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Mass Spectrometric Analysis of Surface-Exposed Regions in the Hexadecameric Phosphorylase Kinase Complex

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Abstract

Phosphorylase kinase (PhK) is a 1.3 MDa ($\alpha\beta\gamma\delta$)₄ enzyme complex, in which $\alpha\beta\gamma\delta$ protomers associate in *D*₂ symmetry to form two large octameric lobes that are interconnected by four bridges. The approximate locations of the subunits have been mapped in low-resolution cryo-electron microscopy structures of the complex; however, the disposition of the subunits within the complex remains largely unknown. We have used partial proteolysis and chemical footprinting in combination with high-resolution mass spectrometry to identify surface-exposed regions of the intact nonactivated and phospho-activated conformers. In addition to the known interaction of the γ subunit's C-terminal regulatory domain with the δ subunit (calmodulin), our exposure results indicate that the catalytic core of γ may also anchor to the PhK complex at the bottom backside of its C-terminal lobe facing away from the active site cleft. Exposed loops on the α and β regulatory subunits within the complex occur at regions overlapping with tissue-specific alternative RNA splice sites and regulatory phosphorylatable domains. Their phosphorylation alters the surface exposure of α and β , corroborating previous biophysical and biochemical studies that detected phosphorylation-dependent conformational changes in these subunits; however, for the first time, specific affected regions have been identified.

Graphical abstract

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Notes

The authors declare no competing financial interest.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bio-chem.5b00682. Figure S1 shows a representative label-free quantification analysis for the release of peptide β 1–15 (m/z 795.91) from the partial proteolysis of PhK by chymotrypsin. This was done by measuring the area under the curve of the base peak from each time point. Insets are chromatograms from a representative time course for reference. Nonactivated (A) and phospho-activated (B) conformers are both shown here. Table S1 gives a complete list of peptides identified by mass spectrometry (PDF)

Phosphorylase kinase (PhK) is a large, hexadecameric enzyme complex in the glycogenolysis cascade. The enzyme as isolated from fast-twitch skeletal muscle, as in this report, consists of four copies each of four different subunits, $(\alpha\beta\gamma\delta)_4$, and has a mass of 1.3 MDa. The α (138.4 kDa, 1237 amino acids¹), β (125.2 kDa, 1092 amino acids¹), and δ (16.7 kDa, 148 amino acids³) subunits are regulatory, inhibiting the kinase activity of the catalytic γ (44.7 kDa, 386 aa⁴) subunit until β and then α are phosphorylated by PKA,^{5,6} which releases the inhibition and allows γ to phosphorylate glycogen phosphorylase in a Ca^{2+} -dependent reaction.⁷ The δ subunit is an intrinsic calmodulin molecule, which accounts for the Ca^{2+} sensitivity of the enzyme.⁸

Because of its large mass, the structure of PhK has been difficult to study; however, several methods have been employed that have produced low-resolution three-dimensional structures, including transmission electron microscopy (EM),^{9–11} cryo-EM,^{12,13} and small-angle X-ray scattering.¹⁴ Structural evidence shows PhK to be tetrahedral, consisting of two opposing lobes and four interlobal connecting bridges, with $\alpha\beta\gamma\delta$ protomers packed in a head-to-head manner in D_2 symmetry.⁹ The approximate locations of the subunits in EM structures of the complex have also been determined. The β subunits are located on the interior face of the lobes and make up the four connecting bridges,^{15,16} whereas the α subunits, which are homologous to β , are nevertheless positioned quite differently at the periphery of the lobes, with their known C-terminal epitope located at the distal ends of the lobes.¹⁷ The γ subunits are proximal to the β subunits, although more central and distal on the interior lobe face.¹⁸ The δ subunits are located near the edge of the lobes¹⁹ and are known to directly interact with the γ subunit,^{20–22} although each subunit type directly interacts with the other three.²³

Although the interactions and relative locations of the subunits within the PhK complex are beginning to be understood, much less is known regarding the exact disposition of the subunits in the complex, i.e., how they are actually situated. To make matters worse, high-resolution structures are available for only δ ²⁴ and the catalytic domain of γ ,²⁵ the two smallest subunits. How those structures might be affected by their quaternary interactions within the complex is unknown, as are the portions of those structures exposed on the surface of the complex, instead of buried and shielded by subunit interfaces within the quaternary structure. In this study, we have employed two complementary techniques, limited proteolysis and chemical footprinting, to map exposed regions of individual subunits in the hexadecameric $(\alpha\beta\gamma\delta)_4$ PhK complex. Both of these methods are useful for

identifying exposed regions of a protein and examining conformational changes between different forms of a protein.^{26–30} While there are numerous reasons why particular regions might be protected from protease cleavage, brief proteolysis of a protein will target regions that are most susceptible, which is highly indicative of surface-exposed loops.^{31,32} Chemical footprinting also identifies solvent-exposed regions and, thus, can corroborate partial proteolysis data. By using partial proteolysis and chemical footprinting in conjunction with mass spectrometry (MS), the released peptides can be sequenced to identify the exact residues that are cleaved or labeled and, thus, surface-exposed. We have mapped exposed loops on the PhK holoenzyme in both its phospho-activated and nonactivated conformers and were able to identify exposed loops in three of the four subunits (α , β , and γ) and to observe changes in the sites of cleavage and iodoacetate labeling when PhK is activated by phosphorylation.

MATERIALS AND METHODS

Enzymes and Reagents

All proteases were from commercial sources: chymotrypsin (Ch), trypsin (Tr), and endoproteinase Arg C (RC) from Worthington Biochemical Corp. (Lakewood, NJ); ficin (Fi) and thermolysin (Th) from Sigma-Aldrich Corp. (St. Louis, MO); protease V8 (V8) from ICN Biomedicals (Irvine, CA); and endoproteinase Lys C (KC) from Promega Corp. (Madison, WI).

[³H]Iodoacetic acid and its nonlabeled counterpart were from American Radiolabeled Chemicals (St. Louis, MO) and Acros Organics (Morris Plains, NJ), respectively. All polyacrylamide gel reagents were from Bio-Rad Laboratories (Hercules, CA).

The catalytic subunit of cAMP-dependent protein kinase (PKA) was purchased from New England Biolabs Inc. (Ipswich, MA).

Nonactivated PhK was purified from New Zealand White rabbit psoas muscle as previously described³³ and stored at -80°C . Phospho-activated PhK was prepared through phosphorylation of the nonactivated enzyme by PKA, specifically by incubating nonactivated PhK (1.2 mg/mL) with $\text{Mg}-(\text{CH}_3\text{CO}_2)_2$ (1.5 mM), ATP (0.3 mM), PKA (2 $\mu\text{g}/\text{mL}$), NaF (50 mM), DTT (0.1 mM), EGTA (0.5 mM), and β -glycerophosphate (100 mM) at pH 6.8 for 10 min at 30°C , which resulted in the incorporation of 0.35 and 0.37 mol of phosphate per mol of α and β subunit, respectively. Phosphorylation was quenched with 200 mM EGTA. The phospho-activated PhK was dialyzed overnight at 4°C in 50 mM Hepes buffer (pH 6.8) containing 10% sucrose and 0.2 mM EDTA and used immediately thereafter.

Partial Proteolysis

To ensure that proteolysis reflected PhK's structure rather than protease selectivity, seven proteases with varying selectivities were used in partial proteolysis experiments. Because PhK's structure is affected by pH, temperature, and buffer conditions, it was important to maintain the same conditions throughout all of the proteolysis experiments; however, given that each protease has different conditions under which it performs optimally, maintaining the same conditions throughout would cause the proteases to hydrolyze at different

efficiencies. To control for this, we determined the concentration for each protease that resulted in <10% of the α and β subunits being cleaved after an appropriate fixed time, ensuring roughly similar amounts of proteolysis for each protease. This standardization process was begun using concentrations of proteases that had been previously optimized using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) after a 10 min partial proteolysis of PhK at 30 °C and pH 6.8,²⁶ but with further optimizations of protease concentrations being made (Table 1) for use in the more sensitive MS analyses. The proteolysis time of 10 min was retained, as it allowed for sufficient proteolysis without significant loss of the activity of PhK and, presumably, the structural integrity of the protein core. Measuring the maximal activity before and after proteolysis by all seven proteases under these standard conditions showed that only ficin digestion caused any significant loss of activity, and by only 20%.

The standard conditions for partial proteolysis were 30 °C and pH 6.8, conditions under which PhK is well characterized. Stock PhK (3.5 mg/mL) was diluted to 0.4 mg/mL in 50 mM Hepes containing 10% sucrose and 0.2 mM EDTA and equilibrated at 30 °C for 2.5 min. Protease was then added at the appropriate concentration (Table 1) and the mixture incubated for 10 min. Reactions were quenched by the addition of 80% acetonitrile with 0.8% formic acid, and mixtures were vortexed for 1 min and centrifuged for 5 min at 14000 rpm. The supernatant was removed to a fresh Eppendorf tube, diluted in H₂O, and flash-frozen in dry ice. Samples were lyophilized overnight, resuspended in 0.1% formic acid, and analyzed by MS. If after repeated experiments a peptide was observed in only one sample, and its presence could not be replicated, it was disregarded. Peptides were required to be present in a minimum of three independent reaction mixtures involving at least two different PhK preparations to be judged as high-confidence peptides. Peptides with good MS/MS spectra that were also observed in two different PhK preparations, but only once in each, or peptides that were observed at least twice in a single preparation are both considered moderate-confidence peptides and are denoted as such in Table 1.

Chemical Footprinting

To map the surface-accessible thiol side chains of Cys in the individual subunits of the ($\alpha\beta\gamma\delta$)₄ complex, conditions were first optimized to ensure that the carboxymethylation of all subunits remained in the linear phase, as has been previously done.²⁶

The optimized, standard reaction was conducted for 20 min at 25 °C; the mixture contained PhK (0.37 μ M $\alpha\beta\gamma\delta$ tetramer), [³H]iodoacetate (3.48 mM, 0.24 Ci/mol), Hepes (50 mM, pH 8.2), and EDTA (100 μ M), and the reaction resulted in the linear incorporation of 0.08, 0.30, and 0.05 mol of carboxymethyl groups per α , β , and γ subunit, respectively. For the mapping of modified residues by MS, nonlabeled iodoacetate was substituted, and the reaction was quenched using a 100-fold molar excess of DTT over iodoacetate. The modified protein was then digested with Tr [26:1 PhK:Tr ratio (w/w)] for 1 h at 37 °C. Aliquots (5% of the total volume) of acetonitrile were then added to the digest every hour to a maximum of 30%, after which proteolysis was conducted for 16 h. The digest was quenched by addition of formic acid to 5% of the total volume. Samples were prepared and analyzed by MS as described below. The procedure described above was repeated with

different preparations of PhK for both non-activated and phospho-activated forms of the complex.

Mass Spectrometry

All samples were lyophilized overnight and brought up to a final volume of 10 μL in 0.1% formic acid. They were loaded onto a C18 reversed-phase peptide trap (100 μm inner diameter fused silica) packed in house with 3 cm of 100 Å, 5 μm , Magic C18 bioparticles (Michrom Bioresources, Auburn, CA). Following a wash with 0.1% formic acid for 30 min at a rate of 1.5 $\mu\text{L}/\text{min}$, peptides were eluted into the connected reversed-phase C18 resolving column (75 μm inner diameter, packed to a length of 12 cm with the same bioparticles used for the peptide trap) at a flow rate of 350 nL/min over a 90 min period using a gradient of 10 to 40% acetonitrile (buffer A consisted of 0.1% formic acid; buffer B consisted of acetonitrile and 0.1% formic acid). The column was mounted on the electrospray stage of a hybrid linear ion trap Fourier transform ion cyclotron resonance MS instrument (LTQ FT, Thermo-Finnigan, San Jose, CA) equipped with a 7 T magnet and controlled by Xcalibur software packaged to continuously perform mass scan analysis on the FT. This was followed by MS/MS scans on the ion trap for the six most intense ions, using dynamic exclusion of two repeats of the same ion, a 30 s repeat duration, and a 90 s exclusion duration. The normalized collision energy for all MS/MS scans was set to 35%. LC-MS data were collected with the source operated at 2.0 kV, with the ion transfer temperature set to 200 °C.

For data analyses, all MS/MS scans were searched using the Sequest algorithm included in Proteome Discoverer (Thermo-Finnigan) using an in-house-built protein database containing the sequences of PhK and common contaminants. The mass range for spectrum selection was 350–5000 Da. A minimum of 6 and a maximum of 144 amino acid residues were used to generate the peptide library to be searched. The ion mass tolerances were 20 ppm for parent ions and 0.6 Da for fragment ions. Protease selectivity was defined as indicated in Table 1. A maximum of two missed cleavages was allowed per peptide. The following variable modifications were selected (phosphorylation of S, T, and Y, deamidation of N and Q, and oxidation of M), and a maximum of three modifications per peptide were allowed. Additionally, for the chemical footprinting experiments, carboxymethylation of Cys was considered as a variable modification. The results of the searches were filtered using the following set of criteria for a high degree of statistical confidence: minimum cross-correlation scores (X_{corr}) of 1.5, 2.0, and 2.5 (partial proteolysis) and 1.5, 2.0, and 2.25 (chemical footprinting) for one, two, and three charged ions, respectively, and a delta correlation score (δ_{corr}) of >0.08. The amino acid sequence assignment of all peptides of interest was subsequently inspected manually. Supplementary Table 1 provides details for all observed peptides.

RESULTS

Mapping Exposed Loops on Nonactivated PhK

Partial proteolysis was used to detect surface-exposed loops on the individual subunits of the PhK complex (Figure 1 and Table 1), and when possible, the solvent exposure of those

regions was verified through chemical footprinting by carboxymethylation (Table 2). Proteolytic cleavage patterns of nonactivated PhK by proteases with different specificities revealed common cleavage sites, indicating the presence of exposed loops in the complex's quaternary structure (Figure 1).^a

With the exception of RC, all proteases targeted the α subunit, and Th did so relatively selectively (Table 1). Two regions of the α subunit were observed with all of the proteases that cleaved it: 663–748 and 967–1041. These entire regions were not observed with any specific protease, but at least one or more peptides were cleaved by each protease, spanning varying lengths within each region. Correspondingly, two cysteine side chains (656 and 697), either proximal or within the first observed loop, were carboxymethylated. The second loop is from residue 967 to 1017 and likely extends to residue 1041, based on peptides detected after digestion with Th (1017–1040) and Fi (1033–1041). Additionally, region 194–210 was observed only in the Fi samples, whereas the stretch from residue 1156 to 1170 was observed during Th proteolysis only.

The β subunit was cleaved by all seven proteases, with KC targeting β selectively. One region was especially susceptible to proteolysis: the N-terminal regulatory domain of residues 1–32 (Table 1),³⁴ which has a quite different sequence in other isoforms of β .³⁵ Peptides from within this region were seen during proteolysis by all seven proteases. Two additional spans in the β subunit that were observed in digests with multiple proteases include residues 683–696 (Tr and KC) and 779–811 (KC and Ch). The latter stretch completely overlaps a region (779–806)³⁵ that is different in the non-muscle isoform of β . Portions of the intervening sequence between residue 696 and 779 are also exposed, as indicated by carboxymethylation of Cys733 and -735 (Table 2), which happen to be the only two cysteines in that stretch.

The catalytic subunit of PhK, γ , does have a determined crystal structure of its catalytic core, composed of residues 1–298.²⁵ The first 13 residues are likely to be highly mobile, as they are not observed with satisfactory electron density in the crystal structure. This region (1–27) was also observed after partial proteolysis by four of the six proteases that cleaved the γ subunit (Table 1). Just C-terminal to this region, another loop (45–68), or an extension of the first, was revealed by partial proteolysis with V8 and modification of two proximal Cys residues [36 and 42 (Table 2)]. As shown in Figure 1C, the C-terminal region also contains two exposed loops that include residues 277–298 (Ch and V8) and 360–386 (Tr, KC, Fi, Ch, and V8).

Comparison of Proteolytic Cleavage Sites in Phospho-Activated and Nonactivated PhK

To observe phosphorylation-induced conformational changes that may affect surface-exposed loops, PhK was phosphorylated by its physiological activator PKA (described in Materials and Methods), and partial proteolysis was performed under the same conditions used for the nonactivated enzyme, but with only four proteases (Tr, KC, V8, and Ch). We

^aIt should be noted that throughout this paper the amino acid residue numbers correspond to their actual positions in the parent subunit polypeptide chain, as opposed to their codon positions in the cDNA for that subunit, i.e., the final presence (α subunit¹) or absence (β^1 and γ^2 subunits) of an N-terminal Met is taken into account.

identified common regions that were cleaved from either form of PhK, regardless of protease specificity, although some peptides represented slightly different cleavage points (Table 1). Thus, activation of the enzyme did not greatly affect most of the exposed and flexible regions on the complex; however, there were nevertheless several differences in the proteolytic patterns. We also compared the carboxymethylation of the two PhK conformers (Table 2).

When PhK is phosphorylated, the α subunit is cleaved in the same loop regions (656–748 and 967–1015) observed for the nonactivated conformer; however, a new region (627–662, Tr) proximal to the former loop was observed. Additionally, two new regions near the C-terminus of α appear, as well: a peptide corresponding to residues 1103–1113 (V8) and one of residues 1217–1224 (Ch). Correspondingly, we observed that Cys1234 was modified only in the phosphorylated complex (Table 2), suggesting that the entire C-terminus from residues 1217 to 1237 may be more exposed in the activated conformer.

In the β subunit, the N-terminal region contains a serine (26) that is targeted by PKA,^{1,36–38} and we have shown previously that this region of β is susceptible to chemical modification in the PhK complex.³⁴ Correspondingly, we find that this region is exposed in both the phospho-activated and nonactivated conformers of PhK (Table 1). Previous studies have shown that the β subunits not only undergo conformational changes in the phosphorylated PhK complex^{18,26,39} but also interact preferentially with themselves in that complex.^{16,23} Phosphorylation of the complex is known to alter the accessibility of residues and/or regions of the β subunits.^{18,26} Corroborating these findings, we detected only in digests of phospho-activated PhK a new peptide comprising residues 713–725 (V8), just C-terminal to Ser700, another target for PKA.¹ In contrast to new regions being observed in the phospho conformer, a proximal loop of β , namely 683–694 (Tr), was detected only in nonactivated PhK, again suggesting conformational changes in this part of the subunit. A similar putative burial of certain regions upon phosphorylation is observed upon moving closer to the N-terminus, as chemical footprinting suggests that the solvent accessibility of cysteines 433, 558, and 574 is altered in the phospho conformer. These three residues were observed to be carboxymethylated in nonactivated PhK, but not in its phosphorylated counterpart (Table 2).

The chemical footprinting and partial proteolysis patterns of the γ subunit were quite similar in the phospho-activated and nonactivated forms of PhK. There were, however, two peptides, 68–78 (Tr) and 184–196 (V8), formed by cleavage of phospho-PhK that were produced by no other proteases with either PhK conformer. There were also some slight changes in proteolysis patterns for all four proteases with the phospho-activated complex.

To gain more information about the relative exposure of the various loops in the two PhK conformers, a time course of proteolysis was performed at 1, 3, and 10 min with three proteases: Tr, V8, and Ch. The most salient observation from these experiments was the rapid cleavage by all proteases of both conformers at both termini of the γ subunit (data not shown) and of the N-terminus of the β subunit (Supplemental Figure 1 for Ch).

DISCUSSION

Previously, it has been shown that partial proteolysis²⁶ and chemical footprinting³⁰ can be used as conformational probes to detect structural differences between nonactivated and activated forms of PhK. In that footprinting study, only the subunits modified were identified, and in the partial proteolysis study, SDS-PAGE was used as the method of detection for proteolytic patterns. As such, exact sites of cleavage were not identified, and sensitivity for smaller released peptides was low. In the study presented here, peptides released by proteolysis were detected by MS, which is able to identify the exact site where cleavage occurs. The use of MS has allowed us to map exposed loops on the intact complex of PhK using both partial proteolysis and chemical footprinting and has provided us with detailed information about specific regions that was not previously available. As such, we are able to use these regions not only to learn more about the disposition of the subunits within the structure but also to enhance and corroborate previously determined information about those subunits.

The fact that the α subunit is readily degraded by a wide variety of proteases in this and previous work²⁶ suggests a peripheral location for this subunit in the PhK complex. The more N-terminal major exposed loop in α [663–748 (Table 1, Tr, KC, Fi, Th, Ch, and V8)] identified in this study ends at the residue, Arg748, previously shown to be targeted by the HIV protease.⁴⁰ This loop coincidentally overlaps with most of the region (654–713) that is deleted in the α' isoform of PhK found in slow-twitch and cardiac muscle.³⁵ The second major loop in α [967–1041 (Table 1, Tr, KC, Fi, Th, Ch, and V8)] encompasses its entire phosphate-rich region (970–1030)⁴¹ containing seven phosphorylated residues. This loop also includes one of two regions in α that is tissue-specific (1012–1062), differing from its largely homologous liver-specific isoform, α_L ,⁴² and there are two regions of alternative splicing within this loop: 1012–1024 and 1025–1041.⁴³ Both of these major loops in α overlap with its only two regions (676–766 and 967–1064) that are unique compared to its homologous β subunit.¹ The fact that both of these regions are relatively hydrophilic prompted speculation that they may be surface-exposed and play a role in mediating PhK's interactions with the environment.¹ One potential interaction may be with PhK's protein substrate, as yeast two-hybrid screening identified the region of α from residue 864 to 1014, which overlaps with the second loop, as containing a site that interacts with glycogen phosphorylase.⁴⁴ The work presented here certainly indicates a surface exposure for both unique regions. The finding that the C-terminal region of α is surface-exposed is further supported by immuno-EM using a mAb against an epitope within this region.¹⁷ Affinity labeling of the PhK complex with exogenous calmodulin, an activator termed δ' , provided even more evidence of C-terminal surface exposure, as only the α subunit was labeled and within a peptide from residue 1080 to 1114.⁴⁵

Although the identification of the four β subunits as the four bridges of the PhK complex¹⁶ places it more precisely than the other three subunits, which areas of it are surface-exposed is less understood. Partial proteolysis of the β subunit revealed three exposed loops, one of which was cleaved by all seven proteases and represents the N-terminus from position 1 to 32. As with the phosphorylation loop of α , this region of β is unique (i.e., not found in α) and contains the major phosphorylation site, Ser26, that accounts for activation of the

complex by PKA.³⁸ This Ser, as well as Ser11, is also autophosphorylated.¹ A second loop in β from residue 683 to 696 (Table 1, Tr and KC) is directly adjacent to a region (697–719) reported to have the highest PEST score (6.2) of any sequence in the PhK complex.⁴⁶ Such a score is suggested to signal a high rate of *in vivo* turnover of a protein.⁴⁷ The third loop from residue 779 to 811 (Table 1, KC and Ch) overlaps with a sequence (770–794) that has been shown to tightly bind (as a synthetic peptide) to isolated calmodulin.⁴⁸ This loop also overlaps a partially defined epitope (704–815)¹⁶ for a mAb against β . Both the first and third loops correspond to regions of β that are different in isoforms produced by alternative RNA splicing in tissues other than skeletal muscle.³⁵ All three exposed loops described above are also on the surface of a recently published atomic model of β derived from intrasubunit chemical cross-linking, threading, MS, and *ab initio* approaches,¹⁶ further corroborating that model.

The α and β subunits are homologous; both have a CaaX box for polyisoprenylation at their extreme C-termini, CAMQ and CLVS, respectively,¹ and both have been reported to be farnesylated on the Cys in the CaaX box.² In the case of α , this is Cys1234; however, we observe the carboxymethylation of this residue in phospho-activated PhK (Table 2), meaning that it was not farnesylated prior to treatment with iodoacetate. We do, however, observe farnesylated peptides in MS spectra from both the α and β subunits, but we observe nonfarnesylated C-termini, as well.^b Although it is likely that some of the nonfarnesylated peptides arise from instability of the farnesylthioether bond in MS,⁴⁹ some clearly represent incomplete post-translational modification within purified PhK.

High-resolution structures are available only for the two smallest subunits of PhK, γ (catalytic) and δ (calmodulin²⁴), and for the former, only the catalytic domain (1–298).²⁵ In the crystal structure, the first 13 residues of the free γ subunit are likely to be highly mobile, as they have poor electron density,²⁵ and this region [1–27 (Table 1, Tr, Fi, Th, and V8)] was also readily cleaved during partial proteolysis, indicating that the N-terminus of γ is mobile and exposed in the solvated, hexadecameric PhK complex, as well. A second exposed loop [45–68 (Table 1, V8)] observed in our data corresponds to another region in γ 's crystal structure that also lacked electron density (53–63).²⁵ A third loop region of γ occurring between residues 277 and 298 (Table 1, Tr, KC, Fi, Ch, and V8) completely encompasses two short helices²⁵ and contains the epitope (277–290) for an anti- γ mAb raised against intact PhK.¹⁸ The final loop between residues 360 and 386 (Table 1, Tr, KC, Fi, and Ch) is consistent with a previous study showing that an antibody raised against a synthetic peptide corresponding to residues 362–386 strongly interacts with the PhK complex.⁵⁰ This region of the γ subunit overlaps significantly with its C-terminal calmodulin-binding domain (332–371) identified using overlapping synthetic peptides.²¹ This finding suggests that the interaction between the γ and δ subunits within the PhK complex may be predominantly through the N-terminal calmodulin-binding domain of γ (287–331),²¹ which matches our finding of zero-length cross-linking between δ and Lys325 of γ in the PhK complex.²⁰

^bIn the initial report of farnesylation of α and β , good yields of recovery (75 and 50%, respectively) for farnesylated peptides were interpreted as both subunits being fully farnesylated in the PhK complex.² In agreement with that previous study, however, we did observe that the final three residues of both subunits were neither removed nor carboxymethylated in farnesylated peptides (data not shown).

Partial proteolysis revealed no exposed loops for the δ subunit, which is not surprising given its small size and compact crystal structure of two Ca^{2+} -binding lobes connected by a central helix.²⁴ Chemical footprinting showed no modification of the δ subunit because it contains no Cys residues.³

Besides the interaction of the C-terminal regulatory domain of γ with the δ subunit discussed above, the γ subunit also directly interacts with α and β .^{23,34,51,52} Information about which regions of the catalytic domain of γ might be interacting with the remainder of the PhK complex can be gained by mapping the surface-accessible sites of proteolysis, carboxymethylation, and mAb binding and the known sites of substrate interaction⁵³ onto the crystal structure of the catalytic domain⁵⁴ and determining the regions that have been omitted. These omitted regions, lacking evidence of surface exposure, are potential sites of interaction between the catalytic domain and the complex proper. Such a map of surface-accessible regions covers several large areas that include the entire N-lobe, part of the interconnecting chain between the lobes, as well as the E (C-terminal three-quarters)- and I- α helices, the activation loop, substrate-binding sites, and catalytic residues of the C-lobe. The region omitted is at the bottom and backside (opposite the active site cleft) of the C-terminal lobe of γ and includes three stretches of residues. Progressing toward the C-terminus, the first stretch includes residues 116–126, which form a loop connecting α helices D and E. The second stretch includes the C-terminus of helix F and part of the loop connecting it to helix G. The third comprises two connecting loops bracketing all of helix H. The overall omitted region forms two connected perpendicular surfaces with a total area of $\sim 507 \text{ \AA}^2$. Given their location and potential function as subunit interaction sites, these surfaces could anchor part of the C-lobe of γ on the interior lobe face of the PhK complex, as if this derriere region of γ is sitting upright on a short chair composed of the remaining subunits (colored red in Figure 2) with the N-lobe and catalytic cleft of γ exposed to solvent.

On the basis of previous work, we thought the β subunit would be the one most likely to show conformational changes resulting from phospho-activation. For one thing, it is the subunit whose phosphorylation is key for activation of the complex.⁵⁵ Moreover, chemical cross-linking of the PhK complex has shown the amount of conjugates formed between the β subunit and other subunits to be significantly affected by phosphorylation.^{52,56,57} Also, top-down MS of phospho-PhK shows a large increase in the degree of self-association of β subunits.²³ Finally, the level of exposure of a β epitope (within 704–815) in the complex is greatly increased by phosphorylation.¹⁸ The only specific region of β previously known to be involved in these conformational changes was that broadly defined epitope, but we can now identify a short loop [713–725 (Table 1, V8)] within that broad region as becoming more exposed. As we move farther toward the N-terminus of β , cysteines 574, 558, and 433 also may become less exposed upon phosphorylation, as they no longer become carboxymethylated (Table 2).

Phosphorylation also brought about several changes in the C-terminal half of the α subunit, one being a newly hydrolyzed loop between residues 627 and 662 (Table 1, Tr), which contains the caspase-3 cleavage site of α , Asp646.⁵⁸ Further toward the C-terminus, new peptides were observed from residue 1103 to 1113 (Table 1, V8) and from residue 1217 to 1224 (Table 1, Ch), as well as new carboxymethylation of Cys1234 (Table 2). The last three

conformational changes occurring in the terminal 135 residues of α coincident with activation by phosphorylation are in the same general region (1060–1237) of that subunit within the PhK complex that has been shown to participate in a Ca^{2+} -sensitive $\alpha \leftrightarrow \gamma \leftrightarrow \delta$ communication network⁵¹ and to undergo a Ca^{2+} -dependent redistribution of mass near the lobe tips,¹⁰ where an anti- α mAb (epitope within 1132–1237) binds.¹⁷ Even though they occur in the same general region of the α subunit, the observed conformational changes associated with activation by phosphorylation and by Ca^{2+} are probably not the same because the activity of phospho-activated PhK remains Ca^{2+} -dependent,⁵⁹ albeit with an altered affinity for Ca^{2+} .^{59–61}

Results from these two simple techniques of partial proteolysis and chemical footprinting implicate specific regions of three of PhK's subunits as being surface-accessible in the complex. More importantly, they corroborate and expand previous studies of the complex that characterized specific regions or amino acids, including sites of mAb binding, affinity labeling, phosphorylation, and single cleavage by several proteases. Further, they suggest roles as sites of target interactions for the distinct regions of variation among the different isoforms of α and β , as well as the unique regions in their otherwise homologous sequences. They also suggest how the catalytic domain of the γ subunit might be anchored to the remainder of the PhK complex. Finally, they give the first indication of regions of conformational change throughout the complex upon phospho-activation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

Ch	chymotrypsin
EM	electron microscopy
Fi	ficin
KC	endoproteinase LysC
mAb	monoclonal antibody
MS	mass spectrometry
PhK	phosphorylase kinase
PKA	cAMP-dependent protein kinase
RC	endoproteinase ArgC

Th	thermolysin
Tr	trypsin
V8	protease V8

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Figure 1.

Proteolytic patterns of nonactivated PhK subunits (A) α , (B) β , and (C) γ . Nonactivated PhK was incubated as described in Materials and Methods with the seven different proteases at the concentrations listed in Table 1. Residue numbers and sites of cleavage for each protease are shown, with the solid lines indicating the N-terminus for each released peptide and its corresponding C-terminus shown by dotted lines. Boxed regions of extensive proteolysis are shown expanded above the parent subunit. Despite the largely different selectivities of the various proteases, they cleave in mainly the same regions of the subunits, showing clearly defined areas of surface exposure. No peptides were observed from the δ subunit.

Figure 2.

Crystal structure (Protein Data Bank entry 1PHK) of the catalytic domain of PhK γ ,²⁵ with surface-accessible regions colored green,⁵⁴ based on known substrate-binding,⁵³ ligand-binding,²¹ and antibody-binding¹⁸ sites. Carboxymethylated cysteines and protease cleavage sites detected herein are colored black with corresponding amino acid side chains. The potential subunit-docking site in the context of the $(\alpha\beta\gamma\delta)_4$ complex is colored red. Note that the C-terminal region of γ known to bind δ (calmodulin) is not present in this γ construct.

Table 1Exposed Regions in Nonactivated and Phospho-Activated PhK^a

protease	abbreviation	selectivity	concentration ($\mu\text{g/mL}$)	subunit	nonactivated	phospho-activated
trypsin	Tr	Arg and Lys	0.08	α	663–690	627–662 ^c
					708–748	663–748 ^d
					972–1015	1000–1015
					1–18 ⁱ	1–18
				β	24–32	24–32
					683–694 ^b	
				γ	1–27	1–27
						68–78 ^d
						276–296 ^d
						367–386
protease V8	V8	Asp and Glu	4.0	α	699–741	699–741
					967–1013	967–1013
						1103–1113
						1–9
				β	1–16	
						713–725
				γ	1–17	1–23
					45–68 ^d	45–73
						184–196
					281–294	280–294
Lys C	KC	Lys	0.4	α	677–721 ^d	677–683
						708–721
					997–1010	997–1006
					1–32	1–13
				β	683–696	683–694
					791–807 ^d	
				γ	360–386	

protease	abbreviation	selectivity	concentration ($\mu\text{g/mL}$)	subunit	nonactivated	phospho-activated
chymotrypsin	Ch	aromatic, acidic, hydrophobic	0.2	α	718–733	703–709
					1006–1017	718–733
						1006–1014
						<i>1217–1224^d</i>
thermolysin	Th	hydrophobic	15	β	1–15	1–15
					779–811 ^d	784–811 ^d
				γ		1–16
					277–298	277–298 ^d
					371–386 ^d	371–386
				α	677–688	
ficin	Fi	nonspecific	0.04		699–746	
					984–1013	
					1017–1040	
					1156–1170	
				β	1–22	
				γ	1–23	
				α	194–210	
					718–748	
Arg C	RC	Arg	8		988–1012	
					1033–1041	
				β	1–22	
					26–32	
				γ	1–26	
Arg C	RC	Arg	8		367–386	
				β	1–20	

^aSequence numbers correspond to either a single peptide or a series of overlapping peptides for any given protease.

^bBold indicates a difference that is observed with but a single protease.

^cItalics denote regions that are different overall between nonactivated and phospho-activated conformers.

^dRegion includes moderate confidence peptides.

See Supplementary Table 1 for more details.

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Table 2
Identification of Carboxymethylated Cysteines in Nonactivated and Phospho-Activated PhK^a

	Cys residue number	nonactivated		phospho-activated	
		trial 1	trial 2	trial 1	trial 2
<i>α</i>	24	+	+	+	+
	121	+	–	–	–
	238	+	+	+	+
	326	+	–	–	–
	488	+	+	+	+
	551	+	+	+	–
	656	+	+	+	+
	697	+	+	+	+
	844	+	–	+	+
	1096	+	+	+	+
<i>β</i>	1199	+	+	+	+
	1234	–	–	+	+
	75	+	+	–	+
	89	+	+	–	–
	259	+	–	–	–
	279	+	+	+	+
	298	+	–	+	+
	362	+	–	–	–
	433	+	+	–	–
	558	+	+	–	–
<i>γ</i>	574	+	+	–	–
	733	+	+	+	+
	735	+	+	+	+
	922	+	+	+	–
	1065	+	+	+	+
	1089	–	+	–	+
	36	+	+	+	+

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Cys residue number	nonactivated		phospho-activated	
	trial 1	trial 2	trial 1	trial 2
42	+	+	+	+
138	+	-	+	-
172	+	+	+	+
308	-	-	-	+

^a Carboxymethylated cysteines detected from a total of 19, 19, and 7 cysteine residues in α , β , and γ , respectively.