Role of the Glycine Triad in the ATP-binding Site of cAMP-dependent Protein Kinase

(Received for publication, January 8, 1997, and in revised form, April 22, 1997)

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A glycine-rich loop in the ATP-binding site is one of the most highly conserved sequence motifs in protein kinases. Each conserved glycine (Gly-50, Gly-52, and Gly-55) in the catalytic (C) subunit of cAMP-dependent protein kinase (cAPK) was replaced with Ser and/or Ala. Active mutant proteins were expressed in Escherichia coli, purified to apparent homogeneity, separated into phosphoisoforms, and characterized. Replacing Gly-55 had minimal effects on steady-state kinetic parameters, whereas replacement of either Gly-50 or Gly-52 had major effects on both $k_m$ and $k_{cat}$ values consistent with the prediction of the importance of the tip of the glycine-rich loop for catalysis. Substitution of Gly-50 caused a 5–8-fold reduction in $K_m$ and $k_{cat}$, whereas replacement of either Gly-50 or Gly-52 had major effects on both $k_m$ and $k_{cat}$. The $K_m$ (ATP) and $k_{cat}$ (peptide) values of C(G50S) and C(G52S) were increased 3–5-fold, respectively, and the $k_{cat}$ was decreased 6-fold. In contrast to catalytic efficiency, the ATPase rates of C(G50S) and C(G52S) were increased by more than an order of magnitude. The thermostability of each mutant was slightly increased. Unphosphorylated C(G52S) was characterized as well as several isoforms phosphorylated at a single site, Ser-338. All of these phosphorylation-defective mutants displayed a substantial decrease in both enzymatic activity and thermal stability that correlated with the missing phosphate at Thr-197. These results are correlated with the crystal structure, models of the respective mutant proteins, and conservation of the Glyx within the protein kinase family.

The eukaryotic protein kinases specific for serine, threonine, and tyrosine all share a conserved catalytic core that folds into a topologically similar three-dimensional structure (2–5). Particularly conserved in this core are the amino acids involved in nucleotide binding and catalysis with one of the most highly conserved features being a GXGXXG motif near the amino terminus. The first and second glycines of this triad are essentially invariant, whereas the third is somewhat more variable but is always a small residue. Our goal is to understand the functional importance of this motif, a hallmark of the protein kinase family.

The catalytic (C) subunit of cAMP-dependent protein kinase (cAPK), one of the most extensively investigated members of the protein kinase family, has served repeatedly as a prototype for studying structure-function relationships in this enzyme family (6, 7). It is a relatively simple protein kinase, and both its regulatory and catalytic subunits can be purified readily as active proteins in Escherichia coli. The glycine-rich motif in the C-subunit, (LG50TG52SFG55RV), displays at each position the amino acid residue found in the majority of protein kinases (3, 4).

The first crystal structure of a protein kinase was a binary complex of the C-subunit and a peptide inhibitor (2, 8). Based on this structure and on subsequent structures of other protein kinases, the conserved features of the unique protein kinase nucleotide fold have been defined (9–13). The glycine-rich sequence is located in the small lobe; specifically, it is part of a $\beta$-strand-$\beta$-strand-$\beta$-strand nucleotide positioning motif (Fig. 1) that is an integral part of the ATP-binding site. This segment serves as a lid to anchor the ATP and to shield the bound nucleotide from solvent. The adenosine is secured beneath the $\beta$-strands, and the phosphates of ATP are anchored by the loop through hydrogen bonds to backbone amides. This loop between $\beta_1$ and $\beta_2$ is referred to here as the “glycine-rich loop,” as opposed to the “P-loop” of mononucleotide kinases (14–16). The P-loop also contains several conserved glycines but has a somewhat different consensus and represents a structurally distinct motif even though the common function of both is to anchor the phosphates of ATP.

The glycine-rich loop is thought to participate in ATP binding as well as to contribute to catalysis based on several lines of evidence (7, 16). The crystal structures of several distinct substrate and product complexes of the C-subunit, for example, are essentially superimposable with the exception of localized changes within the glycine-rich loop, thus indicating that this is a very sensitive and mobile part of the molecule (17). Steady-state kinetics also emphasize the potential importance of this region since the rate-limiting step is diffusion controlled and very likely corresponds to the release of ADP (18–20). Because the phosphoryl transfer step is 20-fold faster than the $k_{cat}$ (21), localized mobility of the glycine-rich loop is likely to be of great functional importance both for cAPK and for protein kinases, in general.

The eukaryotic protein kinases specific for serine, threonine, and tyrosine all share a conserved catalytic core that folds into a topologically similar three-dimensional structure (2–5). Particularly conserved in this core are the amino acids involved in nucleotide binding and catalysis with one of the most highly conserved features being a GXGXXG motif near the amino terminus. The first and second glycines of this triad are essentially invariant, whereas the third is somewhat more variable but is always a small residue. Our goal is to understand the functional importance of this motif, a hallmark of the protein kinase family.

The catalytic (C) subunit of cAMP-dependent protein kinase; PKI, heat stable protein kinase inhibitor; IP20, inhibitor peptide corresponding to PKI(5–24); MOPS, 4-morpholinepropane-sulfonic acid; MES, (N-morpholino)ethanesulfonic acid; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography. Mutants with amino acid substitutions are denoted in the standard one-letter code by the wild-type residue and numbered position within the sequence followed by the amino acid substitution. Residue numbers are given with respect to the cDNA-derived sequence of the murine Cα-subunit (1) assigning the first number to the glycine following the initiating methionine which is cleaved off in the native as well as in the recombinantly expressed enzyme.
Biochemical analysis of protein kinases carrying mutations within the glycine triad has so far been limited but reiterates the importance of this part of the molecule. In a diabetes-related variant of the insulin receptor kinase the third Gly is replaced by Val, thus impairing tyrosine kinase activity (22). When the third Gly is replaced by Ala in p59 

Mutations were introduced into the wild-type murine C-subunit of cAPK was assessed here using site-directed mutagenesis by replacing each Gly with Ala and Ser. After purifying each mutant protein, since correct phosphorylation has been shown previously (29). All mutations were confirmed by sequence analysis. Protein expression checks were done as described previously (29). All proteins were expressed as free catalytic subunits. Some mutants were also expressed as polyhistidine fusion proteins to construct the fusion proteins, the 1.9-kilobase pair NdeI/HindIII cat fragment generated from the Ca/pREN30 construct was subcloned into the NdeI/HindIII sites of pET-15b. Inserts were analyzed for orientation by sequencing, and protein expression checks were done as described previously (29). All mutations were confirmed by sequence analysis.

**Experimental Procedures**

**Construction of Mutant C-subunits**

Wild-type C-subunit was purified as described previously (30, 31). Mutant proteins were purified by two methods. Method A utilized a slightly modified version of the published procedure using chromatography on Mono-S to resolve the isoforms. Alternatively, a newly developed co-lysis method (Method B) was used that is based on rapid holoenzyme formation with a polyhistidine RII-fusion protein, H-RH(R213K) (29). This is an altered form of the type II R-subunit that has a mutation in cAMP-binding site A which lowers the cAMP affinity of the holoenzyme. This rapid method was required for those mutants that were more kinetically impaired.

**Expression and Cell Extraction**

Mutant C-subunits were expressed in *E. coli* BL21-DE3. Cells were grown at 37 °C to an absorbance at 600 nm of 0.5 to 0.8 and then induced with 0.5 mM isopropyl-

**Purification**

Mutations were introduced into the wild-type murine Co-subunit gene by oligonucleotide-directed mutagenesis using single-stranded Kunkel template (28) of a Ca/pREN30 construct as described previously (29). All proteins were expressed as free catalytic subunits. Some mutants were also expressed as polyhistidine fusion proteins. To construct the fusion proteins, the 1.9-kilobase pair NdeI/HindIII cat fragment generated from the Ca/pREN30 construct was subcloned into the NdeI/HindIII sites of pET-15b. Inserts were analyzed for orientation by sequencing, and protein expression checks were done as described previously (29). All mutations were confirmed by sequence analysis.
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Proteins were eluted using a salt gradient (66 ml) from 50 to 400 mM KCl at 1 ml/min.

Method B—This procedure was performed as described elsewhere (29). In summary, cells that expressed the mutant C-subunit were co-expressed with cells that expressed H2RII(R213K), a mutant form of the RI subunit that has a defective cAMP-binding site A. The combined cell pellets, typically using a ratio of 3:2:1, were resuspended in 20 ml of buffer C (30 mM MOPS, pH 8.0, 5 mM ATP, 15 mM MgCl2, 5 mM β-mercaptoethanol) per liter of original culture. The cells were lysed as described above and centrifuged. After incubating for 30 min at 4 °C, Ni2+ resin (Invitrogen) that had been pre-equilibrated in buffer C was added for each liter of culture overexpressing H2RII(R213K). 1 ml of pre-equilibrated Ni2+ resin were added. After incubation for 60 min at 4 °C, the resin was recovered by low speed centrifugation and washed twice in 40 ml of buffer C containing 25 mM KCl. The C-subunit was then specifically eluted by resuspending the resin in the same buffer containing 1 mM cAMP. The last step was performed twice. Dialysis and Mono-S chromatography were carried out as described above.

Protein Determination

C-subunit concentration was determined by absorbance at 280 nm (A280 = 1.2) and by titrations with PKI, cAMP-free recombinant RI subunit (32) and RI(R209K), a mutant RI-subunit that has a defect in cAMP-binding site A (26, 33). The variance of the protein concentrations determined by both methods was typically between 5 and 15%. In a few cases the concentrations determined by absorbance at 280 nm were more accurate than those determined by titration with either PKI or RI(R209K). This most likely was due to the presence of soluble but inactive protein, since the latter method reflects the concentration of active C-subunit, whereas the absorbance measures total C-subunit. Purity was checked by SDS-PAGE, using 12.5% gels (34).

Isoelectric Focusing (IEF) and Two-dimensional Gel Analysis

Isoforms of each mutant C-subunit were analyzed by analytical native IEF as described previously (31) using Pharmacia, LKB Ampholine PAG plates with a pH range from 3.5 to 9.5. Gels were fixed and stained with Coomassie Blue R-250, and isoelectric points (pIs) were determined as described. Two-dimensional gel electrophoresis was performed basically according to O’Farrell (35) with some slight modifications. In summary, the isoelectric focusing was done under denaturating conditions using rod gels (9.2% urea, 3.8% acrylamide, 0.2% bisacrylamide, 5 mM imidodiacetic acid and 0.01 M ethylenediamine, respectively. Prefocusing was performed at 300, 600, and 1000 V for 30 min each, using a buffer containing 9.5 m urea, 2% Nonidet P-40, 2% pharmalytes (Pharmacia) (3-10) in a Bio-Rad chamber. Anode and cathode solutions were 0.01M 0.2% bisacrylamide, 2% Nonidet P-40, and 2% pharmalytes (Pharmacia) with buffer containing 8 M urea, 5% Nonidet P-40, and 1% pharmalytes (Pharmacia) were added as internal controls to map precisely the positions of proteins on the two-dimensional gels.

Peptide Sequencing and Mass Spectrometry

The proteins were digested with trypsin and separated by HPLC as described previously (36). A gas phase sequencer with an on-line phenylthiohydantoin analyzer (Applied Biosystems 470A and 120) was used to sequence the peptides. Electrospray/mass spectrometry (ES/MS) was performed on a Hewlett-Packard 59987A electrospray MS. Proteins were desalted using narrow bore chromatography (Michrom Biosources Inc.) before ES/MS.

Activity Assay

Kinase activity was measured spectrophotometrically (18) using the heptapeptide, LRRALG (Kemptide), as substrate. This assay couples the generation of ADP with the oxidation of NADH by pyruvate kinase and lactate dehydrogenase. Varying amounts of ATP were preequilibrated with C-subunit in buffer containing 100 mM MOPS, pH 7.1, 15 mM DTT, 0.5 mM NADH, 1 mM phosphoenolpyruvate, 12 units of lactate dehydrogenase (Sigma), and 4 units of pyruvate kinase (Sigma) in a total volume of 1 ml at 25 °C. The concentration of MgCl2 was 9 mM in excess of ATP in all assays. Reactions were initiated by the addition of Kemptide. Preincubation of C-subunit with either ATP or Kemptide had no effects on the observed initial velocities, and activities were linearly dependent on the amount of C-subunit added. Titrations with PKI or R-subunit were performed by incubating with inhibitor in the assay mix for 2 min prior to the addition of Kemptide. Kemptide and an inhibitor peptide, LRRALG, were synthesized by the UCSD Peptide and Oligonucleotide Facility and purified by HPLC.

Kinetic Analyses

Kinase activity was measured at varying concentrations of one substrate and saturating concentrations of the other. Saturating conditions were at least 5-fold above the Km, typically 1–10 mM for ATP and 0.2–0.4 mM for Kemptide. The concentration of MgCl2 was always 9 mM in excess of ATP. Km and Vmax values were determined as described previously (37). Plots of initial velocity (v) versus concentration of the varied substrates (S) were analyzed by hyperbolar curve fitting according to the Michaelis-Menten equation.

LRRALG is a competitive inhibitor with respect to Kemptide and noncompetitive with respect to ATP (38). The K for LRRALG was measured by preincubating C-subunit for 5 min with LRRALG in the assay mix prior to initiating the reaction by the addition of Kemptide. Concentrations of LRRALG were varied from 0 to 6.2 mM at several fixed concentrations. Apparent K values were determined by plotting reciprocal velocity against the inhibitor concentration. Each determination was measured at Kemptide concentrations below and above the Km.

ATPase Rates

ATPase activity was measured by incubating C-subunit in the above described spectroscopic assay mixture in the absence of Kemptide at ATP concentrations between 1 and 4 mM (at least 10-fold above the KATPase). MgCl2 was 9 mM in excess of ATP. Using a multivvette holder, the samples (with or without C-subunit) were simultaneously monitored for absorbance at 340 nm. To determine the relative ATPase rate, the C-subunit-dependent ATPase rate was divided by the enzymatic activity, measured as described above using Kemptide as a substrate. All determinations were done in duplicate.

Thermostability

Recombinantly expressed wild-type or mutant C-subunit (2.4–7.2 μM) was dialyzed into 20 mM MOPS, pH 7.0, 150 mM KCl, 1 mM DTT. Bovine serum albumin (Sigma) was then added to a total protein concentration (C + bovine serum albumin) of 3 mg/ml. The solution was incubated in a polymerase chain reaction thermocycler (PTC-100, MJ Research, Inc.) for 3 min at indicated temperatures and then stored on ice and analyzed for catalytic activity at room temperature. Samples were analyzed for thermostability over the range of 25–52 °C either in the absence or presence of 2 mM ATP and 11 mM MgCl2.

RESULTS

Expression and Purification—To investigate the functional importance of the three glycines in the glycine-rich loop, each was replaced with both Ala and Ser. The resulting mutant proteins, referred to as C(G50A), C(G50S), C(G52A), C(G52S), C(G55A), and C(G55S), were expressed in E. coli BL21-DE3. In addition, four mutant proteins were expressed as polyhistidine-tagged proteins and are referred to as H6C(G50A), H6C(G50S), H6C(G52A), and H6C(G52S). Rather conservative mutations were chosen to avoid large structural perturbations.

Although the solubility of most of the mutant proteins was significantly reduced compared with wild-type C, expression at 24 °C typically yielded proteins that were at least partially soluble. C(G55A) and C(G55S) were most similar to wild-type C and were purified using phosphocellulose chromatography (Fig. 2). The isoforms, each representing a distinct phosphorylation state (31, 36), were subsequently resolved by Mono-S chromatography as described under “Experimental Procedures” (Fig. 3). Both proteins were purified to apparent homogeneity.

In contrast, although C(G50S) was soluble and very pure based on SDS-PAGE when eluted from P11 resin, it did not bind readily to the Mono-S column and was mostly inactive. This protein, as well as C(G50A), was therefore purified using a newly developed alternate method (29). This co-lysis method is based on a polyhistidine-tagged RI1 subunit carrying a mutation in cAMP-binding site A that reduced the affinity for
cAMP. When this mutant R-subunit was co-lysed with C, holoenzyme formed immediately in the bacterial extract. C-subunit was then purified rapidly by immobilizing the holoenzyme complex on Ni²⁺ resin and then eluting the free C-subunit with cAMP (Fig. 4). Cation-exchange chromatography on Mono-S was used as a final step to separate distinctly phosphorylated isoforms.

Substituting the most highly conserved glycine (Gly-52) with either Ala or Ser also yielded proteins that could not be purified using the P11 resin. Almost no binding to the P11 resin was observed for these mutant C-subunits. Co-lysis of C(G52S) with H₆RHI(R213K), however, resulted in highly purified C-subunit that was subsequently resolved into isoforms following Mono-S chromatography. Amounts of C(G52A) sufficient for kinetic and biochemical analysis were never obtained.

Characterization of the Phosphorylation State of the Mutant C-Subunits—Wild-type C-subunit expressed in E. coli is comprised of a mixture of three isoforms, I, II and III, that contain 4, 3, or 2 phosphates, respectively (31, 36). In the E. coli-expressed protein, all of these phosphates (Ser-10, Ser-139, Thr-197, and Ser-338) derive from autophosphorylation. Isoforms I–III all have the same specific activity (31) demonstrating that the phosphorylations at Ser-10 and Ser-139 do not effect the enzyme’s activity and are not essential for the folded protein. Replacing Thr-197 of a glutathione S-transferase fused C-subunit with Ala, however, had major effects on activity (26). Thus it was essential to establish the phosphorylation state of each mutant protein not only to properly compare it to wild-type C-subunit but also to make certain that any observed differences were due to the glycine mutation and not to a defect in phosphorylation. To do this, each protein peak eluted from the Mono-S column was analyzed by isoelectric focusing two-dimensional gel electrophoresis, peptide sequence analysis, and mass spectrometry (summarized in Table I).

As was shown previously for wild-type C, proteins that elute early from the Mono-S column are typically phosphorylated to a higher degree than late eluting peaks (31). Whereas wild-type C elutes mostly as isoforms I and II, the mutant proteins showed considerable variability. Most of the mutant proteins, however, could be purified as isoform III which contains two phosphates (Thr-197 and Ser-338). For the following reasons, this isoform was considered to be comparable to fully active wild-type C. (i) Phosphorylations at Ser-10 and Ser-139 were shown to have no affect on the activity of recombinant wild-type C (31). (ii) The active mammalian enzyme is only phosphorylated at Thr-197 and Ser-338 (39). (iii) Phosphorylation of Thr-197 correlates with the posttranslational activation of cAPK (27). These isoforms containing two phosphates were thus considered, for kinetic purposes, to be fully phosphorylated enzymes.

The phosphorylation state of each mutant protein was confirmed first by IEF. The IEF analysis of C(G55S), shown in Fig. 5, indicated that isoform II was predominant. The pI of isoform III for wild-type and mutant Cs was typically 8.2 (31). The phosphorylation state of the proteins was also confirmed by mass spectrometry. Specific sites of phosphorylation were confirmed by sequencing the tryptic peptides and by ES/MS following HPLC. The characterization of each isoform is summarized in Table I.

In addition to isoform III, several mutant C-subunits, C(G50S), H₆C(G50A), C(G52S), were only phosphorylated at a single site based on mass spectrometry (Table I). Native IEF of these isoforms, corresponding to isoform IV, revealed a pI of 8.8 to 8.9. Tryptic digests followed by peptide sequencing and/or mass spectrometry showed in all cases that only Ser-338 was phosphorylated. No phosphorylations at other sites were found. Finally, for the first time, completely unphosphorylated C-subunit, C(G52S)-V, was purified and partially characterized. The singly and nonphosphorylated forms are referred to as underphosphorylated proteins.

Steady-state Kinetic Analysis of Mutant Proteins—In Table II, the steady-state kinetic parameters of the mutant proteins phosphorylated at 2 or more sites are compared with wild-type C-subunit. Each mutation affected catalytic activity; however,
some mutations were significantly more severe than others. Replacements of Gly-55 had the least effect, with little or no change in $K_m$ and only a 3- to 4-fold increase in $K_m/\text{peptide}$. In contrast to Gly-55, substitution of Gly-50 and Gly-52 had more severe consequences both on the $K_m$ values and $k_{cat}$ (Table II). The $K_m/\text{peptide}$ increased approximately 10-fold as a result of the Gly-50 mutations and the $K_m/\text{ATP}$, 5–8-fold. At the same time the $k_{cat}$ was reduced 50–80%. The $k_{cat}$ ($K_m/\text{peptide}/K_m/\text{ATP}$), used here as a criteria for overall catalytic efficiency, was thus reduced by over 2 orders of magnitude when Gly-50 was replaced with either Ala or Ser.

C(G52S)-III was the most defective of the mutant enzymes. The $K_m/\text{peptide}$ and $K_m/\text{ATP}$ were 20- and 8-fold greater, respectively, than wild type C, and the $k_{cat}$ was 17% wild type C. The catalytic efficiency of this enzyme was thus reduced by 3 orders of magnitude. Pure isoforms of C(G52A) could not be isolated in sufficient quantities to allow for kinetic analysis.

To further assess the affinity of some of the mutant enzymes for peptide, the $K_m$ for the heptapeptide LRRAALG, where the phosphorylation site Ser was replaced by Ala, was also determined. The $K_m$ of wild-type C, C(G50A)-III, and C(G52S)-III were 0.83, 1.93, and 0.89 mM, respectively, indicating that the substitution of Gly-50 to Ala but not the substitution of Gly-52 reduced the affinity of the enzyme for the heptapeptide.

The underphosphorylated proteins showed even greater changes in steady-state kinetic parameters than the fully phosphorylated mutant proteins, displaying 30–80-fold elevated $K_m$ values (Table III). The $K_m/\text{peptide}$ ranged between 0.67 and 0.9 mM, and the $K_m/\text{ATP}$ varied between 1.1 and 1.64 mM. These values are very similar to what was reported for the T197A mutant (26, 27). The $k_{cat}$ for these mutant proteins was also decreased by more than an order of magnitude. The completely nonphosphorylated form of C(G52S), isoform V, had a $k_{cat}$ that was significantly lower than the singly phosphorylated isoform, C(G52S)-IV.

**ATPase Activity**—In addition to the catalytic activity, the rate of ATP hydrolysis was also determined for each mutant C-subunit (Table IV). The ratio of the absolute ATPase rate divided by the catalytic rate, subsequently referred to as relative ATPase rate, reflects the probability with which a bound ATP molecule is hydrolyzed versus being transferred to the peptide. Substitution of Gly-50 to Ala caused a slight increase in the absolute ATPase rate, but the relative ATPase rate increased by almost an order of magnitude. Replacing Gly-50 or Gly-52 with Ser increased the ATPase rate by more than an order of magnitude. This combination of an increased ATPase rate with a reduced $k_{cat}$ resulted in a 30-fold increase in the relative ATPase rate for C(G50S) and an increase by almost 2 orders of magnitude for C(G52S).

**Thermostability**—The average $T_m$ values for wild-type C in the absence and presence of MgATP were 45.4 and 48.5 °C, respectively. All type III isoforms of the mutant proteins had a

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**Table I**

| Isoform $^a$ | Phosphorylation sites | pH $^b$ | No. of PO$_4$$^c$ | Molecular weight $^d$ |
|-------------|----------------------|--------|-------------------|----------------------|
|             |                      |        |                   | Observed $^o$ | Calculated |
| wt-C I      | T197, S338, S10, S139| 6.4    | 4                 | 40,763     | 40,760     |
| wt-C II     | T197, S338, S10      | 7.2    | 3                 | 40,682     | 40,680     |
| wt-C III    | T197, S338           | 8.2    | 2                 | 40,600     | 40,600     |
| C(G50A)-III| T197, S338           | 8.2    | 2                 | 40,611 ± 7 | 40,614     |
| C(G50A)-IV | S338                 | 8.8    | 1                 | 40,534 ± 5 | 40,534     |
| H$_6$C(G50A)-III | T197, S338 | ND | 2 | 42,777 ± 5 | 42,778 |
| H$_6$C(G50A)-IV | ND | ND | 1 | 42,699 ± 6 | 42,698 |
| C(G50S)-III| T197, S338           | 8.2    | 2                 | 40,629 ± 7 | 40,630     |
| C(G50S)-IV | S338                 | 8.8    | 1                 | 40,549 ± 5 | 40,550     |
| H$_6$C(G50S)-II | ND | ND | 3 | 42,887 ± 10 | 42,874 |
| C(G52S)-III| ND                   | ND     | 2                 | 40,629 ± 5 | 40,630     |
| C(G52S)-IV | S338                 | 8.8    | 1                 | 40,554 ± 7 | 40,550     |
| H$_6$C(G52S)-V | >9.3 | 0  | 40,468 ± 5 | 40,470 |
| H$_6$C(G52S)-III | ND | ND | 2 | 42,788 ± 10 | 42,794 |
| C(G55A)-III| ND                   | ND     | 2                 | 40,614 ± 5 | 40,614     |
| C(G55S)-III| No phosphate on S10$^e$ | 8.2    | 2 | ND | 40,630 |

$^a$ The isoforms of the mutant C-subunit proteins were numbered based on the isoforms of wild-type C according to order of selection from Mono-S (31). Values for the wild type C (wt-C I-C II and -C III) are taken from Herberg et al. (31).

$^b$ Determined by native isoelectric focusing.

$^c$ The number of phosphates per molecule was determined either by mass spectrometry (ES/MS) and in most cases was confirmed by native IEF in analogy to wild-type C. The fact that C(G55S)-III had the same charge as the isoform III of wild-type C was additionally corroborated by two-dimensional gel analysis (Fig. 5).

$^d$ Measured by ES/MS. At least two measurements were made and averages ± average error are given.

$^e$ Absence of phosphate on Ser-10 was confirmed by sequence analysis of the tryptic peptide. The determination of the phosphorylation state of tryptic peptides was mainly based on ES/MS following HPLC. Matches with less than 2-atmospheric mass unit difference between the calculated and measured mass were accepted for the positive identification of sites. Some of the results were also confirmed by peptide sequencing. ND, not determined.
slightly higher $T_m$ than wild-type C in the absence of Mg-ATP (Table V and Fig. 6). However, in the presence of Mg-ATP, which protects the wild-type enzyme to some extent from thermal denaturation, only C(G55S)-III was more stable than wild-type C. C(G50A)-III and C(G50S)-III showed little or no protection by Mg-ATP.

The thermostability of monophosphorylated mutant proteins, lacking a phosphate at Thr-197, was significantly reduced. The $T_m$ values for C(G50A)-IV and C(G52S)-IV were 4 to 5 °C lower than wild-type C which corresponds to a difference in thermostability of 6 °C due to the presence of the phosphate on Thr-197.

**Steric Constraints Based on Modeling**—To determine the potential steric constraints that might be introduced by these mutations, a ternary complex of C(G50A), C(G52S), and C(G55S) was modeled. After replacing the Gly with Ser, each structure was energetically minimized without ATP and then with ATP. The backbone was fixed, and only the side chains were allowed to move. The results, shown in Fig. 7, indicated that there would be steric interference that couldn’t be accommodated by simply moving the side chains. The steric constraints between Ser-52 and the oxygen that bridges the $\beta$- and $\gamma$-phosphates of ATP were even more significant. Only in the case of the Ser-55 mutation was there no steric interference that couldn’t be accommodated by simply moving the serine side chain. These modeling results are consistent with the observed kinetic consequences of these mutations.

**DISCUSSION**

The protein kinase motif containing the glycine-rich loop is a $\beta$-strand-turn-$\beta$-strand structure that tightly embraces the bound nucleotide by van der Waals attractions and an intricate network of hydrogen bonds (Fig. 1). The adenine ring is sequestered beneath the two $\beta$-strands at the base of the cleft between the two lobes of the conserved core, whereas the phosphates extend outward toward the edge of the cleft interface. It is the phosphates that interact directly with the glycine-rich loop. As summarized in Fig. 1, b and e, the backbone amides of Gly-55 and Phe-54 interact with one of the $\beta$-phosphate oxygens of ATP, whereas the backbone amide of Ser-53 interacts with the $\gamma$-phosphate in the ternary complex of C-PKI-(5–24)-ATP and C-PKI-(5–24)-AMPPNP (9, 10). In addition, the side chain of Ser-53 in these ternary complexes is close enough to hydrogen...
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**TABLE III**

Steady-state kinetic parameters of underphosphorylated C-subunits

Enzymatic activity was measured as described in Table II. $k_{cat}$, $K_{m(peptide)}$, and $K_{m(ATP)}$ values of $H_o(C(G50A))-IV$ were determined to be $2 \text{ s}^{-1}$, $0.69$ and $1.4 \text{ mM}$, respectively.

| Protein       | $k_{cat}$ | $K_{m(peptide)}$ | $K_{m(ATP)}$ | $k_{cat}/K_{m(peptide)}$ | $K_{cat}/K_{m(ATP)}$ | No. of PO$_3$ |
|---------------|-----------|------------------|-------------|--------------------------|-----------------------|--------------|
| wt-C          | 17        | 0.02             | 0.02        | $5 \times 10^4$          | 2–4                   |              |
| C(G50A)-IV    | 0.8       | $0.90 \pm 0.21$  | ND          |                          |                       |              |
| C(G50S)-IV    | 0.7       | $0.88 \pm 0.19$  | $1.23 \pm 0.30$ | 0.6                      | 1                     |              |
| C(G52S)-IV    | 0.3       | $0.67 \pm 0.15$  | $1.10 \pm 0.21$ | 0.4                      | 1                     |              |
| C(G52S)-V     | 0.05      | ND               | $1.64 \pm 0.40$ |                          |                       |              |
| GST-C(T197A)  | 1.4       | 1.10             | 1.40        | 0.9                      |                       |              |
| wt-C$_{C(T197A)}$ | 0.94 | 1.14             | ND          |                          |                       |              |
| C(T197A)      | 1.37      | 1.42             | ND          |                          |                       |              |

$^a$ Data derived from a mutant C-subunit protein that was fused to glutathione S-transferase (26).

$^b$ Taken from Steinberg et al. (27).

$^c$ The indicated proteins were at least partially phosphorylated at a serine residue but not at Thr-197.

**TABLE IV**

ATPase activities of wild-type and mutant C-subunits

To determine the relative rate of ATP hydrolysis in the catalytic transfer of the phosphate to Kemptide, the ATPase rate was divided by the $k_{cat}$. ATPase rates were determined at saturating ATP concentrations.

| Protein       | ATPase rate | ATPase rate/catalytic rate | Fold increase |
|---------------|-------------|----------------------------|---------------|
|               | $s^{-1}$    | $\times 10^{-3}$           |               |
| wt-C          | 0.011       | $0.60 \pm 0.08$            | 1             |
| C(G50A)-III   | 0.016       | $4.11 \pm 0.32$            | 7             |
| C(G50S)-III   | 0.135       | $19.25 \pm 0.46$           | 32            |
| C(G52S)-III   | 0.134       | $44.50 \pm 1.22$           | 74            |
| C(G55A)-III   | 0.001       | $0.07 \pm 0.03$            | 0.1           |
| C(G55S)-III   | 0.005       | $0.44 \pm 0.29$            | 0.7           |

$^a$ The ATPase activity of singly phosphorylated C(G50A)-IV was more than 4 orders of magnitude lower than its catalytic rate.

**TABLE V**

Thermostability of mutant C-subunits

All $T_m$ values of mutant proteins were measured in the absence of Mg-ATP parallel with wild-type C. The difference values were determined by subtracting the $T_m$ of wild-type C from the $T_m$ of the respective mutant protein. Thus negative values reflect reduced thermostability, whereas positive numbers indicate increased thermostability. The values in brackets represent the additional protection provided by Mg-ATP. $T_m$ values are given in °C. The average $T_m$ values for wild-type C subunit in absence and presence of Mg-ATP are 45.4 and 48.5 °C, respectively.

| Protein       | No. of PO$_3$ | $T_m$ (mutant C) – $T_m$ (wild-type C) |
|---------------|--------------|--------------------------------------|
| C(G50A)-IV    | 1            | $-4.9$                               |
| C(G50S)-III   | 2            | $+1.2$                               |
| C(G52S)-IV    | 2            | $+1.5$                               |
| C(G55S)-IV    | 1            | $-4.2$                               |
| C(G55S)-II    | 2            | $+1.7$                               |
| C(G55S)-III   | 2            | $+1.3$                               |

To evaluate the functional importance of each glycine in this motif and to understand why there has been such a strong evolutionary pressure to conserve them.

The three glycines are among the most highly conserved residues in the protein kinase family. Out of 592 protein kinases in the Swiss-Prot (31.0) data base, 94.4, 99.8, and 84.7% have a glycine at the first, second, or third position, respectively (Table VI). Glycines are ideally suited for these positions not only because they provide minimal steric interference but also because they provide maximal flexibility. The inherent flexibility of this loop is indicated by the localized conformational differences seen in this region for different crystal structures of cAPK (17) and by the relatively high B-factors of the glycine loop in the various crystal structures depending on what is occupying the active site cleft. The kinetics also emphasize the importance of this loop for the binding and release of the nucleotide.

Dissecting the functional importance of this loop required a combination of mutagenesis coupled with kinetic and structural analysis. However, because the phosphorylation state of the enzyme is also critical for the active enzyme, it was essential to resolve isoforms prior to any rigorous kinetic comparison. C(G50A), C(G50S), C(G52S), C(G55A), and C(G55S) were thus all purified as type III isoforms. This corresponds to phosphorylation at Thr-197 and Ser-338, similar to what is found in the mammalian enzyme, and allowed for proper comparisons of the steady-state kinetic and other parameters of the mutant proteins with those of the wild-type enzyme.

Substitutions at the third glycine of the triad had the least effect on the steady-state kinetic parameters. The reduced $k_{cat}$ of C(G55S) and the slightly elevated $K_m$ values for peptide of C(G55A) and C(G55S) show that the efficiency of the enzyme is only slightly affected by the mutations. $K_m$ values for ATP of C(G55A) and C(G55S) closely resembled wild-type C. Similar to wild-type C, Mg-ATP also protected C(G55S)-III from thermal denaturation. These data correlate well with modeling of C(G55S) which indicated that a seryl side chain could be accommodated in the structure of the ternary complex without necessitating changing the backbone of the glycine loop, the position of ATP, or the position of other side chains (Fig. 7c).

Although Gly-55 is the least conserved Gly in the triad (85%), more than 99% of protein kinases have a small amino acid (Gly, Ala, or Ser) at this position (Table VI). A truncated form of phosphorylase kinase which naturally has a Ser at the third position had the same activity when the Ser was replaced with Gly (25). On the other hand, a mutant form of the insulin receptor kinase where the third Gly was replaced by Val showed a 90% decrease in activity indicating that residues...
larger than Ser cannot be readily accommodated (22) without interfering with catalytic activity.

In contrast to the mutation of Gly-55, substitutions of either Gly-50 or Gly-52 had more major consequences based on the steady-state kinetic parameters (Table II). C(G50A), C(G50S), and C(G52S) all showed a significant decrease in $k_{cat}$, a 10–20-fold increase in $K_{m(\text{peptide})}$, and a 10–100-fold increase in $K_{m(\text{ATP})}$. For wild-type C, the rate-limiting step in catalysis is ADP release and the concomitant conformational changes in the enzyme associated with product release (21, 40). Based on viscosometric studies the actual phosphoryl transfer step was predicted to be at least 10 times faster than the $k_{cat}$ (20). Subsequent measurement of the presteady-state rate constants confirmed that $k_{cat}$, corresponding to the chemical transfer step, was 500 s$^{-1}$ (21), in contrast to the $k_{cat}$ of 20 s$^{-1}$. Because of these kinetics the $K_{m(\text{ATP})}$ is identical to the $K_{d(\text{ATP})}$, and the $K_m$ for peptide is a true reflection of binding affinity, whereas the $K_m$ for peptide is more complex, does not correlate with the $K_d$, and does not reflect true affinity. Given the published microscopic rate constants and assuming that the changes in $k_{cat}$ of the mutant proteins reflect changes in $k_2$ or ADP release, the $K_{m(\text{peptide})}$ values for the mutants are not explainable without a significant drop in the velocity of the phosphoryl transfer reaction. The measurements of the $K_r$ values of LRRAALG also indicated that the affinity for the peptide was largely unaffected by the mutations.

All of the mutations at Gly-50 and Gly-52 had a major effect on ATP binding, with the larger residues having a more severe effect. C(G50A) and C(G50S) also showed decreased protection from thermal denaturation of C(G50A) by Mg:ATP. This was predicted by modeling each of these replacements. Assuming that the enzyme has to adopt a closed conformation similar to the ternary structure to ensure efficient phosphoryl transfer, strong steric repulsions would result between the inserted side chain atoms and ATP (Fig. 7, a–c). To avoid a shift in the position of ATP, the backbone of the glycine-rich loop would need to adopt a different conformation. It seems reasonable to assume that such steric repulsions would not only influence ATP binding but could also impact the activity of the enzyme to form a catalytically competent complex.

The conservation of the second Gly, greater than 99%, indicates that practically no protein kinase is optimally functional with any other amino acid at this position. Modeling also confirmed that replacement of this glycine would cause steric interference near the oxygen that bridges the β- and γ-phosphates of ATP. Thus the kinetics, conservation throughout evolution, and modeling all support the importance of the second Gly in the triad. A substitution at this position disturbs the enzyme at a most critical position in the immediate vicinity of the terminal phosphoryl group of the nucleotide. Given the microscopic rate constants for wild type C-subunit (20, 26) the kinetic parameters measured for C(G52S) strongly indicate that the phosphoryl transfer step ($S_3$) of this mutant must be strongly affected.

Substitution of the first Gly in the triad had approximately 50% of the effects that were seen when the second Gly was replaced. Modeling showed that there would be some steric repulsion in the vicinity of the ribose ring when Gly-50 was replaced with Ser. This was reflected in the steady-state kinetic parameters and increased ATPase rate of C(G50S).

For an efficient catalysis it is also important to exclude water from the active site. For wild-type C the ATPase rate relative to the catalytic rate is less than 0.1%. The significant increase in the ATPase rate of C(G50S) and C(G52S) indicates that water has much greater access to the γ-phosphate of ATP in these mutant proteins. If the $k_{cat}$ of C(G52S) were rate-limited by the phosphoryl transfer step, up to 4% of the ATP molecules would be hydrolyzed instead of being used for substrate phosphorylation. C(G55A) and C(G55S) both showed an actual decrease in ATPase activity (Table V), whereas the substitution of other side chains at the “tip” of the glycine loop with glycine in-

**Table VI**

**Conservation of glycines in the glycine-rich loop**

According to 592 aligned protein kinase sequences from the Swiss Prot (31.0) database.

| Position | Residue count |
|----------|---------------|
| Gly-50   | Ala 11, Cys 2, Gly 559, His 1, Ile 2, Pro 5, Gln 6, Ser 6, Thr 6 |
| Gly-52   | X 591, 1 |
| Gly-55   | X 502, 58 |

**Fig. 7. Modeling of the mutations in the glycine-rich loop.** The structures of the glycine-rich loop of C(G50S) (a), C(G52S) (b), and C(G55S) (c) were modeled on the basis of the ternary structure shown in Fig. 1. Structures were energetically minimized first without ATP and then again with ATP, allowing only side chains to reposition. Minimization of each model structure, based on 10,000 iterations, was done using the Discover Prot (31.0) database. According to 592 aligned protein kinase sequences from the Swiss Prot (31.0) database.

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increased the ATPase rates of the respective proteins. beetle, the present results support the view that an important function of the glycine-rich loop is indeed to minimize the access of water molecules to the active site and that the conservation of two of the glycines, Gly-50 and Gly-52, but not Gly-55, plays a crucial role in excluding water.

Another explanation for the effects of the mutations on the steady-state kinetics is based on the conformational flexibility of the loop due to the conserved glycines. The region corresponding to the glycine-rich loop displays relatively high B-factors in a variety of structures with the backbone of Gly-52 and Ser-53 showing the greatest variability depending on what is occupying the cleft. Replacement of Gly-52 also had the most severe effect on the enzyme kinetics. If inherent flexibility of the loop is important for the final conformational changes that close the cleft and stabilize the transition state intermediate, substituting the glycines would almost certainly reduce that flexibility and thus directly influence phosphoryl transfer. In this regard it is interesting to note that in absence of ATP all of the fully phosphorylated mutant proteins were slightly more thermal stable than wild-type C (Table V).

Phosphorylation State of the Mutant Proteins—C(G50A), C(G50S), and C(G52S) not only have reduced activity toward the peptide substrate and decreased affinity for ATP but were also less efficient in autophosphorylation. In each case most of the expressed protein was isofrom IV. This isofrom was not phosphorylated at Thr-197, indicating that Ser-338 is the first site to be phosphorylated in E. coli. Lack of the phosphate on Thr-197 resulted in decreased solubility, further losses in activity, and a decrease in $T_m$ of 6°C. This is in accordance with Steinberg et al. (27), who indicated that recombinantly expressed C is already phosphorylated on Ser before activation of the enzyme by phosphorylation at Thr-197 occurs. Whether the phosphorylations at Ser-338 or Thr-197 are due to intramolecular autophosphorylation remains to be determined. Also unclear is whether in eukaryotic cells these phosphorylations result from autophosphorylation or whether a heterologous kinase is involved.

Comparison of completely unphosphorylated C(G52S)-V and singly phosphorylated C(G52S)-IV indicated that although phosphorylation at Ser-338 occurs first, it is not functionally as important as Thr-197 (Table II) (26, 27). The singly phosphorylated mutant proteins, lacking the phosphate at Thr-197, had steady-state kinetic parameters in the range of those of previously published mutant proteins where Thr-197 was replaced with Ala (26) (Table III). It seems likely, therefore, that the missing phosphate and the glycine substitutions have synergetic effects on $k_{cat}$.

In summary this detailed mutational analysis has provided considerable insight into the functional importance of the conserved glycines in the nucleotide-binding site of cAPK. The functional importance (Gly-52 > Gly-50 > Gly-55) is reflected in the extent to which these three residues have been conserved throughout evolution. Further analysis of presteady-state rate constants as well as three-dimensional structures, both static and in solution, are now required to understand this region in greater detail.

Acknowledgments—We acknowledge the technical assistance of S. Garrod and J. Gross for the peptide sequencing and mass spectrometry.

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