Structural Basis for Peptide Binding in Protein Kinase A: 
Role of Glutamic Acid 203 and Tyrosine 204 in the Peptide-Positioning Loop

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Running Title: Peptide Binding in PKA
Abstract

For optimal activity the catalytic (C) subunit of cAMP-dependent protein kinase requires a phosphate on Thr-197. This phosphate anchors the activation loop in the proper conformation and contributes to catalytic efficiency by enhancing the phosphoryl transfer rate and increasing the affinity for ATP (1). The crystal structure of the C-subunit bound to ATP and the inhibitor peptide, IP20, highlights the contacts made by the Thr-197 phosphate as well as the role adjacent residues play in contacting the substrate peptide. Glu-203 and Tyr-204 interact with arginines in the consensus sequence of PKA substrates at the P-6 and P-2 positions, respectively. To assess the contribution that each residue makes to peptide recognition, the kinetic properties of three mutant proteins (E203A, Y204A and Y204F) were monitored using multiple peptide substrates. The canonical peptide substrate, Kemptide, as well as a longer nine-residue peptide and corresponding peptides with alanine substitutions at the P-6 and P-2 positions, were used. While the effect of Glu-203 is more localized to the P-6 site, Tyr-204 contributes to global peptide recognition. An aromatic hydrophobic residue is essential for optimal peptide recognition and is conserved throughout the protein kinase family.
Introduction

The predominant regulatory mechanism used by eukaryotic cells to convey a message from external stimuli is phosphorylation, mediated by protein kinases. These messages control regulation of diverse pathways in response to stress, antigen presentation, and development to name a few. Members of the protein kinase family are related through a structurally conserved catalytic core comprised of two lobes. The smaller N-terminal lobe dominated by β-sheets, is responsible for nucleotide binding, while the larger C-terminal lobe made up primarily of α-helices, relays substrate specificity (2). cAMP dependent protein kinase (PKA) is one of the simplest and best understood members of the protein kinase superfamily. It exists as an inactive holoenzyme complex consisting of a regulatory (R) subunit homodimer, and two catalytic (C) subunits. Upon increased levels of intracellular cAMP, each regulatory subunit cooperatively binds two molecules of cAMP inducing a conformational change resulting in the unleashing of the active catalytic subunits (3). The C-subunit is a 350 amino acid (4), 41kD protein with the conserved kinase core represented by residues 40-300. The core is flanked at the N-terminus by a 39 amino acid helical region and a 50 amino acid C-terminal tail, with each flanking region undergoing co- or posttranslational modifications. The simplicity of this molecule and its ability to define the conserved and active kinase core allows it to serve as a model for other enzymes in the family.

Several specific determinants contribute to recognition of substrates and physiological inhibitors by the active C-subunit. Most prominent is the positioning of Arg at the P-6, P-3 and P-2 positions in the substrate. Most substrates have either a P-6 and P-3 Arg, or a P-3 and P-2 Arg combination (5,6). Additionally, there is a preference for a large hydrophobic residue at the P+1 position, with little constraint placed at the P-1 position. Residues in the C-subunit that
contribute to peptide recognition are located primarily in the large lobe with many of the residues located specifically within the enzyme’s activation segment.

The activation segment, broadly defined as residues 184 to 208, lies on the surface of the large lobe and is essential for organizing the entire enzyme’s active site. The activation segment contains little secondary structure, yet includes several distinct functional regions. First is the Magnesium Positioning Loop, residues 184-187, which positions the magnesium essential for coordinating the γ phosphate of ATP. Residues 188-192 comprise β-sheet 9, the only element of regular secondary structure. This segment interacts with the A-helix outside the core as well as the essential phosphate on Thr-197 in the activation loop. The activation loop follows with residues 194-197. The activation loop is also a site of regulation for most members of the protein kinase family, where phosphorylation on one or two key Tyr, Thr, or Ser residues is required for optimal activity (7). Based on structural comparisons of several active and inactive protein kinases such as cdk (8-10), src (11), and hck (11-13), phosphorylation at these positions appears to change the conformation of the loop and arrange it in a position necessary for optimal activity (14,15). In the C-subunit of PKA the $k_{cat}$ is reduced and the $K_m$ for ATP is increased when Thr-197 is not phosphorylated (1). This leads to a 50-fold decrease in catalytic efficiency ($k_{cat}/K_m$). The activation loop of the kinase resides in the large lobe and the phosphorylated residue in the activation loop of the C-subunit, Thr-197, makes several contacts within the large lobe (Arg-165 and Arg-189), as well as one of the few interactions between the large lobe and the small lobe in the closed conformation (His-87). The next region is the P+1 loop, 198-205. The properly positioned P+1 loop contains regions that interact with the P+1 hydrophobic residue, as well as the P-2 and P-6 arginines of peptide substrates. Examination of a crystallographic molecular model consisting of the C-subunit bound to MgATP and the inhibitor
peptide IP20, residues 5-24 of the protein kinase inhibitor (PKI), lends insight into the molecular nature of these interactions (16). The model demonstrates that a hydrophobic pocket is formed, where the side chains of Leu-198, Pro-202, and Leu-205 make the largest contribution to the pocket (Figure 1A). Also in the P+1 loop Glu-203 forms a hydrogen bond with the P-6 Arg, and Try-204 forms a hydrogen bond with Glu-230 which directly interacts with the P-2 Arg. These residues contribute to recognition nodules where distal parts of the molecule come together. The residues that facilitate recognition of the P-3 Arg, Glu-127 and Try-330, lie outside the activation loop. Finally there are the conserved APE residues, 206-208. These residues serve as an anchor to the large lobe via interaction with Arg-280, another conserved residue in the large lobe.

To further define the role of the P+1 loop in the overall organization of peptide binding, in particular at sites other than the P+1 site, Ala scanning mutagenesis of the entire loop was carried out (17). We focus here on Glu-203 and Tyr-204 for further kinetic analysis. Our goals were to determine whether these residues contribute to localized recognition of the P-2 and P-6 Arg, respectively, or whether they contribute more globally to peptide recognition. An additional mutant, Y204F, was engineered to assess the contributions of the aromatic ring without the hydrogen bonding to Glu-230. The steady-state kinetic parameters of the mutants were measured using several synthetic peptide substrates. The traditional heptapeptide substrate, Kemptide [LRRASLG], was assayed in addition to a longer nine-residue peptide [GRTGRRNSI]. The longer peptide was modified by substituting an Ala for Arg at the P-2 position or P-6 position. The results indicate there is a greater communication among the sites of substrate recognition than was previously appreciated by simple examination of the crystal structure and lead us to define this segment more globally as the peptide-positioning loop.
Materials and Methods

Materials. Reagents were obtained as follows: pRSETB expression vector (Invitrogen, Carlsbad, CA). \(^{32}\)P-\(\gamma\)-ATP (New England Nuclear-DuPonte, Boston, MA). *E. coli* strains BL21(DE3) (Novagen, Madison, WI). P81 filter paper and P-11 phosphocellulose resin (Whatman Inc., Clifton, NJ) Mono S HR 10/10 (Pharmacia, Piscataway, NJ) Muta-Gene site directed mutagenesis kit (BioRad, Hercules, CA) Horseradish peroxidase conjugated anti-rabbit IgG (Amersham, Arlington Heights, IL) H-89 (LC Laboratories, Woburn, MA) SuperSignal West Pico chemiluminescent substrate detection kit (Pierce, Rockford, IL) Oligonucleotides (Genosis-Sigma, St. Louis, MO). The PepTag PKA activity assay kit (Promega, Madison, WI). Mouse monoclonal anti-myc and anti-HA antibodies (Covance, Princeton, NJ). The C-subunit antibodies were generated as described (19) Antibodies to the phosphorylated Thr-197 were originally generated to the phosphorylated Thr-500 of PKC and were a gift from A. Newton (University of California, San Diego) (20). All peptide substrates were synthesized at the Peptide and Oligonucleotide Facility at the University of California, San Diego on a Milligen 9050 PepSyn peptide synthesizer using standard Fmoc methodology activator and purified by high-performance liquid chromatography. All DNA sequencing was performed with the ABI Prism 310 Genetic Analyzer from PE Applied Biosystems.

Site-Directed Mutagenesis of the PKA Catalytic Subunit. cDNA for the murine PKA C\(\alpha\)-subunit in the bacterial expression vector pRESTB was used as a template for Kunkel-based site-directed mutagenesis as described previously (21). All mutations were made using the MutaGene kit as per the manufacturer’s recommendations. DNA sequencing analysis confirmed the presence of the correct mutation.
Expression of Murine PKA Catalytic Subunit. Wild-type and mutant C-subunits were expressed in the *E. coli* strain BL21(DE3). Cells were grown in YT medium containing 100µg/ml ampicillin at 37°C to an optical density at 600nm of 0.5-0.8, induced with 0.5mM isopropyl-β-D-thiogalactopyranoside (IPTG), incubated for an additional 6 hours at 24°C, collected by centrifugation and stored frozen. Cells were lysed with a French pressure cell (American Instruments) at pressures between 1000 and 1500 psi using 15ml of lysis buffer/L culture. Insoluble material was removed by centrifugation at 25,000xg at 4°C for 45 minutes.

Purification of Catalytic Subunit. Wild type and mutant proteins were purified using phosphocellulose chromatography and Mono S FPLC. Briefly, cells pellets were resuspended in lysis buffer (30mM MES pH 6.5, 1mM EDTA, 50mM KCl, 5mM β-mercaptoethanol), lysed, pelleted, diluted with cold water, and batch bound to P11 resin (1 g resin/L culture) overnight at 4°C. Resin was batch washed in running buffer (30mM MES pH6.5, 1mM EDTA, 5mM β-mercaptoethanol) and eluted with running buffer containing potassium phosphate at 0, 50, 90, 250, and 500mM. Wild type C-subunit eluted at 90mM while the mutant proteins typically eluted at 250mM. Elutions were diluted with 3 volumes of cold water and bound to the Mono S 10/10 column. The proteins were eluted in 20mM potassium phosphate pH 6.5, 5mM β-mercaptoethanol with a 0-500mM KCl gradient.

Catalytic Activity Assays. The kinetic values for the proteins were obtained by a direct phosphorylation filter-binding assay using $[^{32}\text{P}]$-γ-ATP (22). The assays were performed as described (23). Briefly, the C-subunit (0.25-1.0nM) was incubated in 50mM MOPS (pH 7.0), 0.1M KCl, 10mM MgCl$_2$, 1mM DTT, 100µg/mL bovine serum albumin, 2.5µCi of $[^{32}\text{P}]$-γ-ATP, 1mM unlabeled ATP and peptide substrate. To determine the $K_m$ (ATP), peptide concentrations were held constant, and the total ATP was varied from 1.0 µM to 2.0 mM. To determine the
K_m’s for peptide substrate, the ATP concentration was fixed and the peptide substrate varied. Reactions were initiated with the addition of peptide substrates and incubated at 30°C in a final volume of 50µL. Reactions were terminated with 20µL of 50% acetic acid. Aliquots were spotted on P81 filter disks and washed together in 0.5% phosphoric acid (4 times, 500mL, 10 minutes). Filter disks were rinsed once with acetone, air-dried and counted in 5mL of EcoLume. Background reactions containing no peptide substrate were subtracted from all data. All reactions were performed in triplicate.

Kinetic data were fitted to the equation \( \nu = \frac{V_{\text{max}}[S]}{[S]K_m} \) where \( \nu \) is the reaction rate, \( V_{\text{max}} \) in the maximum rate, \([S]\) is the concentration of the variable substrate, and the \( K_m \) in the Michaelis constant. Inhibitor constants were determined by assaying at various concentrations of the inhibitor at fixed substrate concentration. Data were fitted to a single-site binding model using GraphPad Prizm version 3.02, and \( K_i \) values were extrapolated from IC_{50} values using the relationship of Cheng and Prusoff: \( K_i = \frac{IC_{50}}{1+[S]/K_m} \) (24).

**Phosphorylation of C-subunit Mutant Proteins.** Experiments were performed as described previously (17). Briefly, wild type, G200A and T201A were expressed in *E. coli*, with the addition of the potent inhibitor, H-89, added at induction for the wild type to prevent autophosphorylation. Bacterial cell pellets were lysed and the soluble fraction was used as substrate for phosphorylation by PDK-1. PDK-1 was obtained by transfecting 293 cells and immunoprecipitating from the soluble cell lysate using and antibody to its N-terminal myc tag. Phosphorylation is assessed using antibodies specific for the phosphorylated Thr-197. The activity of these PDK-1 phosphorylated proteins was assayed using the PepTag PKA activity assay. This assay uses a fluorescent-tagged Kemptide substrate, where a change in net charge
occurs upon phosphorylation. This change is detected by a shift in its direction of mobility when run on an agarose gel.
Results

Prior Ala scanning mutagenesis studies were performed to assess how the residues in the activation segment contribute to phosphorylation on Thr-197 (17). A particularly interesting mutation, highlighted in these experiments (Y204A), yielded an enzyme that did not phosphorylate Kemptide, but was able to autophosphorylate when expressed in *E. coli*. Since autophosphorylation requires similar local recognition factors as those for exogenous peptide substrates, further exploration of this phenomenon was warranted. Mutation of the adjacent residue, Glu-203, generated a mutant enzyme active toward Kemptide and capable of autophosphorylation. Surprisingly, Kemptide does not possess a P-6 residue capable of interacting with this charged side chain. The question of whether Glu-203 would discriminate among substrates as Y204A if there was a P-6 Arg could be determined by using a longer peptide substrate. Based on these properties, E203A and Y204 were selected for a more rigorous kinetic analysis using various peptide substrates.

**Steady-state kinetic parameters for the activation loop mutants.** Since the two mutant enzymes, E203A and Y204A, were both able to autophosphorylate in *E. coli* but showed differences in activity using a qualitative assay, the kinetic parameters of both were analyzed more quantitatively. Direct phosphorylation of the peptide substrate LRRASLG, Kemptide (25), measured by the incorporation of $[^{32}P]$, was used to determine the kinetic properties. Alanine substitution at Glu-203 and Tyr-204 increased the $K_m$ for Kemptide by approximately 10-fold (Table1). Although both mutations equally affected $K_m$, $k_{cat}$ was decreased only for the Y204A mutant. This decrease in $k_{cat}$ from 33s$^{-1}$ to 0.9s$^{-1}$ results in a decrease in catalytic efficiency ($k_{cat}/K_m$) of over 400-fold for the Y204A mutant as compared to a 15-fold reduction for the E203A mutant enzyme. The $K_m$ for ATP was similar for all of the enzymes assayed, indicating
that these mutations are not influencing the nucleotide pocket. Since the $K_m$ for peptide binding does not reflect a true $K_d$ for binding peptide (26), the $K_i$ for each protein was also determined using Ala-Kemptide (LRRAALG), an active site directed inhibitor (27). These values were increased approximately 40-fold for both mutant enzymes relative to the wild type enzyme.

**Steady-state kinetic parameters for a longer peptide substrate.** While much kinetic data are available for Kemptide phosphorylation, this short peptide does not exploit all the subsites in the binding pocket. Since the crystallographic molecular model shows that Glu-203 interacts with the P-6 Arg of the substrate (Figure1A), but there is no P-6 position in the Kemptide substrate, it was necessary to utilize longer peptides to more fully appreciate the role of Glu-203. A nine residue peptide with a P-6 Arg, as well as altered peptides with alanines replacing the two arginines involved in substrate recognition at the P-2 and P-6 position, were thus used as comparative substrates. As expected, the added contacts offered by the larger substrate results in a 30-fold lower $K_m$ compared to Kemptide (Table 2). Alanine substitution at positions 203 and 204 result in 30-fold increases in $K_m$ compared to wild type C. These effects are similar to those observed for Kemptide and the alanine mutants. Finally, both alanine mutants have nominal effects on $k_{cat}$ using GRTGRRNSI as a substrate.

The P-2 substituted peptide, GRTGRANSI, displays the highest $K_m$ with the wild type enzyme compared to the other peptides (Table 2). The $K_m$’s are also elevated for both mutant proteins using this peptide, although the largest effect occurs with Y204A. The $K_m$ for GRTGRANSI is 2- and 30-fold larger for E203A and Y204A, respectively, than for wild type (Table 2). Although the $K_m$ values for the P-2 substituted peptide to wild type and the mutants are higher than those for GRTGRRNSI, the $k_{cat}$ values are similar. The P-6 substituted peptide, GATGRRNSI, shows a similar trend with E203A displaying higher affinity than Y204A. The $K_m$
for GATGRRNSI is 6- and 30-fold larger for E203A and Y204A, respectively, than for wild type (Table 2). The $k_{cat}$ values for the P-6 substituted peptide are close in value to those for GRTGRRNSI. Overall, for all peptides studied, substitution at Tyr-204 has the most profound effects on apparent substrate affinity. This is surprising in light of the crystal structure, which demonstrates that this residue makes no direct contact with the peptide.

**Kinetic Parameters for Y204F.** The results from the above kinetic experiments suggest that Tyr-204 may be contributing more to peptide recognition than the electrostatic interaction between the P-2 Arg and Glu-230. The Y204F mutant was engineered to assess the contributions of the aromatic ring to this phenomenon. Any differences between this mutant and the wild type would be a result of the loss of the Tyr-204 hydroxyl group. Consistent with the other mutant proteins, Y204F did not display a change in the $K_m$ for ATP (Table 1). The $K_i$ value for Ala-Kemptide was similar to the other mutant proteins. Using the Kemptide substrate the $k_{cat}$ was found to be similar to that for wild type. While the $K_m$ is approximately 2-fold higher than that for wild type, this change is much smaller than those for the alanine mutants. These data suggest that the aromatic ring is, indeed, making a significant contribution to peptide recognition. The GRTGRRNSI kinetics display similar trends in $K_m$'s as those for the Kemptide substrate. While the $K_m$ for GRTGRRNSI is 30-fold higher for Y204A than that for wild type, this $K_m$ is only 3-fold higher for Y204F compared to wild type. The P-2 substituted peptide, GRTGRANSI, did have a $K_m$ larger than the Glu-203 mutant protein, but it is still about 5-fold less than the Ala mutant at 204.

**Activity of Phosphorylated G200A and T201A.** The P+1 loop contains other residues necessary for function, but not for direct recognition of substrate side chains. These include Gly-200 and Thr-201, both conserved in Ser/Thr kinases. When alanine mutations are engineered at
these sites and expressed in *E. coli*, the resulting proteins are unphosphorylated on Thr-197 and inactive (17). The mutant proteins analyzed thus far, E203A, Y204A and Y204F, have all been phosphorylated on Thr-197, and perhaps the inactivity of G200A and T201A is the result of under phosphorylation. To test this, G200A and T201A, were expressed in *E. coli* along with a wild type control. Since unphosphorylated C-subunit is the best control, the wild type protein was induced in the presence of H-89, a potent PKA inhibitor that disrupts autophosphorylation. The soluble fraction of these bacterial cell lysates was used as substrate material for phosphorylation by the Thr-197 kinase, 3-phosphoinositide dependent protein kinase-1 (PDK-1). The success of the phosphorylation reaction was tested using antibodies specific for the phosphorylated form of Thr-197. Figure 2A depicts immunoblots of the material in the PDK-1 reaction. The C-subunit antibody shows that each reaction contained the same amount of C-subunit, and the phospho-Thr-197 antibody indicated that each is phosphorylated with similar efficiency. Any lack of activity relative to wild type will not be due to low expression or poor phosphorylation by PDK-1. Aliquots from the phosphorylation reactions were tested for activity toward Kemptide using a qualitative assay. Figure 2B indicates that even when phosphorylated on Thr-197, the perturbation of Gly-200 and Thr-201 abolishes activity.
**Discussion**

After carrying out a qualitative screen of alanine mutants made in the activation loop residues (17), two C-subunit mutants were selected for kinetic analysis based on their unusual kinetic parameters. The Y204A mutant showed reduced Kemptide activity in the qualitative PepTag assay, but was able to autophosphorylate when expressed in *E. coli*. This apparent contradiction led to a closer examination of its kinetic parameters. Replacement of Glu-203 with Ala led to a protein that was active and able to autophosphorylate. The structure suggests that the latter residue is involved in recognition of the P-6 arginine (16), a residue not found in the Kemptide substrate. Kinetic analysis of these two mutants revealed that the P+1 loop, as well as these two specific residues, plays a global role in organizing the binding of peptide substrates. Each residue not only contributes to a local site, but also shows more long range effects. This work redefines the P+1 loop and suggests that it should more appropriately be described as the peptide-positioning loop, as its contributions go well beyond recognition of only the P+1 residue. In addition, the loop contributes either directly or indirectly to recognition of the P-site, the P-2 site and the P-6 site.

The steady-state kinetic parameters presented in this study explain why Y204A displays lower activity compared to wild type and E203A using the Pep-Tag assay. Although both mutants bind Kemptide with equivalent, poor affinity compared to wild type, the larger decrease in $k_{cat}/K_m$, the catalytic efficiency term, for Y204A compared to E203A is due to a lower turnover number, $k_{cat}$ (Table 1). Indeed, the $K_i$ values for Ala-Kemptide are much higher than the $K_m$’s for Kemptide confirming this point.

By studying the longer peptide substrate, GRTGRRNSI, and its derivatives we were able to better evaluate substrate recognition determinants. This peptide contains P-2 and P-6
arginines that can be used to assess the roles of Tyr-204 and Glu-203. While the latter interaction is direct, the former is mediated indirectly via Glu-230 (Figure 1A). In general, the $K_m$ values for GRTGRRNSI are lower for all the enzymes studied, consistent with improved affinities of the longer peptides. Furthermore, the relative changes in $K_m$ values for the mutants follow those for the Kemptide $K_m$'s. Alanine substitution at positions 203 and 204 lead to $K_m$ increases of 10-fold for Kemptide (Table 1) whereas these substitutions lead in $K_m$ increases of between 2- and 30-fold for GRTGRRNSI and its derivatives (Table 2). When either mutant protein was assayed with the substrate peptide lacking the corresponding arginine residue (i.e.-P-2 Ala substitution, Y204A; P-6 Ala substitution, E203A), elevations in $K_m$ are obtained. For example, the $K_m$ for GATGRRNSI is 7-fold larger than that for GRTGRRNSI with wild type (Table 2). Furthermore, the $K_m$ for GRTGRANSI with is approximately 30-fold larger than that for GRTGRRNSI and wild type (Table 2). These results demonstrate that both Glu-203 and Tyr-204 contribute to peptide binding to an extent not predicted by the X-ray structure. For instance, the crystal structure clearly illustrates that the hydroxyl group of Tyr-204 interacts with Glu-230, which in turn is in hydrogen bonding distance from the substrate P-2 arginine. In light of the kinetic parameters for the Y204A mutant, it seemed likely that the aromatic ring of the tyrosine might also be contributing to peptide recognition perhaps through an interaction between the P-2 Arg and the $\pi$ electrons of the aromatic ring. The Y204F mutant protein does indeed demonstrate that the aromatic ring contributes to substrate recognition. The consequence of losing this side chain is seen in the crystal structure of the Y204A mutant protein (28).

Glu-203 and Tyr-204 are involved in peptide recognition of a specific substrate residue, but also contribute to overall substrate recognition indirectly. The direct interactions are clearly shown in the crystal structure, by the proximity of Glu-203 to the P-6 Arg and the network of
interactions involving P2 Arg recognition, termed the P2 nodule (Figure 1B, Table 3). In the P-2 nodule Tyr-204 with Arg-133 is aiding the positioning of Glu-230, which interacts with the P-2 Arg. Additionally, Glu-170 in the catalytic loop helps coordinate the P-2 arginine. When the Glu-203 mutant is assayed with Kemptide the results show an increase in $K_m$ that is quite larger than expected for a mutant whose substrate determinant was not present. This observation suggests that Glu-203 influences peptide binding beyond the P-6 interaction, perhaps through the P+1 loop. The same holds true for Y204A. This mutation disrupts P-2 Arg binding on the substrate, but a peptide with an alanine substituted at this position increased the $K_m$ even more.

Perhaps the P-2 residue itself is contributing to the stability of the enzyme by coordinating those residues that are involved in its binding. Certainly the absence of the P-2 Arg would disrupt the network of the P-2 nodule.

Both Glu-203 and Tyr-204 lie within a sequence that forms a hydrophobic pocket that binds the substrate’s P+1 hydrophobic residue. The positioning of this loop appears to be of critical importance. Hydrophobic residues are contributing to the pocket and the other non-hydrophobic residues contribute to the proper positioning of the loop and of the substrate. Table 4 lists the residues that make up the P+1 loop as well as the interactions in which they are involved. The Glu-203 and the Tyr-204 residues on this loop don’t contribute to its hydrophobicity with their side chains, instead they are directed away from the hydrophobic pocket. The increased $K_m$ for mutant proteins with peptide substrates lacking their corresponding arginine may be due to disruption of the P+1 loop and other interactions it makes. Sequence alignments for members of the kinase family highlight the importance of this P+1 loop in substrate recognition (18). There are distinct sequence differences between Ser/Thr kinases and Tyr kinases in this loop. These changes reflect the need to accommodate the larger substrate
tyrosine. The Thr-201 position is conserved as either a ser or thr in ser/thr kinases but is almost exclusively pro in the tyr kinases. The Glu-203 position is conserved in the AGC superfamily of kinases, but not in other Ser/Thr kinases, where as this position is basic in the Tyr kinases. The Tyr at 204 is somewhat conserved in the Ser/Thr kinases with some substitutions of Phe or Trp, but is always aromatic in the Tyr kinases. The inactivity of G200A and T201A even when phosphorylated, further demonstrates the importance of these individual residues as well as the global nature the contributions this loop makes.

The C-subunit is an enzyme that is poised to phosphorylate its substrates. Crystallographic molecular models show the peptide-positioning loop on the surface of the enzyme, prepared to bind substrate. This is evident in its exhaustive list of substrates, which are involved in wide ranging cellular functions. This characteristic works for the C-subunit because it is also unique as a kinase in its mechanism of regulation. Although there are key sites of phosphorylation that are required for activity, regulation does not occur through the dynamic transfer of a phosphate on/off at the activation loop. Instead regulation occurs through the R subunits, and further through the A-Kinase-Anchoring-Proteins or AKAPs that target the PKA signal to the various parts of the cell. It will be at this level where substrate specificity will next be described.
References

1. Adams, J. A., McGlone, M. L., Gibson, R., and Taylor, S. S. (1995) *Biochemistry* **34**(8), 2447-54

2. Knighton, D. R., Zheng, J. H., Ten Eyck, L. F., Ashford, V. A., Xuong, N. H., Taylor, S. S., and Sowadski, J. M. (1991) *Science* **253**(5018), 407-14

3. Doskeland, S. O., and Ogreid, D. (1984) *J Biol Chem* **259**(4), 2291-301.

4. Shoji, S., Parmelee, D. C., Wade, R. D., Kumar, S., Ericsson, L. H., Walsh, K. A., Neurath, H., Long, G. L., Demaille, J. G., Fischer, E. H., and Titani, K. (1981) *Proc Natl Acad Sci U S A* **78**(2), 848-51.

5. Kemp, B. E., Bylund, D. B., Huang, T. S., and Krebs, E. G. (1975) *Proc Natl Acad Sci U S A* **72**(9), 3448-52.

6. Zetterqvist, O. Z., Ragnarsson, U., and Engstrom, L. (1990) in *Peptides and Protein Phosphorylation* (Kemp, B. E., ed), pp. 171-187, CRC Press, Inc., Boca Raton

7. Johnson, L. N., Noble, M. E., and Owen, D. J. (1996) *Cell* **85**(2), 149-58.

8. De Bondt, H. L., Rosenblatt, J., Jancarik, J., Jones, H. D., Morgan, D. O., and Kim, S. H. (1993) *Nature* **363**(6430), 595-602

9. Jeffrey, P. D., Russo, A. A., Polyak, K., Gibbs, E., Hurwitz, J., Massague, J., and Pavletich, N. P. (1995) *Nature* **376**(6538), 313-20.

10. Russo, A. A., Jeffrey, P. D., and Pavletich, N. P. (1996) *Nat Struct Biol* **3**(8), 696-700.

11. Xu, W., Doshi, A., Lei, M., Eck, M. J., and Harrison, S. C. (1999) *Mol Cell* **3**(5), 629-38.

12. Sicheri, F., Moarefi, I., and Kuriyan, J. (1997) *Nature* **385**(6617), 602-9.

13. Schindler, T., Sicheri, F., Pico, A., Gazit, A., Levitzki, A., and Kuriyan, J. (1999) *Mol Cell* **3**(5), 639-48.

14. Prowse, C. N., and Lew, J. (2001) *Journal of Biological Chemistry* **276**(1), 99-103

15. Hagopian, J. C., Kirtley, M. P., Stevenson, L. M., Gergis, R. M., Russo, A. A., Pavletich, N. P., Parsons, S. M., and Lew, J. (2001) *Journal of Biological Chemistry* **276**(1), 275-80

16. Zheng, J., Knighton, D. R., ten Eyck, L. F., Karlsson, R., Xuong, N., Taylor, S. S., and Sowadski, J. M. (1993) *Biochemistry* **32**(9), 2154-61
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Figure Legends

Figure 1. Interactions Made by Residues of the Peptide Positioning Loop and the P-2 Nodule. As observed in 1atp.pdb (A) The P+1 loop, white, forms a hydrophobic pocket that aids in the binding of the substrate, tan, P+1 hydrophobic residue. Other residues within this sequence are involved in recognition of other substrate determinants: Glu203 with the P-6 position, and Tyr204 through Glu230 in the recognition of the P-2 Arg. (B) The crystal structure of the C-subunit demonstrates that recognition of the P-2 Arg involves residues from different parts of the molecule: Glu270, Arg133, Glu230, and Tyr204.

Figure 2. Activity of Phosphorylated G200A and T201A. The Thr-197 kinase, PDK-1 was used to phosphorylate the indicated proteins in an in vitro reaction. The success of the reactions was assessed by immunoblotting with antibodies specific for the phosphorylated Thr-197 and for total protein with a C-subunit antibody, (A). The blots indicate that there is similar amounts of protein in each reaction, and that they are all are phosphorylated with similar efficiency. Panel B indicates the results of the PepTag activity assay. When the fluorescent-tagged Kemptide substrate gets phosphorylated, it undergoes a change in net charge of +1 to −1, altering its direction of migration on an agarose gel. Here phosphorylated peptide migrates toward the top of the page, indicating activity. Only the wild type protein demonstrates activity.
Table 1: Steady-State Kinetic Parameters for P+1 Mutant Proteins Using Kemptide as Substrate

| Parameter                  | Wild Type | Y204A   | Y204F   | E230Q<sup>a</sup> | E203A   |
|----------------------------|-----------|---------|---------|-------------------|---------|
| $K_m$ (μM)                 | 31.1±3    | 341±40<sup>b</sup> | 74.2±20 | 1400±200          | 292±20  |
| $k_{cat}$ (s<sup>-1</sup>) | 33.1±3    | 0.9±0.5 | 31.1±3  | 10.0±0.4          | 20.4±2  |
| $k_{cat}/K_m$ (μM<sup>-1</sup>s<sup>-1</sup>) | 1.07      | 0.0025  | 0.4     | 0.007             | 0.07    |
| $K_m$ (ATP) (μM)           | 21.2±4    | 17.3±3  | 22.2±4  | 9.8±0.9           | 26.5±4  |
| $K_i$ (Ala-Kemp) (μM)      | 181.8±20  | 7,869±700 | 3,441±400 | 4,600±700          | 6,728±900 |

<sup>a</sup> E230Q results are from Grant et. al. (29)

<sup>b</sup> Values for the $K_m$ (Kemptide) were calculated from a Lineweaver-Burke Plot
Table 2: Kinetic Values for P+1 Loop Mutant Proteins Using Variations of a Longer Peptide Substrate

| Parameter | Wild Type | Glu203Ala | Tyr204Ala | Tyr204Phe |
|-----------|-----------|-----------|-----------|-----------|
| GRTGRRNSI |           |           |           |           |
| $K_\text{m} (\mu\text{M})$ | 1.0±0.3 | 33.2±4    | 32.6±6    | 3.4±0.9   |
| $k_{\text{cat}} (\text{s}^{-1})$ | 18.0±2   | 22.7±2    | 11.4±3    | 26.7±0.9  |
| $k_{\text{cat}} / K_\text{m}$ | 18.0     | 0.7       | 0.3       | 7.9       |
| GRTGRANSI |           |           |           |           |
| $K_\text{m} (\mu\text{M})$ | 66.9±5   | 145.9±30  | 1978±90   | 407±30    |
| $k_{\text{cat}} (\text{s}^{-1})$ | 22.2±2   | 27.6±2    | 28.4±6    | 19.9±0.7  |
| $k_{\text{cat}} / K_\text{m}$ | 0.3      | 0.2       | 0.01      | 0.05      |
| GATGRRNSI |           |           |           |           |
| $K_\text{m} (\mu\text{M})$ | 8.9±1    | 59.5±7    | 283±40    | n.d.      |
| $k_{\text{cat}} (\text{s}^{-1})$ | 31.7±3   | 25.5±3    | 5.2±3     | n.d.      |
| $k_{\text{cat}} / K_\text{m}$ | 3.6      | 0.4       | 0.02      | n.d.      |
| Residue   | Location       | Interaction               |
|-----------|----------------|---------------------------|
| Arg 133   | D-Helix        | IP20 P-2 Arg              |
| Glu 170   | Catalytic Loop | IP20 P-2 Arg              |
| Tyr 204   | P+1 Loop       | H-Bond to Glu 230         |
| Glu 230   | F-Helix        | IP20 P-2 Arg              |
| Residue  | Interaction                                                                 |
|----------|-----------------------------------------------------------------------------|
| Leu 198  | P+1 Pocket                                                                  |
| Cys 199  | Water Molecule, CH$_3$ of Thr 197 and δC of His 87                           |
| Gly 200  | Hydrogen Bond to P+1 Backbone Amide                                         |
| Thr 201  | Hydrogen Bond to Catalytic Base Asp 166                                     |
| Pro 202  | P+1 Pocket                                                                  |
| Glu 203  | P-6 Arg of IP20                                                             |
| Tyr 204  | P-2 Arg of IP20                                                             |
| Leu 205  | P+1 Pocket                                                                  |
Structural basis for peptide binding in protein kinase A: Role of glutamic acid 203 and tyrosine 204 in the peptide-positioning loop
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