–10 μCi of [32P]-γ-ATP, unlabeled ATP, and peptide substrate. For Km 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 Km’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% H3PO4 (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 = Vmax[S]/(S + Km) where v is the reaction rate, Vmax is the maximum rate, [S] is the concentration of the variable substrate, and Km 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; LRRALG, 10 μM–10 mM) at several fixed substrate concentrations. Data were fitted to a single-site binding model using GraphPad Prizm Software, and Ki values were extrapolated from IC50 values using the relationship of Cheng and Prusoff: Ki = IC50/(1 + [S]/Km) (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 viscogogen did not affect the binding of the peptide to P81.

RESULTS

Expression and Purification of Ser53 Mutants. To examine the role of Ser53 in catalysis, substrate specificity, and the ability of cAPK C-subunit to interact with pseudosubstrate inhibitors, this residue was subjected to site-directed mutagenesis. Ser53 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 [His6RII(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 His6RII(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 KC1 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 Ser53
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 Ser53 mutants with the heptapeptide, LRRASLG (Kemptide), were examined. Both C(S53T) and C(S53G) 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 $\beta$-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 Ser53 mutants maintain an ordered kinetic mechanism (34, 35).

Ser53 Influences the P-Site Specificity of cAPK C-Subunit. To further characterize the role of Ser53 in the ability of the C-subunit to phosphorylate peptide substrates, the steady-state kinetic parameters of wild-type C-subunit and the Ser53 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 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.

| Table 1: Mass Determination of Mutant cAPK Catalytic Subunit Mutants |
|----------------|----------------|----------------|----------------|
| mutant (isoform) | no of PO4 | expected mass (Da) | experimental mass (Da) |
| wild-type (I) | 4 | 40760 | 40763 |
| wild-type (II) | 3 | 40680 | 40684.2 (6.9) |
| wild-type (III) | 2 | 40600 | 40601 |
| Ser53Gly (I) | 4 | 40730 | 40725.5 (6.1) |
| Ser53Gly (II) | 3 | 40650 | 40644.1 (6.4) |
| Ser53Gly (III) | 2 | 40570 | 40575.2 (7.0) |
| Ser53Thr (I) | 4 | 40774 | 40779.7 (6.4) |
| Ser53Thr (II) | 3 | 40694 | 40693.8 (7.2) |
| Ser53Thr (III) | 2 | 40614 | 40618.5 (6.4) |
| Ser53Pro (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.
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Table 2: Steady-State Kinetic Parameters of Wild-Type and Mutant cAPK Catalytic Subunits

<table>
<thead>
<tr>
<th>parameter</th>
<th>ligand</th>
<th>wild-type</th>
<th>C(S53T)</th>
<th>C(S53G)</th>
<th>C(S53P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_{cat}$ (s$^{-1}$)</td>
<td>LRRASLG</td>
<td>21.7 ± 0.7</td>
<td>20.8 ± 0.3</td>
<td>31.9 ± 1.5</td>
<td>10.4 ± 0.2</td>
</tr>
<tr>
<td>$K_m$ (µM)</td>
<td>LRRASLG</td>
<td>21.9 ± 2.4</td>
<td>14.5 ± 0.9</td>
<td>22.2 ± 3.5</td>
<td>489.5 ± 20.7</td>
</tr>
<tr>
<td>$k_{cat}/K_m$ (µM$^{-1}$ s$^{-1}$)</td>
<td>LRRASLG</td>
<td>1.0</td>
<td>1.4</td>
<td>1.4</td>
<td>0.021</td>
</tr>
<tr>
<td>$k_3$ (s$^{-1}$)</td>
<td>LRRATLG</td>
<td>7.9 ± 0.5</td>
<td>9.1 ± 0.5</td>
<td>24.5 ± 1.0</td>
<td>nd</td>
</tr>
<tr>
<td>$K_a$ (µM)</td>
<td>LRRATLG</td>
<td>654.0 ± 108.3</td>
<td>745.6 ± 102.0</td>
<td>365.7 ± 48.4</td>
<td>nd</td>
</tr>
<tr>
<td>$k_{cat}/K_m$ (µM$^{-1}$ s$^{-1}$)</td>
<td>LRRATLG</td>
<td>0.012</td>
<td>0.012</td>
<td>0.067</td>
<td>nd</td>
</tr>
<tr>
<td>$K_m$ (µM)</td>
<td>ATP</td>
<td>12.7 ± 0.5</td>
<td>12.5 ± 0.4</td>
<td>25.1 ± 0.9</td>
<td>108 ± 18.1</td>
</tr>
<tr>
<td>$K_i$ (µM)</td>
<td>ADP</td>
<td>14.6 ± 0.3</td>
<td>12.5 ± 0.4</td>
<td>30.7 ± 0.9</td>
<td>43.5 ± 1.1</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Scheme 1</th>
<th>LRRASLG</th>
<th>LRRATLG</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_{cat}$</td>
<td>C(S53G)</td>
<td>C(S53P)</td>
</tr>
<tr>
<td>0.99 ± 0.06</td>
<td>1.00 ± 0.03</td>
<td>0.09 ± 0.01</td>
</tr>
<tr>
<td>0.05 ± 0.02</td>
<td>0.09 ± 0.03</td>
<td>0.00 ± 0.06</td>
</tr>
<tr>
<td>500 ± 60$^a$</td>
<td>fast$^a$</td>
<td>11.4$^a$</td>
</tr>
<tr>
<td>21.7</td>
<td>31.9</td>
<td>111.6</td>
</tr>
</tbody>
</table>

$^a$ Values were derived as follows: $k_3 = k_{cat}/(1-K_m)$; $k_i = k_{cat}/k_{cat}$. Values for $k_{cat}$ and $(k_{cat}/K_m)$ 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 $(k_{cat}/K_m)$, determination of microscopic rate constants $k_2$ and $k_4$ according to Scheme 1 was not possible (36). Parameters determined by pre-steady-state kinetic quench-flow analysis (36).

Scheme 1

$E$:$ATP + S \xrightleftharpoons[k_2]{k_1} E$:$ATP:S \xrightarrow{k_4} E$:$ADP + P \xrightarrow{k_i} E$:$ADP$

(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 ($\eta$). 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 $k_3$ (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 $K_m$, the peptide binding constant. To circumvent this problem, the $K_i$ for an inhibitory version of Kemptide, LRRATLG, where the P-site serine is replaced with alanine, was examined with wild-type C-subunit and 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 $K_i$, 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 $k_{cat}$.

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$\text{K}_524$ 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 $\beta$-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

<table>
<thead>
<tr>
<th>inhibitor C-subunit</th>
<th>wild-type (C53T)</th>
<th>C53G</th>
<th>C53P</th>
</tr>
</thead>
<tbody>
<tr>
<td>RI (10^{-8} M)</td>
<td>0.15 ± 0.03</td>
<td>0.20 ± 0.05</td>
<td>0.12 ± 0.04</td>
</tr>
<tr>
<td>RII (10^{-8} M)</td>
<td>0.23 ± 0.04</td>
<td>0.19 ± 0.08</td>
<td>0.30 ± 0.05</td>
</tr>
<tr>
<td>PKI (10^{-7} M)</td>
<td>0.40 ± 0.06</td>
<td>0.27 ± 0.06</td>
<td>0.12 ± 0.07</td>
</tr>
</tbody>
</table>

All values were determined using varying inhibitor concentrations and at least three fixed Kemptide concentrations above and below their respective IC50 values. Ki values were calculated from IC50 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.

In this report, Ser53 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 Ser53 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 Ser53 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 (I4). When Val59, which is equivalent to Ser53 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—Ser53 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 (kcat/Km nearly 100-fold lower than that of wild-type C-subunit). These effects were manifested as a lower kcat and reduced Km's for both ATP and Kemptide. The Ki 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 Ser53 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

suggest the hydrogen bonds between the γ-phosphate of ATP and both Ser53 and Lys168 (5, 10, 11) in the catalytic loop are the more important interactions for efficient phosphoryltransfer.

Effect of Mutations on the Interaction of the C-Subunit with the Regulatory Subunits. To determine if Ser53 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 Ser53 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 IC50 of 40-fold, is affected most dramatically. The effect on PKI inhibition is even greater, with the Ki increased by 250-fold. Therefore, while the potential hydrogen bond between the β-hydroxyl of Ser53 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 (I4) and the cAPK C-subunit (I) indicate that these residues play a critical role in catalysis. In the cAPK C-subunit, Ser53 lies at the distal tip of the loop very near the site of phosphate transfer. Structural models indicated that this residue can form hydrogen bonds to both the γ-phosphate of ATP and the P-site carboxyl 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).
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 to a proline did result in a 5-fold increase in \( k_1 \) (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 \( \gamma \)-phosphate with the backbone amide of Ser 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 also lies very close to the P-site of the peptide substrate in the cAPK C:ATP:PKI and C:AMPNNPKS 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 (DARP-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 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 \( \gamma \)-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 \( \beta \)-hydroxyl of Ser 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 \( \gamma \)-phosphate of ATP reduces the efficiency of the kinase. Therefore, the role of Ser\(^{53} \) seems to be to interact with the \( \gamma \)-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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