An Inhibitory Segment of the Catalytic Subunit of Phosphorylase Kinase Does Not Act as a Pseudosubstrate*

The C terminus of the catalytic γ subunit of phosphorylase kinase contains two autoinhibitory calmodulin binding domains designated PhK13 and PhK5. These peptides inhibit truncated γ (1-300). Previous data show that PhK13 (residues 302–326) is a competitive inhibitor with respect to phosphorylase b, with a $K_i$ of 1.8 μM (1). This result suggests that PhK13 may bind to the active site of truncated γ (1-300). Variants of PhK13 were prepared to localize the determinants for interaction with the catalytic fragment γ (1-300). PhK13-1, containing residues 302–312, was found to be a competitive inhibitor with respect to phosphorylase b with a $K_i$ of 6.0 μM. PhK13 has been proposed to function as a pseudosubstrate inhibitor with Cys-308 occupying the site that normally accommodates the phosphorylatable serine in phosphorylase b. A PhK13-1 variant, C308S, was synthesized. Kinetic characterization of this peptide reveals that it does not serve as a substrate but is a competitive inhibitor. Additional variants were designed based on previous knowledge of phosphorylase kinase substrate determinants. Variants were analyzed as substrates and as inhibitors for truncated γ (1-300). Although PhK13-1 does not appear to function as a pseudosubstrate, several specificity determinants employed in the recognition of phosphorylase b as substrate are utilized in the recognition of PhK13-1 as an inhibitor.

The catalytic activity of many protein kinases is regulated by an autoinhibitory mechanism known as intrasteric regulation (2, 3). Many autoinhibitory domains were discovered by the observation that proteolysis or truncation of these protein kinases resulted in activation. The autoinhibitory domain often, but not always, resembles the consensus sequence of an enzyme’s substrate. Protein kinase A was the first described case of intrasteric regulation by a pseudosubstrate domain located on the regulatory subunit (4–6). Substantial evidence exists implicating this mechanism in protein kinase G, protein kinase C, myosin light chain kinase, calmodulin-dependent protein kinase I, and phosphorylase kinase (2, 3, 7, 8).

Phosphorylase kinase is a member of the Ca$^{2+}$/calmodulin-dependent protein kinase family. Members of the Ca$^{2+}$/calmodulin-dependent protein kinase family, myosin light chain kinase, calmodulin-dependent protein kinases I, II, IV, and phosphorylase kinase contain an autoinhibitory domain that overlaps the calmodulin binding regions (2, 3, 9–11). A possible mechanism for this intrasteric regulation is that the autoinhibitory domain occupies the enzyme’s active site and that binding of calmodulin in the presence of calcium induces a conformational change, releasing the autoinhibitory domain from the active site and thus activating the enzyme. Alternatively, the autoinhibitory domain could bind outside of the active site and influence the active site indirectly. Binding of calmodulin to the autoinhibitory domain in the presence of calcium would result in a conformational change that is transmitted to the active site and results in the activation of the enzyme.

Phosphorylase kinase catalyzes the conversion of phosphorylase b (inactive) to phosphorylase a (active) by phosphorylating serine residue 14. This reaction is highly specific. Phosphorylase kinase is the only protein kinase known to catalyze this reaction in vivo. Holoenzyme phosphorylase kinase has a molecular mass of 1,300 kDa. It is comprised of a hexadecamer, containing four of each of the four subunits (αβγδ),. The α and β subunits are regulatory, the δ subunit is equivalent to bovine brain calmodulin and confers calcium sensitivity to the enzyme, and the γ subunit is the catalytic subunit.

The N-terminal 300 amino acids of the γ subunit encompass the catalytic domain of phosphorylase kinase. The C-terminal 86 amino acids contain two non-contiguous high affinity calmodulin binding segments, PhK13 (residues 302–326) and PhK5 (residues 342–366) (12). The catalytic γ subunit is unique in that the δ subunit remains tightly bound in the absence of calcium, and the autoinhibitory C-terminal domain is comprised of two calmodulin binding domains as opposed to one. Dasgupta and Blumenthal (13) determined the $K_1$ values for PhK13 and PhK5 (300 nM and 20 μM, respectively) with holoenzyme phosphorylase kinase. These values fall into the range of $K_i$ values obtained with other protein kinase autoinhibitory domains (2, 3). The peptides PhK13 and PhK5 also differentially inhibit truncated γ (1-300). Previous data show that PhK13 is a competitive inhibitor of γ (1-300), with respect to phosphorylase b, with a $K_i$ of 1.8 μM (1). This suggests that PhK13 may act as a pseudosubstrate with cysteine 308 occupying the site normally occupied by the serine of the phosphorylatable substrate.

Kinetic studies using two truncated γ (1-300) mutants, E110K and E153R with charge reversals of the 9-18 peptide, demonstrated that these residues recognize the P$^{-3}$ (lysine) and P$^{-2}$ (glutamine) positions/residues of substrate, respectively (1). The crystallization of truncated γ (1-300) with a peptide substrate confirms this assignment (14). PhK13 contains a lysine at P$^{-3}$ and a valine at P$^{-2}$. Analysis of PhK13 binding to...
Inhibition of Phosphorylase Kinase by Peptide Variants

Fig. 1. Time course of phosphorylase b phosphorylation in the presence of increasing concentrations of PhK13-1. Phosphorylation in the absence of PhK13-1 (○); phosphorylation in the presence of 5 μM PhK13-1 (●); phosphorylation in the presence of 12.5 μM PhK13-1 (▲); phosphorylation in the presence of 25 μM PhK13-1 (●). Assay conditions are described under "Experimental Procedures."

The truncated γ(1-300) mutants E110K and E153R resulted in increasing Kᵢ values for PhK13 compared with wild-type truncated γ(1-300). The Kᵢ increased 14-fold with E110K and 8-fold with E153R (1). These results are consistent with PhK13 binding to the active site of truncated γ(1-300).

The aim of this work was to determine whether PhK13 functions as a pseudosubstrate inhibitor. Conversion of PhK13 from an inhibitor to a substrate by the single conservative amino acid substitution of serine for cysteine would be indicative of active site binding. In lieu of structural information, this would support a model in which the C terminus of phosphorylase kinase interacts directly with the active site. In addition, the specificity determinants of PhK13 that are important for the inhibition of phosphorylase kinase were examined in an effort to further our understanding of this molecular interaction.

EXPERIMENTAL PROCEDURES

Materials—[γ-32P]ATP was purchased from ICN Biomedicals (Costa Mesa, CA). All other reagents were commercially available from Fisher, Sigma, or Roche Molecular Biochemicals (Indianapolis, IN). Phosphorylase b was purified as described (15). PhK13 (γ residues 302–326), PhK13-1 analogs (residues 302–314), and 9-18 peptide (residues corresponding to the phosphorylatable region of phosphorylase b) were synthesized at the Iowa State University Protein Facility. All peptides were purified by reverse-phase high pressure liquid chromatography using a C18 column.

Peptide Concentration Determinations—Peptide concentrations were determined by amino acid analysis at the Iowa State University Protein Facility and by 5,5'-dithiobis-2-nitrobenzoic acid (DTNB)1 titration for the cysteine-containing peptides (16). After DTNB titration to verify the free sulfhydryl, all cysteine-containing peptides were stored frozen in the presence of 5 mM dithiothreitol (DTT). Thus, all cysteinyl-containing peptides utilized had one free sulfhydryl.

Expression, Purification, and Renaturation of Truncated γ Subunit—Truncated γ(1-300) was expressed, purified, and renatured as previously described (17). Recombinant truncated γ(1-300) was purified to homogeneity as judged by SDS-polyacrylamide gel electrophoresis (data not shown). Protein concentrations were determined by Bradford assay with commercially prepared reagents from Bio-Rad (18).

Activity Assay—The protein kinase activity of truncated γ(1-300) was determined by 32P incorporation into phosphorylase b as previously described (with minor modification) (19, 20). The standard activity assay contained 50 mM PIPES, 50 mM Tris, pH 8.2, 10 mM MgCl₂, 10 μg/ml phosphorylase b, 5 mM DTT, 1 mM [γ-32P]ATP, and 0.5 μg/ml truncated γ(1-300). The reaction was performed at 30 °C and was initiated by the addition of enzyme. After incubation, 15 μl of reaction mixture was spotted on ET-31 Whatman filter paper and washed with trichloroacetic acid (21). The radioactivity incorporated was determined by liquid scintillation counting.

Substrate Analyses—All peptides were evaluated as substrates for truncated γ(1-300) using the standard activity assay but with non-radioactive ATP and peptide concentrations of 1–2 mM. These samples were analyzed in the Iowa State University Protein Facility by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) using a Finnigan Lasermat 2000 with equal molar ratios of an α-cyano-4-hydroxycinnamic acid matrix to peptide. The second evaluation of peptides as substrates for truncated γ(1-300) utilized the standard activity assay with [γ-32P]ATP.

Inhibition Studies—In the inhibition assays the standard activity assay was used except for the addition of PhK13-1, and the final concentration of phosphorylase b was 4 μM. In the kinetic analyses, the concentration ranges of phosphorylase b varied from 4–90 μM, inhibitor peptide concentrations varied from 1 μM–1 mM, and the concentration of ATP was fixed at 1 mM. For 9-18 peptide phosphorylation the standard phosphorylation assay was used with 0.5 mM 9-18 peptide, 10 and 50 μM PhK13-1, and 0.1 μg/ml truncated γ(1-300).

Kinetic data were analyzed with either Enzfitter (Elsevier Science Publishers) or Enzyme Kinetics (Trinity Software). The inhibition data were fitted to equations for competitive or noncompetitive inhibition. The inhibition constants were determined from the linear secondary plots (data not shown). All analyses were repeated a minimum of three times in triplicate on at least three separate occasions.

ATPase Assays—ATPase assays were performed at 30 °C as described (22). Final concentrations of reagents in the assay were as follows: 50 mM Hepes, pH 8.2, 0.1 mM DTT, 20 mM MgCl₂, 1 mM ATP. Reactions were initiated by the addition of truncated γ (1-300) to a final concentration of 10 μg/ml PhK13-1 concentrations varied from 0.1–500 μM. At the specified time 20-μl aliquots were removed from the assay mixture and pipetted into vials containing 20 μl of a mixture of 2.8 N sulfuric acid, 4.3% silicotungstic acid, plus 100 μl of an organic mixture containing benzene and isobutyl alcohol (1:1). After mixing the solution, 20 μl of 10% ammonium molybdate was added, and the sample was vortexed for 10 s. An aliquot of 20 μl was removed from the upper organic phase and spotted onto ET-31 Whatman filter paper. Liquid

1 The abbreviations used are: DTNB, 5,5'-dithiobis-2-nitrobenzoic acid; DTT, dithiothreitol; MALDI-TOF-MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; PIPES, piperazinediethanesulfonic acid; AMP-PNP, 5'-adenylly-β,γ-imidodiphosphate.
scintillation counting was used to determine the amount of $^{32}$P
inorganic phosphate released.

RESULTS

Identification of the Inhibitory Section of PhK13—A variant
of PhK13 was prepared to localize the determinants for inter-
action with truncated $\gamma$(1-300). A dodecamer of the N
terminus of PhK13, PhK13-1 (GFKKFVICTLVA) containing
cysteine 308, inhibited truncated $\gamma$(1-300) (Fig. 1). A peptide of the C
terminus of PhK13 (LASVRIYYQYRRVKP) did not inhibit (13),
suggesting that the major interaction occurred through the seg-
ment containing cysteine. PhK13-1 concentrations of 6–8 $\mu$M
inhibited 50% of truncated $\gamma$(1-300) activity (Fig. 2A). Sub-
sequent kinetic analysis demonstrated that PhK13-1 was a com-
petitive inhibitor of truncated $\gamma$(1-300) with respect to phos-
phorylase b and had a $K_i$ of 6 $\mu$M (Fig. 3 and Table I).

Competitive inhibition is indicative of active site binding but
is not definite proof. Because phosphorylase b is a large protein
it was important to test whether PhK13-1 would also inhibit
phosphorylation of a smaller peptide substrate. Inhibition of
peptide phosphorylation by PhK13-1 would support PhK13-1
blocking the active site, consistent with the observed competitive
inhibition kinetics. We examined phosphorylation of the
9-18 peptide in the presence of PhK13-1. Increasing amounts of
PhK13-1 inhibited the phosphorylation of 9-18 peptide sub-
strate by truncated $\gamma$(1-300). 50 $\mu$M PhK13-1 inhibited 9-18
peptide phosphorylation 60% over control (data not shown).
Additional kinetic analysis was not carried out. Although no
data currently exist in support of cysteine phosphorylation by
phosphorylase kinase, cysteine phosphorylation of PhK13-1
was feasible. Analysis of PhK13-1 as a substrate for truncated
$\gamma$(1-300) by MALDI-TOF-MS, and $^{32}$P incorporation shows no
evidence of cysteine phosphorylation by truncated $\gamma$(1-300)
(data not shown).

Pseudosubstrate Analysis of PhK13—Alanine is the most
common amino acid occupying the phosphate acceptor site
in autoinhibitory and/or pseudosubstrate domains (3). In many
autoinhibitory protein kinases the substitution of this alanine
residue with serine resulted in conversion of the inhibitor to a
substrate. If cysteine 308 occupies the phosphate acceptor site,
does the equivalent serine-substituted peptide then serve as a
substrate? We made the PhK13-1 variant, PhK13-1(C308S),
changing Cys-308 to serine. Based on our knowledge of phos-
phorylase kinase substrate specificity, this PhK13-1(C308S)
peptide was expected to function as a substrate. The variant
was analyzed as a substrate using 9-18 peptide as a compari-
son, but we were unable to detect any phosphorylation in this
peptide using MALDI-TOF-MS (Fig. 4). Analysis of PhK13-
1(C308S) as an inhibitor demonstrated that this variant is a
competitive inhibitor of truncated $\gamma$(300) with respect to
phosphorylase b. The change of C308S increased the $K_i$, 30-fold,
from 6 to 175 $\mu$M (Table I).

Inhibitor Specificity Determinants—To learn more about the
contribution of other residues in the sequence of PhK13-1 to the
interaction with $\gamma$, additional PhK13-1 variants were designed
based on prior knowledge of phosphorylase kinase substrate
determinants. Residues previously shown to affect the specific-
ity of phosphorylase kinase for phosphorylate were systemati-
cally substituted (23). Table I lists the PhK13-1 variants de-
signated. PhK13-1(F304R/C308S) adds a basic residue in the P
position since phosphorylase b contains an arginine at P$^{-4}$. The
optimal sequence derived from peptide library work places a
phenylalanine at P$^{-4}$, implying that phenylalanine can be
accommodated in this position (24). PhK13-1(V306Q/F304R)
replaces valine with glutamine at P$^{-2}$. The recent crystal struc-
ture of truncated $\gamma$(1–298) complexed with a modified version
of the optimal library sequence indicates that valine should fit
in place of glutamine (14). Arginine in the P$^{-2}$ position has
been shown to be a positive determinant for phosphorylase
kinase recognition, and this substitution resulted in peptide
PhK13-1(C308S/T310R). Each of these peptides was analyzed
for its ability to serve as a substrate of truncated $\gamma$(1-300).
With every PhK13-1 variant listed in Table I we were able to function as substrates for
truncated $\gamma$(1-300) and $^{32}$P incorporation, none of the PhK13-1 variants
inhibited 50% of truncated $\gamma$(1-300) versus PhK13-1 varied from 0–50 $\mu$M. Phospho-
ylase b was used as substrate. Assay conditions are described under
"Experimental Procedures." B, inhibition of truncated $\gamma$(1-300) by
PhK13-1(1/RG). Percent activity of truncated $\gamma$(1-300) varied from 0–50 $\mu$M. Phos-
phorylase b was used as substrate. Assay conditions are described under
"Experimental Procedures." The amounts of PhK13-1(1/RG) are as indicated.

FIG. 2. Inhibition of truncated $\gamma$(1-300) by PhK13-1 and PhK13-
1(1/RG). A, inhibition of truncated $\gamma$(1-300) by PhK13-1. Percent activity
of truncated $\gamma$(1-300) versus PhK13-1 varied from 0–50 $\mu$M. Phos-
phorylase b was used as substrate. Assay conditions are described under
"Experimental Procedures." B, inhibition of truncated $\gamma$(1-300) by
PhK13-1(1/RG). Percent activity of truncated $\gamma$(1-300) in the presence of peptide PhK13-1(1/RG) is shown. Assay conditions are described under
"Experimental Procedures." The amounts of PhK13-1(1/RG) are as indicated.
FIG. 3. Inhibition pattern of phosphorylase b by PhK13-1. Kinetic assay conditions are described under "Experimental Procedures." Inhibition of phosphorylase b by PhK13-1 with phosphorylase b as the variable substrate is shown. The final concentrations of PhK13-1 in the assay were 0 μM (□), 5 μM (▲), 11.5 μM (●), and 23 μM (▲). The inset represents the secondary plot of slope versus PhK13-1 concentration.

**TABLE I**

| Sequence comparisons of peptide substrates and inhibitors of γ(1–300) | Mode of inhibition | Constant (K<sub>i</sub>) (μM) |
|---|---|---|
| 9–18 | not applicable | | |
| PhK13-1 | Competitive | 6 ± 1 |
| PhK13-1(C308S) | Competitive | 175 ± 3 |
| PhK13-1(F304R/C308S) | Competitive | 802 ± 1.5 |
| PhK13-1(V306Q/C308S) | Competitive | 80 ± 5 |
| PhK13-1(K309S/T310R/V311G) | Competitive | 44 ± 4 |
| PhK13-1(RG(T310R/V311G)) | not applicable | | |

modulin, was incorporated into PhK13-1 (25). The first glycine variant PhK13-1(C308S/T310R/V311G), at concentrations of up to 1 mM no longer inhibited truncated γ(1-300) (data not shown). This peptide variant was phosphorylated as analyzed by MALDI-TOF-MS, using 9-18 peptide as a positive control (data not shown). Phosphorylation of this peptide proceeded at a much reduced rate (~100-fold less) when compared with 9-18 peptide (Fig. 5). Additional kinetic analysis was not performed. The conversion of this peptide from inhibitor to substrate may be due to the incorporation of glycine, which could change the orientation of the serine. If incorporation of glycine does change the orientation of serine, then the corresponding peptide with cysteine would also change in orientation. This potential change in orientation could weaken the interaction with truncated γ(1-300), and we may no longer observe inhibition with the peptide PhK13-1 (T310R/V311G). The initial results with peptide PhK13-1 (T310R/V311G) showed no inhibition of truncated γ(1-300) with peptide concentrations up to 400 μM (Fig. 2B).

**ATPase Activity**—Substitution of serine for cysteine resulted in much weaker inhibition. The difference in size between cysteine and serine could be a factor in the differences in inhibition. The distance between the γ phosphate of ATP and the acceptor residue might be changed in the presence of the larger cysteine residue. Although there is no evidence for cysteine phosphorylation by a protein kinase-catalyzed reaction, phosphorylation could occur, but because a cysteine phosphate would be very labile it could break down and re-form, making the product extremely difficult to detect. If the cysteine residue in PhK13-1 is in closer proximity to the γ phosphoreryl group of ATP, it may affect the ATPase activity of truncated γ(1-300). One way to test this hypothesis is to examine the ATPase activity of truncated γ(1-300) in the presence and absence of PhK13-1. Protein kinases have a small intrinsic hydrolytic activity toward ATP. They are able to utilize the hydroxyl group of water as a phosphate acceptor at a very slow rate. ATPase activity is monitored by detecting the release of Pi. Thus, if truncated γ(1-300) was attempting to use the cysteine as a phosphate acceptor, we should have detected an increase in the amounts of Pi released in the presence of PhK13-1 when compared with the ATPase activity of truncated γ(1-300) alone. Alternatively, the peptide PhK13-1 could block the access of water into the active site, thus preventing ATPase activity. The results of the analyses of ATPase activity revealed that PhK13-1 inhibits the ATPase activity of truncated γ(1-300) (Fig. 6). This result indicated that PhK13-1 may bind tightly, filling the active site, and either excluding water from entering or disrupting the γ phosphate orientation so that ATPase activity is diminished.

**DISCUSSION**

Many protein kinases are believed to be regulated by an autoinhibitory mechanism, better referred to as intrasteric regulation (2, 3). The autoinhibitory domain may resemble the consensus sequence of an enzyme’s substrate. Our data suggest a model in which PhK13-1 binding blocks the active site of truncated γ(1-300) but does not act as a pseudosubstrate.

Competitive kinetics obtained for the PhK13-1 variants are consistent with these peptides binding at the active site. PhK13-1 is a competitive inhibitor with a K<sub>i</sub> of 6 μM. The low K<sub>i</sub>
of PhK13-1 compared with the $K_i$ for PhK13 (1.8 mM) indicates that most of the residues important for the inhibition of truncated $\gamma$(1-300) are located in the N-terminal half of PhK13. In addition to the ability of PhK13-1 to inhibit phosphorylation of the 9-18 peptide substrate of phosphorylase kinase, the data fit simple linear competitive kinetics, and the slope plots are linear. There is no evidence of the peptide forming a disulfide with truncated $\gamma$(1-300) or with PhK13-1 itself. Work with a cysteine-substituted 9-18 peptide showed that this peptide does not function as a substrate and does not inhibit truncated $\gamma$(1-300) in the presence of DTT (26). All the experiments conducted in the present study contained 5 mM DTT. In addition, the free sulphydryl in all cysteine-containing peptides was verified through amino acid analysis and DTNB titration. Previous data from this laboratory utilizing charge reversal mutants of truncated $\gamma$(1-300) with PhK13 also support its binding at the active site. PhK13 exhibited higher $K_i$ values with the mutants E110K and E153R than truncated $\gamma$(1-300), and PhK13 remained a competitive inhibitor.

**Fig. 4.** MALDI Spectra of 9-18 compared with PhK13-1(C308S). MALDI spectra of 9-18 peptide compared with the PhK13-1(C308S) peptide before and after incubation with $\gamma$(1-300). Reaction conditions are described under "Experimental Procedures." A, 9-18 before reaction, showing the predicted weight of 1180, and B, 9-18 after reaction, with the expected phosphorylated product peak at 1259, the PhK13-1(C308S) peptide before reaction. D, the PhK13-1(C308S) after reaction, indicating that no phosphorylation has occurred.

**Fig. 5.** $[^{32}P]$ incorporation into peptides 9-18 compared with PhK13-1(S/RG). Phosphorylation of peptides 9-18 (■) and PhK13-1(S/RG) (●). The inset illustrates the reaction with PhK13-1(S/RG) (●). Assay conditions are described under "Experimental Procedures." The concentration of 9-18 peptide was 1 mM and PhK13-1 S/RG was 2 mM.

**Fig. 6.** ATPase inhibition by PhK13-1. Percent ATPase activity in the presence of increasing amounts of PhK13-1. Assay conditions are described under "Experimental Procedures." The concentrations of PhK13-1 are as indicated.
The inability to convert the peptide PhK13-1 to a substrate for truncated γ(1-300) by changing one amino acid (Cys-308 to serine) supports this model. The cysteine side chain is larger than the side chain of serine. If upon PhK13-1(C308S) binding the seryl group could not reach the terminal γ phosphoryl group of ATP, this peptide would not serve as a substrate, although it may act as a competitive inhibitor. Indeed, PhK13-1(C308S) competitively inhibits truncated γ(1-300). The Kᵢ of PhK13-1(C308S) increases 30-fold over the Kᵢ of the parent peptide, PhK13-1, from 6 to 175 μM.

Further analysis of the specificity determinants in PhK13-1 identified other potentially important residues in addition to cysteine 308 that are involved in the inhibition of truncated γ(1-300) by PhK13-1. In fact, most changes in PhK13-1 to make it more substrate-like actually weakened the binding. The increase in Kᵢ from 175 μM in PhK13-1(C308S) to 802 μM in PhK13-1(F304R/C308S) indicates that phenylalanine in the P₋₄ position is an important specificity determinant for inhibition. This phenylalanine is equivalent to the conserved hydrophobic anchoring residue found in many Ca²⁺/calmodulin-dependent protein kinases. This hydrophobic anchoring residue is important for calmodulin binding as well as inhibition. The crystal structure of truncated γ(1–298) contains a conserved hydrophobic binding pocket, and it has been speculated that this pocket might be important for binding the conserved anchoring hydrophobic residue in Ca²⁺/calmodulin-dependent protein kinases (27). The Kᵢ for 9-18 peptide, containing arginine in the equivalent position, is 1 mM. Substitution of arginine for phenylalanine raised the Kᵢ close to the mM range. However, substitution of the arginine at P₋² decreased the Kᵢ down to an 8-fold difference when compared with PhK13-1, emphasizing the preference phosphorylase kinase has for arginine at P₋².

The inability of the glycine variant PhK13-1(C308/PG) to inhibit truncated γ(1-300) is consistent with previous results with a cysteine-substituted 9-18 peptide substrate, KRR-QICVRGL, which does not inhibit truncated γ(1-300) and does not serve as a substrate (26). Previous work utilizing a cysteine-substituted Kemptide, LRRACGL, showed that this peptide was unable to function as a substrate for protein kinase A (28). Although this peptide could serve as an inhibitor of protein kinase A with respect to Kemptide, the Kᵢ of 1.9 mM is very high compared with the micromolar Kᵢ of Kemptide (28). This suggests that the valine-leucine pair (residues 311–312) found in PhK13-1 and several of the variants causes steric interference and changes the position of the substituted serine residue. The substitution of glycine for valine (P₋₃) could relieve this interference, enabling phosphorylation of the peptide substrate.

PhK13-1 is a much better inhibitor than PhK13-1(C308S). What characteristics of cysteine make it a better inhibitor than serine? Cysteine may interact with the aromatic side chains of amino acids. The crystal structure of truncated γ(1-300) complexed with peptide substrate illustrates that such a possibility might occur in the interaction of Ph-e170 with the cysteine residue found in PhK13 (14, 29). The change from cysteine to serine would alter this potential interaction and support PhK13 binding to the active site of truncated γ(1-300).

Many protein kinase pseudosubstrates contain alanine in the phosphocceptor position. The side chain of cysteine is similar to that of alanine with regards to apolarity. The side chain of serine is more hydrophilic, although the size of an alanine side chain would be closer to that of serine (30). The larger cysteine may change the distance between the γ phosphate of ATP and the putative acceptor residue. This could result in the SH of cysteine causing steric interference with the γ phosphate of ATP. Alternatively, PhK13-1 binding to truncated γ(1-300) could fill the active site, excluding water. Elimination of water or a change in orientation of the γ phosphate in the active site would prevent ATPase activity. The inhibition of ATPase activity in the presence of PhK13-1 supports its binding to the active site of truncated γ(1-300).

Conversely, if truncated γ(1-300) were attempting to utilize PhK13-1 as a substrate, then we would expect an apparent increase in ATPase activity because the cysteine phosphate product would be very labile. The lack of ATPase enhancement supports our model that PhK13-1 does not function as a pseudosubstrate, in that truncated γ(1-300) does not attempt to (or is unable to) phosphorylate PhK13-1.

Another possible explanation is that binding of PhK13-1 could alter the conformation or orientation of the N- and C-terminal lobes of the catalytic kinase core, thus altering the active site. A change in conformation around the active site could alter metal ion binding, ATP binding, the orientation of the catalytic base, and/or the binding of substrate. The crystal structure of twitchin kinase reveals that the C-terminal autoinhibitory domain extends through the active site, makes extensive contacts with the surface of the large lobe of the catalytic core, but then it turns away from the activation loop and travels along the small lobe (32). Recent work from small x-ray and neutron scattering resulted in a structure for myosin light chain kinase complexed with calmodulin. Calmodulin was near the catalytic core but away from the active site, suggesting displacement of the autoinhibitory domain. The additional structure has been expanded to include the substrates AMP-PNP and a peptide substrate derived from the regulatory light chain (33). Here the overall complex becomes more compact (i.e. “closed”), yielding the active conformation of a protein kinase. It is not currently possible to definitely distinguish which of these modes of binding will ultimately be utilized by the autoinhibitory C terminus of phosphorylase kinase. Thus, the exact binding of PhK13-1 awaits structural determination.

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