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Blueprint of the Intramolecular Regulatory Mechanism of Eukaryotic Protein Kinases

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Blueprint of the Intramolecular Regulatory Mechanism of Eukaryotic Protein Kinases

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in

Biomedical Sciences

by

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2015
The Dissertation of Hiruy Sibhatu Meharena is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

2015
DEDICATION

For my family
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LIST OF ABBREVIATIONS

µL. Microliter

Å. Angstrom

"AGC. Group of kinases including PKA, PKG, and PKC"

ATP. Adenosine triphosphate

"cAMP. Adenosine 3’,5’-cyclic monophosphate"

C-subunit. The Catalytic Subunit of PKA

C-terminus. Carboxy-terminus

DNA. Deoxyribonucleic Acid

DTT. Dithiothreitol

E. coli. Escherichia coli

EDTA. Ethylenediaminetetraacetic acid

EGTA. [ethylenebis(oxyethylenenitriilo)] tetraacetic acid

H87. Histidine 87

IP20. Residues 5-24 of PKI

kDa. Kilodaltons

mg. milligram

Mg2. Magnesium

mM. millimoles/liter

NaCl. Sodium chloride

N-terminus. Amino-terminus
PAGE. Polyacrylamide gel electrophoresis

PBS. Phosphate buffered saline

PDB. Protein data bank

PDK1. Phosphoinositide-dependent protein kinase

PKA. Protein Kinase A or cyclic-AMP Dependent Protein Kinase

PKC. Protein kinase C

PKI. Protein kinase A inhibitor

pT197. Phosphorylated Threonine 197

RS0. Aspartate 220

RS1. Tyrosine 164

RS2. Phenylalanine 185

RS3. Leucine 95

RS4. Leucine 106

SDS. Sodium dodecyl sulfate

Sh1. Valine 104

Sh2. Gatekeeper or Methionine 120

Sh3. Methionine 118

TBS. Tris-buffered saline

Tris. Tris Hydroxymethylaminoethane

WT. Wild type

αCE. αC-helix Glutamate

β3K. Catalytic Lysine
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ABSTRACT OF THE DISSERTATION

Blueprint of the Intramolecular Regulatory Mechanism of Eukaryotic Protein Kinases

by

Hiruy Sibhatu Meharena

Doctor of Philosophy in Biomedical Sciences

University of California, San Diego, 2015

Professor Susan S. Taylor, Chair

Eukaryotic protein kinases (EPKs) regulate numerous signaling processes by phosphorylating targeted substrates through a highly conserved catalytic domain. Previous computational studies proposed a model stating that a properly assembled non-linear motif termed the Regulatory (R) spine is essential for catalytic activity of EPKs. Here we define the required intramolecular interactions and biochemical properties of the R-spine and the newly identified “Shell” that surrounds the R-spine.
using site-directed mutagenesis and various \textit{in vitro} phosphoryl transfer assays using cyclic AMP-dependent protein kinase as a representative of the entire kinome. Analysis of the 172 available Apo EPK structures in the protein data bank (PDB) revealed 4 unique structural conformations of the R-spine that correspond with catalytic inactivation of various EPKs. Elucidating the molecular entities required for the catalytic activation of EPKs and the identification of these inactive conformations opens new avenues for the design of efficient therapeutic EPK inhibitors.

The catalytic core of EPKs oscillates between inactive and active states as well as toggling between open and closed conformations when active. Currently, the intramolecular interactions that regulate this dynamic behavior are not well understood. Here, we show that there are at least two possible mechanisms regulating this dynamics. The first mechanism involves the highly conserved salt bridge between a lysine from the $\beta_3$-strand and a glutamate from the $\alpha_C$-helix as well as a hydrogen bond that only forms when the activation loop is phosphorylated. The second mechanism involves an ensemble of hydrophobic interactions within the nonlinear motifs known as the Regulatory spine and Shell. Our findings also show that the highly conserved $\beta_3$-lysine serves as a “catalytic synchronization hub” that aligns and positions the dynamic components required for catalysis.
Chapter 1

Introduction
The cellular profile of an organism is in a constant dynamic state where every individual cell is adjusting to and impacting the surrounding environment while maintaining its fundamental property. This is achieved through a highly regulated process known as cell signaling or signal transduction. Cell signaling is a multi-step process that can be divided into two major categories; extracellular and intracellular signaling (Krauss, 2006). First the signaling molecules are synthesized and released by endocrine, paracrine and autocrine signaling cells. Second, these signaling molecules are detected by specific surface or intracellular receptor protein in targeted cells. These receptors include four major classes; G-protein (metabotropic) coupled receptors, ionotropic receptors, enzyme-linked receptors and nuclear receptors. Finally the receptor-signal complexes are amplified by secondary messengers that induce an intracellular signaling cascade which elicits a physiological response. The prominent secondary messengers include cyclic adenosine monophosphate (cAMP), cyclic guanidine monophosphate (cGMP), calcium ion (Ca^{2+}), inositol 1,4,5-trisphosphate (IP3), Diacyl glycerol (DAG) and nitric oxide (NO). Signal amplification is also facilitated by posttranslational modifications and protein-protein interactions. Protein phosphorylation, dephosphorylation, acetylation, myristylation, neddylation, palmytylation, sulfonation, and ubiquitination are crucial mechanisms in amplifying signaling events. Protein phosphorylation is a ubiquitous signal amplification mechanism that alters the enzymatic activity, protein-protein interactions, and global protein conformations in ~30% of eukaryotic proteins which provides a diverse array for the molecular regulation of cell signal cascades (Olsen et al., 2006; Ptacek et al.,
In eukaryotes, protein phosphorylation is accomplished through a family of proteins known as Eukaryotic Protein Kinases (EPKs).

1.1 History and Classification of Eukaryotic Protein Kinases

In 1906 Levene and Alsberg observed unusual levels of phosphate in Vitellin and in 1932 Lipmann and Levene identified that these phosphates where modifications of a serine residue (Levene and Alsberg, 1906; Lipmann and Levene, 1932). In 1954 Burnett and Kennedy identified and elucidated the first EPK that phosphorylated casein on a serine residue (Casein Kinase [CK1]) (Burnett and Kennedy, 1954). But in 1943 Gerty Cori and Arda Green had already isolated the first kinase without realizing the specific functional role but as the protein that converts phosphorylase $b$ to phosphorylase $a$ and it wasn’t until 1956 that Krebs and Fisher deciphered the role of this protein’s involvement in the reversible, covalent attachment of a phosphate group to phosphorylase $b$ in the presence of adenosine triphosphate (ATP) and they called this protein phosphorylase kinase (Cori and Green, 1943; Krebs and Fischer, 1956a). In 1969, studies of cAMP-dependent protein kinase (PKA) unveiled that phosphorylation is not tissue or species specific (Kuo and Greengard, 1969). Currently more than 15,000 EPKs have been identified in the Domain Eukaryota and more than 500 EPKs have been identified in humans based on a highly conserved catalytic core. EPKs are divided into two major groups; the serine/threonine kinases and the tyrosine kinases (Lehti-Shiu and Shiu, 2012; Manning et al., 2002b). In humans, the relative amount of phosphorylation is $\sim$86.4% on a serine, $\sim$11.8% on a threonine and $\sim$1.8%
Figure 1.1. The Human Kinome. The seven super-families of EPKs represented on the kinome tree adopted from Manning et al. (2002) Science 298:1912.
on a tyrosine (Olsen et al., 2006). These two groups are further divided into seven super-families based on their sequence similarity (Fig 1.1) (Manning et al., 2002a)

I. **AGC kinases** are serine/threonine kinases that are named after PKA, Protein Kinase G (PKG), and Protein Kinase C (PKC).

II. **CAM kinases** are serine/threonine kinases that are Calcium and Calmodulin-regulated, and have several other sequentially related members.

III. **CMGC kinases** are serine/threonine kinases that are named after Cyclin-Dependent protein Kinase (CDK), Mitogen Activated Protein Kinase (MAPK), Glycogen Synthase Kinase (GSK) and Cell Kinase 2 (CK2).

IV. **CK1 kinases** are small group serine/threonine kinases that are sequentially similar to Cell Kinase 1 (CK1).

V. **STE Kinases** are serine/threonine kinases that regulate the MAPKs and have three sub-families the STE7, STE11 and STE20.

VI. **Tyrosine Kinases (TK)** are EPKs that exclusively phosphorylate a tyrosine residue. This group is divided into two major sub-families;

a. Receptor Tyrosine Kinases (RTKs) are activated by binding of an extracellular molecule, usually a protein growth factor.

b. Cytoplasmic Tyrosine Kinases (CTKs) possess a Sh2 domain with the exception of Activated CDC42 kinase (ACK), Focal Adhesion Kinase (FAK) and Janus Activated Kinase (JAK).

VII. **Tyrosine Kinase Like (TKL)** kinases are serine/threonine kinases that sequentially resemble tyrosine kinases but do not phosphorylate a tyrosine
residue. Prominent members of this super-family are the RAF family EPKs that regulate the MAPK pathway.

1.2 The EPK Core Architecture

Due to the extensive biological, biochemical and biophysical studies, PKA serves as the prototype for our understanding of the EPK structure and function. In the late 70’s and early 80’s the biophysical studies of PKA’s active site yielded some insight to some of the major components required for catalytic activity. Even though genetic sequencing identified some of these residues as highly conserved in all EPKs, it wasn’t until 1991 with the resolution of the first PKA structure that an in depth understanding of the functional and structural properties of the EPK core started unraveling. Currently, it is established that the EPK core consists of two lobes, the Amino-terminus (N)-lobe and Carboxyl-terminus (C)-lobe, and consists of approximately 250 amino acid residues (Hanks and Hunter, 1995; Knighton et al., 1991b). The smaller, N-lobe is primarily involved in anchoring and orienting ATP (Figure 1.2). This lobe is predominantly constructed of five antiparallel \( \beta \)-sheet structures (\( \beta_1-\beta_5 \)) that are unique among nucleotide binding proteins and a highly conserved \( \alpha \)C-helix between \( \beta_3 \) and \( \beta_4 \). A highly conserved glycine-rich loop joins the \( \beta_1 \) and \( \beta_2 \) strands and interacts with the ATP phosphates. A short loop known as the “hinge region” is the only structure that connects these two lobes. The deep cleft between the two lobes forms the active site where the phosphoryl transfer process occurs. Both the
**Figure 1.2. Global Architecture of the EPK Core.** The EPK core was classified into 11 subdomains by Hanks and Hunter based on their sequence (Hanks and Hunter, 1995). The N-lobe is split into three subdomains [subdomains-I (red), subdomain-II (orange) and subdomain-III (yellow)]. Subdomain-IV (green) is the only subdomain that is shared by both the N- and C-lobe. The C-lobe consists of six subdomains [subdomain-V (light blue), subdomain-VI (blue), subdomain-VII (magenta), subdomain-VIII (pink), subdomain-IX (brown) and subdomain-X/XII (purple).
N-and C-lobes participate in the binding of ATP with 2 magnesium ions (Mg$^{2+}$). The C-lobe binds the protein substrate bringing it in close proximity to ATP resulting in the phosphorylation of the protein substrate. The C-lobe is mostly α-helical (αD-αl) and is more stable as compared to the N-lobe. Within the C-lobe there are two crucial sequential motifs known as the activation loop and the catalytic loop. As their names suggest the activation loop is involved in the activation and deactivation in most kinases whereas the catalytic loop consists of the residues required for phosphoryl transfer.

In the last decade, computational analysis of the EPKs suggested that the core is organized around three major elements; a large hydrophobic αF-helix in the middle of the C-lobe and two non-linear motifs termed “spines”: the Catalytic (C) spine and the Regulatory (R) spine (Kornev et al., 2006; Kornev et al., 2008). The spines are anchored to the αF-helix and secure the position of ATP, substrate and amino acid residues that are important for catalysis (Figure 1.3). The spines are unusual structural motifs as they consist of amino acid residues that come from different parts of the EPK sequence and do not form a conventional sequential motif. A unique feature of the C-spine is that the adenine ring of ATP is part of this spine and connects the hydrophobic residues from the N- and the C-lobes. Thus the geometry of the C-lobe is dynamic, where the C-spine is completed by the binding of ATP and is broken with the release of adenosine diphosphate ADP. The C-spine consists of eight residues; two from the N-lobe (Val 57 and Ala 70) and six from the C-lobe (Met 128, Leu 171, Leu 172, Ile 173, Leu 227 and Met 231 in PKA). Whereas the geometry of the R-spine is relatively stable as it remains intact throughout the phosphoryl transfer process. The
Figure 1.3. EPK Spines. Two non-linear motifs span the two lobes of the EPK core and provide a firm but flexible connection between the N- and C-lobes. The regulatory spine [R-spine (maroon)] and the Catalytic spine [C-spine (yellow)] contain residues from various subdomains and the R-spine anchored to the αF-helix by conserved Asp220 and the catalytic spine (C-spine) is completed by ATP.
R-spine consists of two residues from the C-lobe (Tyr 164 and Phe 185), and two from the N-lobe (Leu 95 and Leu 106 in PKA).

### 1.3 Phosphoryl Transfer

Unlike other enzymes, EPKs are unique as they do not have a single active and inactive conformation. The active state of an EPK is highly dynamic where the core toggles between the open, intermediate and closed conformations as it traverses the catalytic cycle. The transition from the open to the closed conformation is initiated by the binding of ATP which transitions the core from the open to an intermediate conformation (Figure 1.4). This transition is primarily driven by the interaction of the adenosine portion of ATP with the C-spine and the hinge region (Narayana et al., 1997a). The C-lobe portion of the active site of most EPKs has a negatively charged electrostatic surface and the binding of ATP requires two magnesium ions (Mg$^{2+}$) to neutralize the negative charge of the phosphates (Figure 1e) (Zheng et al., 1993). These Mg$^{2+}$ ions bind to two conserved residues from the C-lobe, an aspartate (D184 in PKA) from the DFG motif on the AL and an asparagine (N171) from the CL, to position the $\gamma$-phosphate of ATP for phosphoryl transfer (Figure 1f) (Buechler and Taylor, 1988). The $\alpha/\beta$-phosphates of ATP interact with the highly conserved lysine residue from the $\beta$3-strand (K72) which forms a salt bridge with a conserved glutamate from the $\alpha$C-helix (E91) (Zoller et al., 1981). The Glycine-rich Loop (G-loop) of the N-lobe interacts with ATP, substrate and the C-lobe sealing the active site chamber in the closed conformation. The substrate (serine, threonine or tyrosine)
Figure 1.4. EPK Active Site. The EPK active site interactions before phosphoryl transfer with ATP and substrate bound in the active site (PDBID:4DG2).
interacts with a conserved aspartate from the HRD motif on the CL (D166) and phosphoryl-transfer is achieved (Valiev et al., 2003). Finally the phosphorylated substrate and adenosine diphosphate (ADP) are released and the EPK transitions back to the open conformation where it is poised to bind another ATP molecule to repeat this process (Bastidas et al., 2013).

1.4 EPKs and Human Diseases

Of the more than 500 EPKs identified in the human genome, approximately 180 are associated with human diseases, either as causative agents or as therapeutic intervention points (http://www.cellsignal.com/reference/kinase_disease.html). EPK mutations have been correlated with numerous diseases including cancer, cardiovascular disease, diabetes and neurodegenerative diseases and thus EPKs have now become the second most sought after family for therapeutic intervention after G-protein coupled receptors (GPCRs) (Cohen, 2002; Johnson, 2009). Currently, 30 small molecule EPK inhibitors are FDA approved and numerous compounds are in clinical trials but the lack of structural diversity of the active EPK core has created a bottleneck for designing successful therapeutic inhibitors (http://www.brimr.org/PKI/PKIs.htm). Even though EPKs lack diversity in their active state, recent findings indicate that there is a higher level of structural diversity in their inactive states (Figure 1.5). This greater degree of diversity has led to the identification of the majority of the FDA approved EPK inhibitors that bind to the inactive EPK core including imatinib, sorafenib, and lapatinib. The lack of an in-depth
understanding of all the possible inactive conformations has hindered the design of more successful EPK inhibitors with minimal off-target effects.

1.5 Goals of the Thesis

Structural and computational studies suggest that the EPK core is highly dynamic but also requires a precisely and harmoniously formed active site to achieve phosphoryl transfer. This precision requires both the N- and C-lobes as well as all the catalytic residues to be perfectly oriented and positioned to achieve catalysis. Even though this precision is appreciated by the scientific community how this precision is achieved is still not well understood. In this thesis I will aim to address two major questions; first, “what is the role of the R-spine in EPK activation and inactivation?” and second, “what are the intramolecular interactions that stabilize and drive the active state?” To address these questions we utilized an E. coli expression system, site-directed mutagenesis, western blotting, radioactive phosphoryl transfer assay, computational structural analysis, molecular dynamics (MD) simulations, molecular modeling and a thermal shift assay using PKA as a model system.
Figure 1.5. EPK Inhibitors. Representation of the off-targets of various EPK inhibitors that target the active and inactive conformations mapped on the Kinome (Collins and Workman, 2006). Inhibitors that have been FDA approved are labeled with respect what disease they were first approved for and the EPK were approved for targeting.
Chapter 2

Deciphering the Structural Elements of Eukaryotic Protein Kinase Regulation
2.1 Introduction

Eukaryotic protein kinases (EPKs) phosphorylate a serine, threonine or tyrosine residue in approximately 30% of human proteins and thus regulate numerous cellular and metabolic processes (Manning et al., 2002b). Abnormal catalytic activity of EPKs is implicated in numerous human diseases, including cancer, cardiovascular diseases and diabetes. Therefore, EPKs are considered to be one of the most promising therapeutic drug targets. Of the more than 500 EPKs identified in the human genome, approximately 180 are associated with human diseases, either as causative agents or as therapeutic intervention points. Currently, 24 small molecule EPK inhibitors are FDA approved and numerous compounds are in clinical trials (Knight et al., 2010). Some of the major challenges for designing efficient therapeutic drugs include the promiscuous nature of these inhibitors targeting multiple members of the family as well as patient relapse due to mutations that drive drug resistance (Krishnamurty and Maly, 2010).

EPKs have a highly conserved structural core that consists of two lobes: a small N-terminal lobe (N-lobe) and a larger C-terminal lobe (C-lobe) (Hanks and Hunter, 1995; Hanks et al., 1988). The smaller, N-lobe is primarily involved in anchoring and orienting the nucleotide (Figure 2.1a). This lobe is predominantly constructed of antiparallel β-sheet structures that are unique among nucleotide binding proteins. A short loop known as the “hinge region” is the only structure that connects these two lobes. The deep cleft between the two lobes forms the active site where the phosphoryl transfer process occurs. Both the N-and C-lobes participate in the binding
Figure 2.1. The Architecture of the EPK Core. A) The conserved EPK structural core is shown mapped on the catalytic subunit of PKA (PDBID:1ATP). The N-lobe (Grey) is mostly composed of β-sheets and the C-lobe (tan) is mostly α-helical. ATP (black) and two atoms of magnesium (purple) are bound in the cleft between the lobes. B) R-spine (maroon) and C-spine (yellow) are bound to the large αF-helix (black) in the center of the C-lobe. They span the whole kinase core and the C-spine is completed by the adenine ring of ATP (yellow). Activation loop phosphorylation at residue T197 (pT197 (red)) is crucial for the complete activation of PKA and pT197 forms a H-bond with H87 (blue) in the αC-helix from the N-lobe. C) The different components of the R-spine are labeled as RS1 from catalytic loop (tan), RS2 from the activation loop (tan), RS3 from the αC-helix (grey), RS4 from the β4(grey) and is anchored by RS0 (light blue) from the αF-helix (black). D) A cartoon representation of the R-spine and the major components of the EPK core.
of ATP with 2 magnesium ions. The C-lobe binds the substrate bringing it in close proximity to ATP resulting in the phosphorylation of the substrate.

Previous computational analysis of EPKs proposed that the core is organized around three major elements (Figure 2.1b); a large hydrophobic $\alpha_F$-helix in the middle of the C-lobe and two non-linear hydrophobic motifs termed “spines”: the Catalytic (C) spine and the Regulatory (R) spine (Kornev et al., 2006; Kornev et al., 2008). The spines are anchored to the $\alpha_F$-helix and secure the position of ATP, substrate and amino acid residues that are important for catalysis. The spines are unusual structural motifs as they consist of amino acid residues that come from different parts of the EPK sequence and do not form a conventional sequence motif. A unique feature of the C-spine is that the adenine ring of ATP is part of this spine and connects the hydrophobic residues from the N- and the C-lobes (Figure 2.1b). The geometry of the R-spine is relatively stable as it remains intact throughout the phosphoryl transfer process.

Unlike other enzymes, EPKs are unique as they do not have a single active and inactive conformation (Taylor et al., 2004). The active state of the enzyme is highly dynamic where the core toggles between the open and closed conformations. The inactive state has traditionally been divided into two general groups defined by the positioning of the phenylalanine of the DFG motif from the activation loop (Huse and Kuriyan, 2002; Kannan and Neuwald, 2005; Levinson et al., 2006). If the DFG-phenylalanine moves far enough from its active position, it is classified as the “DFG-out” conformation which is currently the major target for therapeutic drug design. The second inactive conformation known as the “DFG-in” conformation is when the
phenylalanine does not move substantially from the active conformation. The most common inactive DFG-in conformation is caused by the movement of the αC-helix, but other less understood inactive conformations, not caused by the movement of the DFG-motif or αC-helix, also belong to this group.

Since the R-spine is a geometrically preserved motif that spans both lobes of all EPKs in the active state we sought to elucidate the properties required for a catalytically functional R-spine (Figure 2.1b). Using an *E. coli* expression system, site-directed mutagenesis, western blotting and a radioactive phosphoryl transfer assay, we elucidated the biochemical and biophysical properties required for a catalytically viable R-spine using cyclic AMP-dependent protein kinase (PKA) as a model system. We identified three additional hydrophobic residues around the R-spine that we refer to as the “Shell”, which play a crucial role in supporting the R-spine’s ability to maintain catalytic function. We experimentally tested the relationship between the phosphorylation state of the activation loop, the R-spine and the catalytic activity in PKA. Additionally, qualitative structural analysis of 172 available Apo EPK structures from the protein data bank (PDB) lead to the identification of 4 distinct ways the R-spine is disassembled corresponding with catalytic inactivation of EPKs.

2.2 Experimental Procedures

Sequence alignment

Representative EPK sequences from major taxonomic groups and families (~13690 sequences) were identified and multiply aligned using the MAPGAPS
program (Neuwald, 2009). The aligned columns were used to calculate amino acid frequencies at each of the R-spine positions (RS0-RS4) and the Shell positions (Sh1-3).

**Site-directed mutagenesis**

QuikChange II site-directed mutagenesis kit (Agilent Technologies) was used to introduce various mutations.

**Western blot phosphoryl transfer assay**

The His6-tagged murine Ca-subunit of cAMP dependent protein kinase (PKA) in pET15b was expressed in E. coli (BL21 (DE3)). Cultures were grown at 37 °C to an A600 of ~0.6 and induced with 0.5 mM isopropyl β-d-thiogalactopyranoside (IPTG). The cultures were allowed to grow overnight at 16 °C before harvesting. The expression of PKA was confirmed using PKA C-subunit antibodies from BD Biosciences, and the phosphorylation state of the activation loop was confirmed using a polyclonal pT197 antibody from Invitrogen.

**Radioactive phosphoryl transfer assay**

The His6-tagged wild type and mutation containing PKA in pET15b as well as mutants co-expressed with GST-tagged PDK1 was expressed in E. coli (BL21 (DE3)). Cultures were grown at 37 °C to an A600 of ~0.6 and induced with 0.5 mM IPTG. The cultures were allowed to grow overnight at 16 °C before being harvested. The pellet was resuspended in lysis buffer (50 mM KH2PO4, 20 mM Tris-HCl, 100 mM NaCl, 5 mM β-mercaptoethanol, pH 8.0) and lysed using a microfluidizer (Microfluidics) at 18,000 p.s.i. The cells were clarified by centrifugation at 15,000 rpm at 4 °C for 60 min in a Beckman JA20 rotor, and the supernatant was incubated
with TALON His-Tag Purification Resin (Clontech) overnight at 4 °C using gravity. The resin was washed twice (20X bed volume) with the lysis buffer and twice with using two different concentrations of imidazole in the wash buffer (50 mM KH2PO4, 20 mM Tris-HCl, 100 mM or 1 m NaCl, 50mM/100mM imidazole, and 5 mM β-mercaptoethanol, pH 7). A 250mM imidazole elution buffer was used to elute the His-tagged protein (supplemental figure 1a).

The kinetics was carried out with common reaction mix containing 50 mM MOPS pH 7.4, 1mM Kemptide, 10mM MgCl2, 1mM ATP, and 32 γP radiolabelled ATP (specific activity 500-1000 cpm/pmole) in a final volume of 20μL. The reaction was initiated by addition of PKA with final concentration of 50nM in a volume of 10μl to 10 μl of the reaction mix described above. The reaction was carried out as an end point assay with 3 min as fixed time and at the end-point the reaction was quenched with 90 μl of 30% Acetic acid. 50μl of the quenched reaction was then spotted on p81 phosphocellulose paper, washed 3 times for 5 min each with 5% phosphoric acid and finally washed with acetone (1X), air dried and counted on liquid scintillation counter. The background counts were subtracted from the experimental time points and plotted to compare their activities. The wild type protein was used as positive control and E91A mutant as the negative control. Each reaction was carried out in triplicates and data plotted are mean percent of wildtype ± standard error. The plots were made using MS Excel.
2.3 Results

PKA is one of the well-studied EPKs, and, therefore, it is often used as a prototype for understanding the biophysical and biochemical properties of the entire kinome. In PKA, the R-spine has two residues from the C-Lobe; tyrosine 164 (RS1) of the YRD/HRD motif from the catalytic loop and phenylalanine 185 (RS2) of the activation loop DFG motif (Figure 2.1c, d). It is completed by two N-lobe residues; leucine 95 (RS3) of the $\alpha$C-helix and leucine 106 (RS4) of $\beta$4-strand. This non-linear motif is anchored to the $\alpha$F-helix through aspartic acid 220 (RS0). PKA has a phosphorylation site, threonine 197 (T197) on the activation loop, which is required for the completion of the catalytic activation process (Figure 2.1a, b d) (Yonemoto et al., 1997). The phosphorylation of T197 (pT197) initiates an intricate hydrogen bond network observed in the C-lobe, which is required for substrate binding (Steichen et al., 2012). pT197 also forms a hydrogen bond (H-bond) with histidine 87 (H87) from the $\alpha$C-helix, and previous studies demonstrated that mutating H87 to alanine (H87A) increases the catalytic activity by 2-3 fold (Cox and Taylor, 1995). T197 is a trans-auto phosphorylation site as well as a substrate for 3-phosphoinositide-dependent protein kinase 1 (PDK1) (Cheng et al., 1998). When PKA is expressed in bacteria, auto phosphorylation of pT197 is a qualitative indicator for the catalytic activity of PKA (Yonemoto et al., 1993). The catalytic activity is negatively controlled by mutating glutamic acid 91 into an alanine (E91A) resulting in the loss of the salt bridge with the catalytic lysine 72 which is essential for the phosphoryl transfer process.
Table 2.1. Summary of the alignment of the more than 13,000 EPK sequences.
Percentile of aromatic, aliphatic, hydrophobic and representative amino acids for each R-spine and Shell from alignment of more than 13,000 EPK sequences.

<table>
<thead>
<tr>
<th>Amino Acids</th>
<th>RS0</th>
<th>RS1</th>
<th>RS2</th>
<th>RS3</th>
<th>RS4</th>
<th>Sh1</th>
<th>Sh2</th>
<th>Sh3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aromatic</td>
<td>0.23</td>
<td>99.45</td>
<td>91.57</td>
<td>8.18</td>
<td>20.37</td>
<td>5.06</td>
<td>20.52</td>
<td>7.43</td>
</tr>
<tr>
<td>Aliphatic</td>
<td>99.77</td>
<td>0.55</td>
<td>8.43</td>
<td>91.82</td>
<td>79.63</td>
<td>94.94</td>
<td>79.48</td>
<td>92.57</td>
</tr>
<tr>
<td>Hydrophobic</td>
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<td>8.71</td>
<td>99.67</td>
<td>89.37</td>
<td>96.31</td>
<td>89.68</td>
<td>82.37</td>
<td>98.24</td>
</tr>
<tr>
<td>Alanine (A)</td>
<td>0.08</td>
<td>0.01</td>
<td>0.11</td>
<td>2.73</td>
<td>1.83</td>
<td>2.86</td>
<td>0.18</td>
<td>0.41</td>
</tr>
<tr>
<td>Cysteine (C)</td>
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<td>0.01</td>
<td>0.04</td>
<td>1.15</td>
<td>1.50</td>
<td>1.31</td>
<td>0.51</td>
<td>0.25</td>
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<tr>
<td>Aspartic acid (D)</td>
<td>96.38</td>
<td>0.40</td>
<td>0.01</td>
<td>0.07</td>
<td>0.02</td>
<td>0.02</td>
<td>0.02</td>
<td>0.01</td>
</tr>
<tr>
<td>Glutamic acid (E)</td>
<td>0.43</td>
<td>0.02</td>
<td>0.01</td>
<td>0.09</td>
<td>0.04</td>
<td>0.08</td>
<td>0.41</td>
<td>0.17</td>
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<tr>
<td>Phenylalanine (F)</td>
<td>0.00</td>
<td>0.53</td>
<td>88.64</td>
<td>1.45</td>
<td>10.50</td>
<td>0.11</td>
<td>10.90</td>
<td>6.64</td>
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<tr>
<td>Glycine (G)</td>
<td>0.04</td>
<td>0.00</td>
<td>0.01</td>
<td>0.55</td>
<td>0.02</td>
<td>0.08</td>
<td>0.11</td>
<td>0.01</td>
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<td>Histidine (H)</td>
<td>0.01</td>
<td>91.04</td>
<td>0.01</td>
<td>3.13</td>
<td>0.17</td>
<td>0.00</td>
<td>0.19</td>
<td>0.07</td>
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<tr>
<td>Isoleucine (I)</td>
<td>0.00</td>
<td>0.09</td>
<td>0.15</td>
<td>5.28</td>
<td>7.39</td>
<td>22.47</td>
<td>2.59</td>
<td>26.54</td>
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<tr>
<td>Lysine (K)</td>
<td>0.04</td>
<td>0.01</td>
<td>0.01</td>
<td>0.18</td>
<td>0.06</td>
<td>0.05</td>
<td>0.05</td>
<td>0.02</td>
</tr>
<tr>
<td>Leucine (L)</td>
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<td>0.22</td>
<td>6.76</td>
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<td>54.74</td>
<td>8.32</td>
<td>18.42</td>
<td>46.02</td>
</tr>
<tr>
<td>Methionine (M)</td>
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<td>0.70</td>
<td>21.26</td>
<td>5.21</td>
<td>1.33</td>
<td>38.36</td>
<td>10.92</td>
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<tr>
<td>Asparagine (N)</td>
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<td>0.07</td>
<td>0.10</td>
<td>0.75</td>
<td>0.07</td>
<td>0.20</td>
<td>0.23</td>
<td>0.02</td>
</tr>
<tr>
<td>Proline (P)</td>
<td>0.02</td>
<td>0.04</td>
<td>0.05</td>
<td>0.08</td>
<td>0.37</td>
<td>4.89</td>
<td>0.02</td>
<td>0.06</td>
</tr>
<tr>
<td>Glutamine (Q)</td>
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<td>0.04</td>
<td>0.00</td>
<td>1.96</td>
<td>0.14</td>
<td>0.14</td>
<td>2.04</td>
<td>0.23</td>
</tr>
<tr>
<td>Arginine (R)</td>
<td>0.02</td>
<td>0.01</td>
<td>0.01</td>
<td>0.31</td>
<td>0.02</td>
<td>0.16</td>
<td>0.26</td>
<td>0.02</td>
</tr>
<tr>
<td>Serine (S)</td>
<td>0.07</td>
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<td>0.01</td>
<td>0.83</td>
<td>0.52</td>
<td>0.39</td>
<td>1.31</td>
<td>0.11</td>
</tr>
<tr>
<td>Threonine (T)</td>
<td>2.13</td>
<td>0.02</td>
<td>0.07</td>
<td>1.51</td>
<td>0.76</td>
<td>3.00</td>
<td>12.49</td>
<td>0.31</td>
</tr>
<tr>
<td>Valine (V)</td>
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<td>0.02</td>
<td>0.39</td>
<td>1.80</td>
<td>7.31</td>
<td>54.53</td>
<td>2.50</td>
<td>6.98</td>
</tr>
<tr>
<td>Tryptophan (W)</td>
<td>0.00</td>
<td>0.01</td>
<td>1.30</td>
<td>0.33</td>
<td>0.08</td>
<td>0.04</td>
<td>0.02</td>
<td>0.06</td>
</tr>
<tr>
<td>Tyrosine (Y)</td>
<td>0.21</td>
<td>7.13</td>
<td>1.61</td>
<td>3.19</td>
<td>9.26</td>
<td>0.02</td>
<td>9.40</td>
<td>0.67</td>
</tr>
</tbody>
</table>
Is the aromatic or aliphatic property of the R-spine essential for catalytic activity?

In most EPKs the R-spine consists of two aromatic residues from the C-lobe and two aliphatic residues from the N-lobe. Alignment of more than 13,000 EPK sequences showed that RS1 is conserved as an aromatic residue in ~99% of EPKs (Table 2.1). Previous studies in Drosophila Src64 showed that mutating RS1 from a histidine into a leucine does not eliminate the catalytic activity (Strong et al., 2011). To examine whether PKA can tolerate an aliphatic residue instead of a tyrosine at the RS1 position we inserted a mutation converting RS1 to a methionine (RS1M). The western blot assay illustrates that the mutant remains catalytically active even though activity is reduced (Figure 2.2a). The second R-spine residue from the C-lobe, RS2, is conserved as a phenylalanine in approximately ~90% of EPKs, but this residue is also a leucine (aliphatic) in about ~6.7% of EPKs. To test if this naturally occurring variant is tolerable in PKA we mutated RS2 to a leucine (RS2L). We observed that RS2L remains as catalytically active as the wild type PKA (WT-PKA) (Figure 2.2a). The N-lobe residues RS3 and RS4, on the other hand, are conserved as aliphatic residues in approximately 90% and 80% of EPKs, respectively. In order to determine if PKA can tolerate an aromatic residue instead of an aliphatic residue, we individually mutated RS3 and RS4 from a leucine into a phenylalanine (RS3F and RS4F). The results show that RS3F and RS4F mutants have normal levels of catalytic activity in comparison to the WT-PKA (Figure 2.2a).
Figure 2.2. Understanding the properties required for a catalytically active R-spine. PKA mutants were expressed in *E. coli* and the catalytic activity was analyzed using western blot assay to determine the effect of A) aromatic and aliphatic properties of the R-spine and B) specific interactions of RS1. A qualitative western blot assay (C) and quantitative radioactive phosphoryl transfer assay (D) were carried out for PKA mutants containing hydrophilic R-spine mutations. A qualitative western blot assay (E) and quantitative radioactive phosphoryl transfer assay (F) were carried out for PKA mutants containing removal of side chain atoms of the R-spine residues.
Is the aromatic or aliphatic property of the R-spine essential for catalytic activity?

In most EPKs the R-spine consists of two aromatic residues from the C-lobe and two aliphatic residues from the N-lobe. Alignment of more than 13,000 EPK sequences showed that RS1 is conserved as an aromatic residue in ~99% of EPKs (Table 2.1). Previous studies in *Drosophila* Src64 showed that mutating RS1 from a histidine into a leucine does not eliminate the catalytic activity (Strong et al., 2011). To examine whether PKA can tolerate an aliphatic residue instead of a tyrosine at the RS1 position we inserted a mutation converting RS1 to a methionine (RS1M). The western blot assay illustrates that the mutant remains catalytically active even though activity is reduced (Figure 2.2a). The second R-spine residue from the C-lobe, RS2, is conserved as a phenylalanine in approximately ~90% of EPKs, but this residue is also a leucine (aliphatic) in about ~6.7% of EPKs. To test if this naturally occurring variant is tolerable in PKA we mutated RS2 to a leucine (RS2L). We observed that RS2L remains as catalytically active as the wild type PKA (WT-PKA) (Figure 2.2a). The N-lobe residues RS3 and RS4, on the other hand, are conserved as aliphatic residues in approximately 90% and 80% of EPKs, respectively. In order to determine if PKA can tolerate an aromatic residue instead of an aliphatic residue, we individually mutated RS3 and RS4 from a leucine into a phenylalanine (RS3F and RS4F). The results show that RS3F and RS4F mutants have normal levels of catalytic activity in comparison to the WT-PKA (Figure 2.2a).
Table 2.2. Radioactive phosphoryl transfer assay of different PKA mutants. The activity is represented by the percent of catalytic activity of each mutant relative to the wild type for triplicate experiments using a radioactive phosphoryl transfer assay.

<table>
<thead>
<tr>
<th></th>
<th>Activity (%)</th>
<th>Relative Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>100.00</td>
<td>10.42</td>
</tr>
<tr>
<td>E91A</td>
<td>1.83</td>
<td>0.26</td>
</tr>
<tr>
<td>RS1N</td>
<td>2.15</td>
<td>0.46</td>
</tr>
<tr>
<td>RS2N</td>
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<td>0.02</td>
</tr>
<tr>
<td>RS3N</td>
<td>84.50</td>
<td>6.34</td>
</tr>
<tr>
<td>RS4N</td>
<td>96.83</td>
<td>10.55</td>
</tr>
<tr>
<td>RS1G</td>
<td>0.17</td>
<td>0.12</td>
</tr>
<tr>
<td>RS2G</td>
<td>0.21</td>
<td>0.07</td>
</tr>
<tr>
<td>RS3G</td>
<td>97.58</td>
<td>15.13</td>
</tr>
<tr>
<td>RS4G</td>
<td>92.65</td>
<td>2.42</td>
</tr>
<tr>
<td>RS3G</td>
<td>97.58</td>
<td>7.56</td>
</tr>
</tbody>
</table>
Which interactions of RS1 are required for catalytic activity?

The RS1 residue is conserved as a histidine in ~91% and tyrosine in ~8% of EPKs (Table 2.1). The side chain of RS1 has the ability to interact with the neighboring amino acid residues in three different ways; first, the hydrophobic interaction of tyrosine with RS2, which we demonstrated to be sufficient for maintaining some catalytic activity (RS1M) (Figure 2.2b). Next is the CH-π interaction with RS2, which is conserved in approximately 90% of EPKs. To assess whether this interaction is sufficient for maintaining catalytic activity, we replaced RS1 with phenylalanine (RS1F). The western blot results show that RS1F is sufficient for maintaining catalytic activity, and the introduction of the aromatic ring improves the catalytic activity when compared to RS1M (Fig 2.2b). Since RS1 is conserved as histidine (RS1H) in 91% of EPKs we mutated RS1 from a tyrosine to histidine and western blot analysis shows that the mutant remains catalytically active. Finally, the polar interaction of RS1 with RS0; here the main chain of RS1 interacts with the side chain of RS0 and this interaction is conserved in more than 95% of EPKs. To examine if this polar interaction is required to maintain the proper assembly of the R-spine, we mutated RS0 to alanine (RS0A) and western blot results show that catalytic activity was abolished (Figure 2.2b). This is consistent with recent studies on Aurora kinase, where mutation of RS0 to an alanine (RS0A) abolished Aurora kinase activity (Oruganty et al., 2013). To test if loss of this polar interaction could be rescued through a hydrophobic interaction we replaced RS0 with a leucine (RS0L); results show that some catalytic activity was rescued (Figure 2.2b).
Is the hydrophobic property of the R-spine residues mandatory for catalytic activity?

Based on sequence alignment (Table 2.1) the three R-spine residues (RS2, RS3 and RS4) are highly conserved as a hydrophobic residue, whereas only ~8% of EPKs including PKA have a hydrophobic residue at the RS1 position. To address if the hydrophobic property is required for catalytic activity, we introduced the hydrophilic residues aspartic acid (RS1D, RS2D, RS3D and RS4D) or asparagine (RS1N, RS2N, RS3N and RS4N) to each of the four R-spine positions individually. Using western blotting techniques and radioactive phosphoryl transfer assays, we discovered that the two C-lobe residues (RS1 and RS2) were highly sensitive to the introduction of a hydrophilic residue. Western blot analysis demonstrates that the catalytic activity was abolished when RS1 or RS2 were substituted with a hydrophilic residue (RS1D, RS2D, RS1N and RS2N) (Figure 2.2c). Quantitative analysis of the hydrophilic non-charged asparagine mutation using the radioactive phosphoryl transfer assay confirmed that the catalytic activity of RS1N and RS2N was reduced by more than 95% (Figure 2.2d and Table 2.2). In contrast, when the N-lobe R-spine residues were mutated to hydrophilic residues (RS3D, RS4D, RS3N and RS4N), the mutants remained catalytically active (Figure 2.2c). The enzyme retained 85% and 95% of its activity when the RS3 and RS4 positions were mutated to asparagine (RS3N and RS4N), respectively (Figure 2.2d and Table 2.2).
Are the atoms of the side chains of the R-spine residues crucial for catalytic activity?

To evaluate whether the side chain of each R-spine residue is required for catalytic activity, we individually mutated each residue into an alanine or a glycine. After mutating the RS1 and RS2 (RS1A, RS2A RS1G and RS2G), the catalytic activity was abolished, as illustrated by the western blots (Figure 2.2e). The radioactive phosphoryl transfer assay confirmed that the catalytic activity was reduced by more than 99% for RS1G and RS2G (Figure 2.2f and Table 2.2). Whereas, the western blots of RS3 and RS4 to alanine and glycine mutants (RS3A RS4A, RS3G and RS4G) showed that these mutants had comparable levels of catalytic activity as the WT-PKA (Figure 2.2e). The quantitative data for RS3G and RS4G confirm that the catalytic activity was only reduced by ~15% and ~5%, respectively (Figure 2.2f and Table 2.2).

Why is the N-lobe region of the R-spine unaffected by the alteration of the side chains?

To understand why the catalytic activity was unaffected by the introduction of a hydrophilic residue or the removal of the side chain to the N-lobe region of the R-spine we analyzed the amino acid residues that are within 4Å of RS3 and RS4 in PKA. Looking at the previous Local Spatial Pattern (LSP) alignment data (Kornev et al., 2008), only 3 out of the 14 amino acid residues surrounding RS3 and RS4 are highly conserved. We termed these 3 residues as the Shell, as they seemed to be supporting the N-lobe region of the R-spine (Figure 2.3a). In PKA these residues are valine 104...
Figure 2.3. The role of the shell for catalytic activity. A) The 3-dimensional structure of the shell (teal) is shown surrounding the R-spine (maroon) in PKA (PDBID:1ATP). B) The R-spine and shell are represented as a cartoon. C) A radioactive phosphoryl transfer assay was carried out on various mutants elucidating the relation of the shell with the R-spine and the required role for catalytic activity.
(Sh1) which is conserved as a hydrophobic residue in ~90% of EPKs, the gatekeeper residue (methionine 120 (Sh2)) which is conserved as a hydrophobic residue in ~82% of EPKs, and methionine 118 (Sh3), conserved as a hydrophobic residue in ~98% of EPKs (Table 2.1 and Figure 2.3b). To understand the role of the shell for catalytic activity, we made multiple mutations followed by radioactive phosphoryl transfer assays in PKA.

Above, we showed that RS3G has comparable catalytic activity to the WT-PKA (Figure 2.3c and Table 2.3). To destabilize this mutant we decided to introduce an alanine mutation at the Sh2 position (RS3G/Sh2A). Results showed that the catalytic activity was significantly reduced by ~96% (Figure 2.3c and Table 2.3). This indicates that RS3 is essential in the absence of Sh2. Next, to understand the role of Sh1 on the catalytic activity, we mutated Sh1 into a glycine (Sh1G). In the absence of the Sh1 side chain, the catalytic activity of the EPK was reduced by ~94% (Figure 2.3c and Table 2.3). Sh1 is a crucial residue for catalytic activity as previous studies describe that the αC-β4-loop is crucial for anchoring the αC-helix (Kannan et al., 2008).

To understand the significance of each of the 3 residues (RS4, Sh3 and Sh2), we mutated the RS4 residue to a glycine and SH2 and SH3 residues to an alanine (RS4G/Sh2A/Sh3A). This catalytically dead triple mutant serves as the reference point for understanding the role of each residue on the catalytic activity of the EPK (Figure 2.3c and Table 2.3). We then reintroduced each residue individually into the triple mutant to assay for rescue of catalytic activity. When returning Sh2 to a methionine in the triple mutant (RS4G/Sh3A) we were able to rescue ~13% of the catalytic activity.
Table 2.3. Radioactive phosphoryl transfer assay of elucidating the role of the Shell residues. The activity is represented by the percent of catalytic activity of each mutant relative to the wild type for triplicate experiments using a radioactive phosphoryl transfer assay.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Activity (%)</th>
<th>Relative Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>100.00</td>
<td>10.42</td>
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<tr>
<td>E91A</td>
<td>1.83</td>
<td>0.26</td>
</tr>
<tr>
<td>RS3G/Sh2A</td>
<td>3.63</td>
<td>2.44</td>
</tr>
<tr>
<td>Sh1G</td>
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<td>1.83</td>
</tr>
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<td>2.44</td>
</tr>
<tr>
<td>Sh2A/Sh3A</td>
<td>0.06</td>
<td>0.06</td>
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</tbody>
</table>
(Figure 3c, 3d and Table S2). Next, we mutated RS3 to a glycine (RS4G/Sh3A/RS3G) to address whether Sh2 could maintain activity through Sh1. Here we were able to rescue the catalytic activity by ~23% which indicates that Sh1 plays a role in maintaining a viable hydrophobic interaction between the N- and C-lobes through Sh2 (Figure 2.3c and Table 2.3). Next, we attempted to rescue some of the catalytic activity by mutating Sh3 back to a methionine in the triple mutant (RS4G/Sh2A), and we were able to rescue ~47% of the catalytic activity (Figure 2.3c and Table 2.3). Above, we showed that in the absence of Sh2 and RS3 the catalytic activity was abolished, this indicates that Sh3 requires the presence of RS3 to maintain catalytic activity (Figure 2.3c and Table 2.3). Finally, when we returned RS4 back to a leucine in the triple mutant (Sh3A/Sh2A), we were unable to recover any catalytic activity (Figure 2.3c and Table 2.3). This indicates that RS4 requires the presence of either Sh2 or Sh3 to maintain catalytic activity.

What is the association between the R-spine and phosphorylation of the activation loop?

Complete activation of PKA is achieved after phosphorylation of T197 (pT197) on the activation loop (Steinberg et al., 1993). pT197 initiates a major hydrogen bonding network in the C-lobe (Steichen et al., 2012) and forms a H-bond between the activation loop and the αC-helix through H87. Previous studies showed that eliminating the H-bond between pT197 and H87 improves the catalytic activity by 2-3 fold (Cox and Taylor, 1995). We hypothesized that destabilization of the R-spine through hydrophilic mutations would cause disorientation of the N- and C-lobes, and
Figure 2.4. Phosphorylation of the activation loop is involved in stabilizing the assembled R-spine. A) A comparison of the phosphorylated (PDBID:1ATP) and unphosphorylated (PDBID:4DFY) structures of PKA showed that the H-bond between pT197 and H87 is disrupted in the unphosphorylated state and the two lobes move away from each other and twist. B) The inactivation of the C-subunit by twisting, separation of the two lobes, and disruption of the H-bond is shown as a cartoon representation. C) The hydrophilic R-spine mutants were coexpressed with PDK1 to ensure complete activation loop phosphorylation their activity measured using a radioactive phosphoryl transfer assay. Then H87A mutation was introduced to the hydrophilic R-spine mutants and coexpressed with PDK1 and activity was measured.
Table 2.4. Radioactive phosphoryl transfer assay of elucidating the collaboration between the R-spine and pT197-H87. The activity is represented by the percent of catalytic activity of each mutant relative to the wild type for triplicate experiments using a radioactive phosphoryl transfer assay.

<table>
<thead>
<tr>
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<th>Activity (%)</th>
<th>Relative Standard Deviation</th>
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<td>WT</td>
<td>100.00</td>
<td>10.42</td>
</tr>
<tr>
<td>E91A</td>
<td>1.83</td>
<td>0.26</td>
</tr>
<tr>
<td>RS1N + PDK1</td>
<td>43.59</td>
<td>3.99</td>
</tr>
<tr>
<td>RS1N/H87A + PDK1</td>
<td>19.75</td>
<td>5.41</td>
</tr>
<tr>
<td>RS2N + PDK1</td>
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<td>2.42</td>
</tr>
<tr>
<td>RS3N/H87A + PDK1</td>
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<td>8.84</td>
</tr>
<tr>
<td>RS4N + H87A</td>
<td>39.61</td>
<td>0.15</td>
</tr>
</tbody>
</table>
this loss of catalytic activity can be rescued through the H-bond formed between pT197 and H87 (Figure 2.4a, b). To test this hypothesis we began by rescuing the catalytic activity of RS1N and RS2N by co-expressing these constructs with PDK1 which phosphorylates PKA on T197 and introduces the pT197-H87 H-bond. Using a radioactive phosphoryl transfer assay, we observed a ~43% rescue of catalytic activity from ~2% for RS1N and ~75% rescue from less than 1% catalytic activity for RS2N (Figure 2.4c and Table 2.4). As a control we co-expressed RS3N with PDK1 (RS3+PDK1) and results show that the catalytic activity is comparable with RS3N. To understand if this rescue of function was due the intricate hydrogen bond network formed in the C-lobe or due to pT197-H87 we introduced the H87A mutation into all the asparagine mutants and co-expressed these double mutants with PDK1 (RS1N/H87A+PDK1, RS2N/H87A+PDK1, RS3N/H87A+PDK1 and RS4N/H87A+PDK1). The results from the radioactive phosphoryl transfer assay show that the catalytic activity for RS2N/H87A+PDK1 was reduced by ~95% in comparison to RS2N+PDK1 and the catalytic activity for RS3N/H87A+PDK1 was reduced by ~73% with respect to RS3N+PDK1 (Figure 2.4c and Table 2.4). However, the catalytic activity of RS1N/H87A+PDK1 and RS4N/H87A+PDK1 was reduced by ~50% and ~55%, when compared to RS1N+PDK1 and RS4N, respectively (Figure 2.4c and Table 2.4). Although the effect is not as drastic for RS1 and RS4, these results demonstrate that any instability of the R-spine affects the catalytic activity.
2.4 Discussion

In 2006, the R-spine hypothesis for EPK regulation was proposed based on the computational comparison of 23 EPK structures (Kornev et al., 2006). Despite the lack of solid biochemical validation of this model, it quickly became popular and has been widely accepted as a framework for analysis of EPKs (Azam et al., 2008; Bossi et al., 2010; Jura et al., 2011; Roskoski, 2013). Nevertheless, many questions related to the properties of the R-spine residues remained unanswered. In this work we present the first systematic study of the R-spine in PKA, an EPK that has served as a prototype for the entire kinome for more than two decades. Our findings establish that the hydrophobic nature of the R-spine and the non-polar CH-π interaction of RS1 with RS2 are mandatory for catalytic activity. The interaction of RS0 with RS1 is crucial for catalytic activity but we demonstrate that a hydrophobic interaction can maintain the anchoring of the R-spine to the αF-helix. We also revealed that the N-lobe region of the R-spine is supported by a 3 residue hydrophobic ensemble which we termed the “Shell” (Sh1, Sh2 and Sh3). The absence of Sh1 causes catalytic inactivation, indicating that the interaction of Sh1 with RS3 is crucial for anchoring the αC-helix in the active conformation. Sh1 is also capable of maintaining some catalytic activity in the absence of RS3 by completing the R-spine through the gatekeeper residue (Sh2). Previous studies showed that Sh2 and Sh3 are equally important for catalytic activity because either residue has the ability to compensate for the absence of the other, as in IL2-inducible T-cell kinase (Itk), and that at least one was mandatory for maintaining catalytic activity (Joseph and Andreotti, 2011). Here we confirm that the absence of
Figure 2.5. The R-spine and Shell configuration in the inactive state of EPKs. The four inactive conformations of the EPKs are shown with representative structures as well as cartoons in order to illustrate the configurations of the R-spine and Shell. The structures are inactive I (AKT; PDBID:1GZK) representing the DFG-out configuration; inactive II (Src; PDBID:1FMK) representing the C-helix out configuration; inactive III (AMPK; PDBID:3H4J) representing the HRD-out configuration; inactive IV (P38 MAPK; PDBID:1WFC) representing the twisted lobe configuration. The active EPK conformation (PKA; PDBID:1ATP) is shown for comparison (center).
Table 2.5. Amino acid numbers of each R-spine and Shell residues for the representatives of each conformation. A list of the R-spine and Shell residues for PKA, AKT, Src, AMPK, and P38-MAPK.

<table>
<thead>
<tr>
<th></th>
<th>PKA</th>
<th>AKT</th>
<th>Src</th>
<th>AMPK</th>
<th>P38-MAPK</th>
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<tr>
<td><strong>RS0</strong></td>
<td>D220</td>
<td>D332</td>
<td>D444</td>
<td>D213</td>
<td>D205</td>
</tr>
<tr>
<td><strong>RS1</strong></td>
<td>Y164</td>
<td>Y273</td>
<td>H384</td>
<td>H154</td>
<td>H148</td>
</tr>
<tr>
<td><strong>RS2</strong></td>
<td>F185</td>
<td>F294</td>
<td>F405</td>
<td>F175</td>
<td>F169</td>
</tr>
<tr>
<td><strong>RS3</strong></td>
<td>L95</td>
<td>L204</td>
<td>M314</td>
<td>L86</td>
<td>L75</td>
</tr>
<tr>
<td><strong>RS4</strong></td>
<td>L106</td>
<td>L215</td>
<td>L325</td>
<td>L97</td>
<td>L86</td>
</tr>
<tr>
<td><strong>Sh1</strong></td>
<td>V104</td>
<td>T213</td>
<td>V323</td>
<td>I95</td>
<td>I84</td>
</tr>
<tr>
<td><strong>Sh2</strong></td>
<td>M120</td>
<td>M229</td>
<td>T338</td>
<td>I111</td>
<td>T106</td>
</tr>
<tr>
<td><strong>Sh3</strong></td>
<td>M118</td>
<td>F227</td>
<td>I336</td>
<td>M109</td>
<td>L104</td>
</tr>
</tbody>
</table>
Sh3 and Sh2 in PKA abolish catalytic activity and returning either one enables the partial rescue of catalytic function. This is supported by the numerous disease-driving bulky hydrophobic single-nucleotide polymorphisms of the gatekeeper (Sh2) residue that boost activity (Azam et al., 2008). The absence of a perfectly assembled R-spine results in loss of catalytic activity. However, this loss of function can be rescued by phosphorylating the activation loop, thus creating the pT197-H87 H-bond that stabilizes the assembled conformation of the R-spine.

Since the assembly of the R-spine is required for catalytic activity, we searched for naturally occurring disassembled conformations of the R-spine that correlated with catalytic inactivation. From the available 172 Apo EPK structures available in the PDB, we identified 4 different ways the R-spine can be disassembled corresponding to catalytically inactive EPKs (Table 2.5). The first two inactive groups were described as the DFG-out and DFG-in inactive conformations, respectively (Levinson et al., 2006). Inactive I or the DFG-out conformation is where the side chain of the RS2 of the DFG motif is misplaced from the active conformation as illustrated by the structure of Protein kinase B (AKT) (Yang et al., 2002) (Fig 2.5 and Table 2.5). This inactive conformation was previously described in ABL kinase, and we were able to mimic this conformation in PKA through the RS2G mutant. Inactive II or the αC-helix out conformation, which was previously described in Src, occurs when RS3 is removed from the active conformation due to the αC-helix twisting out and moving away from the active site (Fig 2.5 and Table 2.5). The RS3G+Sh2A mutant mimics this inactive conformation as the Sh2 residue in Src is the small hydrophilic residue threonine. In the inactive III or the YRD/HRD-out conformation, represented by 5'
Figure 2.6. Inactive conformation stabilizing FDA approved EPK inhibitors. A) Imatinib (Gleevec) bound to Bcr-Abl tyrosine-kinase (3K5V.pdb) in the DFG-out conformation (Inactive I) and B) Lapatinib (Tykreb) bound to Receptor tyrosine-protein kinase erbB-4 (3BBT.pdb) in the αC-helix-out conformation (Inactive II). C) Table summarizing the R-spine and Shell residues in PKA, Bcr-Abl and erbB4.
AMP-activated protein kinase (Chen et al., 2009), we observe that RS1 from the YRD/HRD motif of the catalytic loop is no longer anchored to the αF-helix (Figure 5, Table S3, S3). We were able to mimic this conformation through the RS0A mutant in PKA. The last inactive conformation is the inactive IV or the twisted lobe conformation, which occurs when the two lobes move away from each other and twist causing the R-spine to split in half (Figure 5, Table S3, S3). This conformation is represented by the structure of P38 mitogen-activated protein kinases (Wilson et al., 1996). Identification of the inactive I and inactive II conformations enabled the design of successful drugs such as Imatinib (Zhang et al., 2010) and Lapatinib (Qiu et al., 2008), respectively (Figure S2). We believe that the identification and the functional understanding of the R-spine and shell will generate novel approaches to designing more efficient therapeutic EPK inhibitors as well providing insight towards understanding some of the disease causing mutations.

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Chapter 3

Intramolecular Interactions Regulating the Mechanics of Eukaryotic Protein Kinases
3.1 Introduction

Eukaryotic Protein Kinases (EPKs) were first discovered in 1943, and their functional role in phosphorylation was elucidated in 1956 (Cori and Green, 1943; Krebs and Fischer, 1956b; Wosilait and Sutherland, 1956). In 1969, studies of cAMP-dependent protein kinase (PKA) unveiled that phosphorylation is not tissue or species specific (Kuo and Greengard, 1969). PKA has since served as the prototype for our understanding of EPK structure and function. EPKs are ubiquitously expressed in all eukaryotes and approximately 2% of the human genome encodes for EPKs (Lehti-Shiu and Shiu, 2012; Manning et al., 2002b). EPKs are involved in most biological processes and have been associated with numerous human diseases, making EPKs key candidates for therapeutic intervention (Lahiry et al., 2010).

EPKs have a highly conserved catalytic core that mediates the transfer of the $\gamma$-phosphate of adenosine triphosphate (ATP) to a protein substrate (Hanks and Hunter, 1995). Structurally, the core consists of two lobes, the N-lobe and C-lobe (Figure 3.1a) (Knighton et al., 1991a). Within the core there are two nonlinear motifs known as the Catalytic (C)-spine and the Regulatory (R)-spine that span both the N- and C-lobes (Figure 3.1b) (Kornev et al., 2008). The R-spine has two residues from the C-lobe [RS1 from the YHRED motif on the catalytic loop (CL) and RS2 from the DFG motif on the activation loop (AL)], and two from the N-lobe [RS3 from the $\alpha$C-helix and RS4 from the $\beta$4-strand] (Figure 3.1c). The R-spine is anchored to the $\alpha$F-helix through a highly
Figure 3.1. Global architecture of the EPK core. A) The structural core mapped on the catalytic subunit of PKA (PDBID:1ATP). The N-lobe (grey) is mostly composed of \(\beta\)-sheets and the C-lobe (olive) is mostly \(\alpha\)-helical. The active site is between these lobes and ATP binds with two magnesium ions. B) R-spine (maroon), Shell residues (teal) and C-spine (yellow). C) Orientation of the specific residues of the R-spine (maroon) (labeled as RS1 from catalytic loop (olive), RS2 from the activation loop (olive), RS3 from the \(\alpha\)C-helix (grey), RS4 from the \(\beta\)4(grey), which is anchored to the \(\alpha\)F-helix (olive) by RS0 (light blue) and Shell (teal) (Sh1 from the \(\alpha\)C-\(\beta\)4 loop (grey), Sh2 and Sh3 from the \(\beta\)5-strand (grey)) and a table showing the R-spine and Shell residues of PKA and Src as representatives of serine/threonine and tyrosine kinases respectively. D) A structural representation of the open (green (PDBID:4NTS)), intermediate (yellow (PDBID:1BKX)) and closed (red (PDBID:1ATP)) confirmations of PKA measured by the distance from the center of the \(\alpha\)F-helix (A223-\(\alpha\)) to the G-loop (S53-\(\alpha\)) and \(\alpha\)C-Helix (H87-\(\alpha\)). E) Electrostatic potential of the catalytic core shows that the N-lobe active site (top right) is mostly positive whereas the C-lobe active site (bottom right) is highly negative. F) Active site interactions before phosphoryl transfer with ATP and substrate bound in the active site (PDBID:4DG2).
conserved aspartate (RS0) (Meharena et al., 2013). The R-spine is supported by an ensemble of conserved hydrophobic residues known as the Shell [Sh1 from the αC-β4 loop, Sh2 (gatekeeper) and Sh3 from the β5-strand] (Liu et al., 1998; Meharena et al., 2013). EPKs are typically in equilibrium between the active and inactive states where the R-spine is assembled and disassembled respectively (Huse and Kuriyan, 2002; Meharena et al., 2013). After the assembly of the R-spine, the active state of an EPK toggles between the open, intermediate and closed conformations as it traverses the catalytic cycle (Knighton et al., 1991a; Skora et al., 2013; Zheng et al., 1993). The transition from the open to the closed conformation is initiated by the binding of ATP which transitions the core from the open to an intermediate conformation (Figure 3.1d). This transition is primarily driven by the interaction of the adenosine portion of ATP with the C-spine and the hinge region (Narayana et al., 1997a). The binding of a substrate allows the final transition from the intermediate to the closed conformation (Narayana et al., 1997b).

The C-lobe portion of the active site of most EPKs has a negatively charged electrostatic surface and the binding of ATP requires two magnesium ions (Mg$^{2+}$) to neutralize the negative charge of the phosphates (Figure 3.1e) (Zheng et al., 1993). These Mg$^{2+}$ ions bind to two conserved residues from the C-lobe, an aspartate (D184 in PKA) from the DFG motif on the AL and an asparagine (N171) from the CL, to position the γ-phosphate of ATP for phosphoryl transfer (Figure 3.1f) (Buechler and Taylor, 1988). The α/β-phosphates of ATP interact with the highly conserved lysine residue from the β3-strand [β3K (K72)] which forms a salt bridge with a conserved glutamate from the αC-helix [αCE (E91)] (Kamps et al., 1984; Zoller et al., 1981). It
was initially assumed β3K was required for binding ATP and the salt bridge with αCE was required for stabilizing β3K. However, biochemical data showed that mutation of these residues abolished catalytic activity but had no impact on the binding of ATP, and therefore the role of these residues in catalysis remained unclear (Carrera et al., 1993). The Glycine-rich Loop (G-loop) of the N-lobe interacts with ATP, substrate and the C-lobe sealing the active site chamber in the closed conformation. The substrate (serine, threonine or tyrosine) interacts with a conserved aspartate from the HRD motif on the CL (D166) and phosphoryl-transfer is achieved (Valiev et al., 2003). Finally the phosphorylated substrate and adenosine diphosphate (ADP) are released and the EPK transitions back to the open conformation where it is poised to bind another ATP molecule to repeat this process (Bastidas et al., 2013). Even though the end points of the phosphoryl transfer process have been elucidated, the interactions that facilitate the conformation transitions of the catalytic cycle remain unknown. Using an E. coli expression system, site-directed mutagenesis, western blotting, radioactive phosphoryl transfer assay, molecular dynamics (MD) simulations, molecular modeling and a thermal shift assay we have deciphered the interactions required for the dynamics of opening and closing as well as the role that the β3K, αCE, R-spine and Shell residues play in the mechanics of EPK activation and catalytic activity, using PKA as a model system.
3.2 Experimental Procedures

Site-Directed Mutagenesis

QuikChange II site-directed mutagenesis kit (Agilent technologies) was used to introduce mutations.

Phosphoryl transfer assays

The His6-tagged murine Ca-subunit of cAMP-dependent protein kinase (PKA) in pET15b was expressed in E. coli (BL21 (DE3)). Cultures were grown at 37°C to an A600 of ~0.6 and induced with 0.5 mM isopropyl β-d-thiogalactopyranoside (IPTG). The cultures were allowed to grow overnight at 25°C before harvesting. Western blots were utilized to check for the expression of PKA, the phosphorylation state of the activation loop (pT197) and c-terminal tail (pS338) using antibodies as previously described (Iyer et al., 2005b). Harvested cultures were purified and were used to preform radioactive phosphoryl transfer assay as described previously (Meharena et al., 2013).

Structural representations and molecular modeling

All images, electrostatic potential and structural distance calculations were done using the PyMOL molecular graphics system (Schrödinger, LLC). For molecular modeling, mutants were generated in PyMol and were then submitted to the YASARA minimization server (Krieger et al., 2009). Comparative figures were aligned by the αF-helix unless specifically stated.
**Molecular Dynamics system preparation and simulations**

Starting with a high-resolution structure of PKA in the closed conformation with ATP and two Mn2+ ions, PDB ID:3FJQ, the Protein Local Optimization Program was used to pre-process the PDB file and optimize or mutate/optimize the side-chain at position β3K, and then the structure was imported into Maestro (Schrodinger, Inc.). The remainder of the preparation was done using the procedure described previously (McCleland et al., 2014).

The program AMBER14 was used for the initial energy-minimization, heating, and equilibration steps, using the pmemd.cuda module with hydrogen mass repartitioning. Each system was energy-minimized, first with position restraints on the protein and ATP/Mg2+, and then without restraints. Constant volume simulations using particle mesh Ewald with a 10 Å cutoff for range-limited interactions were used. Molecular dynamics with a 2 fs time-step was performed to heat the systems from 0 K to 300 K, linearly over 5.0 ns, with 10.0 kcal/mol/Å position restraints on the protein and ATP/Mg2+. Temperature was maintained by the Langevin thermostat, with a collision frequency of 1.0 ps-1. Then constant pressure dynamics was performed with isotropic position scaling, first with position restraints for 100 ps and a relaxation time of 360 ps, and then without position restraints for 2.4 ns.

Production runs in the NVT ensemble were conducted using AMBER14 with particle mesh Ewald using an 8Å cutoff for range-limited interactions. Temperature was maintained by the Langevin thermostat at 300K, with a collision frequency of 2.0ps-1. A time-step of 2fs was used and snapshots collected every 100ps for the first 2ns, and then the time-step was changed to 4fs for the remainder of the production run,
with snapshots collected every 240ps. Triplicate simulations were completed for each system, with simulation times for each replicate (including equilibration) of 1.13 milliseconds (WT-C, β3K/A, β3K/M), 712 nanoseconds (β3K/H) and 794 nanoseconds (β3K/R). The distances and dihedral angles were calculated for a single simulation using Gromacs or VMD and the data was exported into Microsoft-excel where traces, line graphs, histograms, average distances, and standard deviations were calculated for the first 712ns unless specified. Density distribution histograms were generated for the all triplicate simulations.

**Thermal shift assay**

The WT-C and different β3K mutants were expressed and purified as previously described (Steichen et al., 2012). Differential Scanning Fluorimetry was used to estimate the shift in melting temperature of the WT-C and mutants upon binding of ATP and /or the substrate (peptide inhibitor IP20). 2.0µM (4.5µg in 50µl) of protein was used in 25mM HEPES pH 7.0, 100mM NaCl, 10mM MgCl₂ and 1mM DTT (Herberg et al., 1999). Duplicate samples were prepared for Apo protein, protein with ATP, protein with ATP+substrate and protein with substrate. ATP and substrate were used at final concentrations of 1mM and 25µM respectively. Samples were incubated on ice for 10min prior to adding 5X working concentration of SYPRO orange fluorescent dye (Invitrogen). All samples were read on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad). Temperature scan mode was used to heat the samples from 10-90°C at a rate of 1°C/min with a halt-time of 1min after every 1°C. Fluorescence from the protein-bound SYPRO orange was read using FAM (492nm)
and ROX (610nm) filters for excitation and emission respectively (Niesen et al., 2007). Sigmoidal curves were obtained according to the Boltzmann equation:

\[ y = LL + \frac{(UL-LL)}{(1+\exp[(Tm-x)/a])} \]

where LL and UL are the upper and lower limits of fluorescent intensities and a is the slope of the curve within the melting temperature Tm. Tm values for thermal unfolding could be estimated from the first derivative curves as fitted by the CFX96 machine. For more precise Tm values, curves were fitted to a sigmoid equation to estimate its inflexion point using the non-linear regression module of Graph-pad software. Data was exported to Microsoft-excel where histograms were generated.

3.3 Results

Is the salt bridge required for stabilizing β3K or the αC-helix?

PKA has two phosphorylation sites; a *trans*-autophosphorylation site on the AL (T197) and a *cis*-autophosphorylation site outside of the core on the C-terminal tail (S338)(Yonemoto et al., 1993). The phosphorylation of the AL has been shown to be the mechanism for regulating the transition from the inactive to active state(Adams, 2003). The dephosphorylated AL in PKA induces disassembly of the R-spine as well as the twisting of the N-lobe away from the C-lobe (Figure 3.2a, b) (Steichen et al., 2012). The dephosphorylated form maintains ~5% catalytic activity, which suggests that the dephosphorylated form of PKA is oscillating between the inactive and active states (Figure 3.2c). We hypothesize that in the absence of the salt bridge the αC-helix is not synchronized and will not transition into the active conformation with the rest of
Figure 3.2. Role of the salt bridge in catalytic activity. A) Structural representation of the inactive form of PKA where the R-spine (maroon) is disassembled and the Shell residues (teal). B) A structural comparison of the active (grey and olive (PDBID:1ATP)) and inactive (tan and black (PDBID:4DFY)) PKA structures showing the twisted conformation of the inactive conformation. C) Structural representation of the required transition of the αC-helix from the inactive (tan) to the active (grey) conformation. The interaction between the αC-helix (H87) with the AL (pT197), the salt bridge between the αC-helix (αCE) and β3-strand (β3K) as well as the R-spine and Shell residues in the active closed conformation. D) Western blot of the different mutants showing the expression (PKA-C) and autophosphorylation state of the AL and C-tail (pT197 and pS338 respectively). E) Level of radioactive phosphoryl transfer of the different mutants as compared to the WT-C.
Table 3.1. Radioactive phosphoryl transfer assay for the rescue of the β3K-αCE salt bridge rescue. The activity is represented by the percent of catalytic activity of each mutant relative to the wild type for triplicate experiments using a radioactive phosphoryl transfer assay.

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<th>Activity (%WT)</th>
<th>Relative Standard Deviation</th>
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</thead>
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<td>WT</td>
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<td>7.36</td>
</tr>
<tr>
<td>αCE/A</td>
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<td>0.10</td>
</tr>
<tr>
<td>αCE/A(PDK1)</td>
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<td>1.30</td>
</tr>
<tr>
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<td>44.64</td>
<td>7.24</td>
</tr>
<tr>
<td>αCE/A+H87/A(PDK1)</td>
<td>42.21</td>
<td>5.99</td>
</tr>
<tr>
<td>αCE/A+RS3L/F+H87/A(PDK1)</td>
<td>197.06</td>
<td>19.71</td>
</tr>
<tr>
<td>H87/A</td>
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<td>1.30</td>
</tr>
<tr>
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<td>4.84</td>
</tr>
<tr>
<td>RS3L/F+H87/A</td>
<td>3.91</td>
<td>0.35</td>
</tr>
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</table>
the N-lobe. As previously shown, the αCE to alanine [αCE/A] mutation is unable to undergo autophosphorylation (Figure 3.2d), and radioactive phosphoryl transfer assay with a small peptide (kemptide) shows less than 0.5% catalytic activity in comparison to the wildtype PKA catalytic subunit (WT-C) (Figure 3.2e and Table 3.1) (Gibbs and Zoller, 1991).

To synchronize the αC-helix through the assembly of the R-spine we co-expressed αCE/A with 3-phosphoinositide-dependent protein kinase 1 (PDK1) (Mora et al., 2004). PDK1 only trans-phosphorylates T197 and the phosphorylation of T197 initiates a hydrogen bond network in the C-lobe which orients RS2 into its active conformation, this nucleates a hydrophobic docking site for RS3. When αCE/A is co-expressed with PDK1 [αCE/A(PDK1)] it is able to undergo cis-autophosphorylation of S338 (pS338) as well as an enhanced radioactive phosphoryl-transfer of ~13%. We then decided to further stabilize the αC-helix in the active conformation by introducing a bulkier hydrophobic phenylalanine at RS3 [RS3L/F]. The non-phosphorylated form of αCE/A+RS3L/F remains inactive but αCE/A+RS3L/F(PDK1) has ~45% catalytic activity as compared to WT-C. Therefore, the salt bridge is required for stabilizing the αC-helix and introducing a bulky residue into the R-spine partially rescues the absence of the salt bridge.

**Is the salt bridge required for opening?**

Previous studies showed that the rate limiting step for phosphoryl transfer is the release of ADP (Zhou and Adams, 1997). We hypothesize that this is due to stabilization of the closed conformation which is facilitated by the interaction of
pT197 of the AL with the αC-helix through Histidine 87 (H87) and this interaction needs to be broken to transition from the closed to the open conformation to allow for release of ADP (Figure 3.2c). We propose, in the absence of the salt bridge [αCE/A] the catalytic activity is diminished due to the inability to synchronize the opening of the αC-helix, which remains anchored to the AL through the pT197-H87 interaction. To address this, we replaced H87 with an alanine [H87/A] in the salt bridge deficient mutant [αCE+H87/A]. This enhanced the catalytic activity to over 40% when co-expressed with PDK1 but remained inactive when expressed independently (Figure 3.2d, e and Table 3.1). We then further stabilized the αC-helix in the αCE/A+H87/A mutant through an RS3L/F mutation [αCE/A+RS3L/F+H87/A]. This mutant had ~200% catalytic activity when co-expressed with PDK1. A previous study showed that the H87/A mutant had a 2-3 fold increase in catalytic activity which correlated with our results where H87/A had 267% catalytic activity as compared to the WT-C (Cox and Taylor, 1995). The equilibrium of closing and opening as well as the stability of the R-spine is a delicate balance as the introduction of RS3L/F or RS3L/F+H87/A into the WT-C reduces the catalytic efficiency to ~30% and ~4% respectively.

Is the αC-helix regulated by hydrophobic or electrostatic interactions?

The αCE/A+RS3L/F+H87/A(PDK1) mutant is ~2-fold more active than WT-C in the absence of the salt bridge and the pT197-H87 electrostatic interactions (Figure 3.2d, e and Table 3.1). To address if the catalytic activity in this mutant is regulated by the hydrophobic interactions of the Shell residues we mutated Sh2 (M120/gatekeeper)
and Sh3 (M118) from the β5-strand simultaneously to alanine [αCE/A+RS3L/F+H87/A+Sh2M/A+Sh3M/A]. This mutant was inactive when expressed independently and when co-expressed with PDK1 indicating that a stable R-spine is not sufficient for catalytic activity (Figure 3.3a, b, c and Table 3.2). Next, to understand if catalytic activity can be achieved with just the electrostatic interactions, we introduced the Sh2M/A+Sh3M/A mutations into the WT-C. Previously we have shown that this mutant is catalytically inactive when expressed independently (Meharena et al., 2013) but when co-expressed with PDK1 to introduce the pT197-H87 interaction to the already existing salt bridge [Sh2M/A+Sh3M/A(PDK1)] the catalytic activity was rescued to ~115%. Removal of the pT197-H87 interaction through the H87/A mutation [Sh2M/A+Sh3M/A+H87/A] abolished catalytic activity when expressed independently or co-expressed with PDK1. These results indicate that in the absence of the Shell residues the pT197-H87 interaction is required for regulating the αC-helix as compared to the H87/A mutant which is regulated by the R-spine and Shell. To address whether the R-spine stabilizing mutant (RS3L/F) can rescue catalytic activity in the absence of the Shell residues and the pT197-H87 interaction, we generated another mutant [Sh2M/A+Sh3M/A+H87/A+RS3L/F].
Figure 3.3. Hydrophobic vs. Electrostatic interactions. a, Western blot of the different mutants showing the expression (PKA-C) and autophosphorylation state of the AL and C-tail (pT197 and pS338 respectively). b, Level of radioactive phosphoryl transfer of the different mutants as compared to the WT-C. c, Cartoon representation of the mutants shown in panel b.
Table 3.2. Radioactive phosphoryl transfer assay elucidating the role of hydrophobic and electrostatic interactions. The activity is represented by the percent of catalytic activity of each mutant relative to the wild type for triplicate experiments using a radioactive phosphoryl transfer assay.

<table>
<thead>
<tr>
<th></th>
<th>Activity (%WT)</th>
<th>Relative Standard Deviation</th>
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<tr>
<td>WT</td>
<td>100.00</td>
<td>7.36</td>
</tr>
<tr>
<td>αCE/A+RS3L/F+H87/A(PDK1)</td>
<td>197.06</td>
<td>19.71</td>
</tr>
<tr>
<td>αCE/A+RS3L/F+H87/A+Sh2M/A+Sh3M/A(PDK1)</td>
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<td>0.02</td>
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<tr>
<td>Sh2M/A+Sh3M/A(PDK1)</td>
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<td>9.28</td>
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<td>0.54</td>
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<tr>
<td>Sh2M/A+Sh3M/A+H87/A+RS3L/F(PDK1)</td>
<td>2.36</td>
<td>0.54</td>
</tr>
<tr>
<td>Sh2M/A+Sh3M/A+RS3L/A(PDK1)</td>
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<td>6.37</td>
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<td>1.17</td>
<td>0.73</td>
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</table>
In this case, the enhanced hydrophobicity of the R-spine was insufficient to rescue catalytic activity when expressed independently or co-expressed with PDK1. We then hypothesized that in the shell deficient mutant the αC-helix is positioned by the two electrostatic pairs (salt bridge and pT197-H87) independent of the R-spine. To test this, we mutated RS3 to an alanine (RS3L/A) in the Shell deficient mutant [Sh2M/A+Sh3M/A+RS3L/A]. This mutant maintained catalytic activity comparable WT-C (101.41%) when co-expressed with PDK1. Finally, to address whether the presence of all Shell residues are required for catalytic activity a triple Shell mutant Sh2M/A+Sh3M/A+Sh3V/G was generated. Catalytic activity in the absence of all Shell residues is abolished when expressed independently or co-expressed with PDK1. Our previous studies showed that Sh1 is a crucial residue that is uniquely positioned to interact with both the N- and C-lobes and any alterations to this residue abolishes catalytic activity (Meharena et al., 2013). These results collectively indicate that two systems regulate catalytic activity, the hydrophobic ensemble (R-spine+Shell) and the two electrostatic pairs (salt bridge+pT197-H87). While we have teased apart the mechanics regulating the dynamics, in their natural states these two systems are in harmony.

What are the consequences of altering β3K?

Previous studies have indicated that mutation of β3K to alanine [β3K/A], methionine [β3K/M], histidine [β3K/H] and arginine [β3K/R] have no catalytic activity (Iyer et al., 2005b; Kamps and Sefton, 1986; Robinson et al., 1996; Weinmaster et al., 1986). To understand if the loss of catalytic activity of these
Figure 3.4. Catalytic activity of the different β3K mutants and WT-C. A) Western blot of the different mutants showing the expression (PKA-C) and autophosphorylation state of the AL and C-tail (pT197 and pS338 respectively). B) Level of radioactive phosphoryl transfer of the different mutants as compared to the WT-C.
Table 3.3. Radioactive phosphoryl transfer assay attempting to rescue the β3K mutants. The activity is represented by the percent of catalytic activity of each mutant relative to the wild type for triplicate experiments using a radioactive phosphoryl transfer assay.

<table>
<thead>
<tr>
<th>Mutant / Additional Mutations (PDK1)</th>
<th>Activity (%WT)</th>
<th>Relative Standard Deviation</th>
</tr>
</thead>
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<tr>
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<td>7.36</td>
</tr>
<tr>
<td>β3K/A (PDK1)</td>
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<td>0.52</td>
</tr>
<tr>
<td>β3K/M (PDK1)</td>
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<td>0.70</td>
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<td>β3K/R (PDK1)</td>
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<td>0.46</td>
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<td>0.07</td>
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<td>0.11</td>
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<td>β3K/R+RS3L/F+H87/A (PDK1)</td>
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<td>0.34</td>
</tr>
<tr>
<td>β3K/R+αCE/A (PDK1)</td>
<td>3.37</td>
<td>0.36</td>
</tr>
<tr>
<td>β3K/R+RS3L/F+H87/A+αCE/A (PDK1)</td>
<td>2.92</td>
<td>0.18</td>
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</table>
mutants was due to the loss of the salt bridge we first co-expressed these mutants with PDK1 (Figure 3.4a, b and Table 3.3). The β3K/A, β3K/M and β3K/H mutants remained inactive for cis-autophosphorylation, trans-autophosphorylation and for steady state catalysis. In contrast, the β3K/R mutant was able to undergo cis-autophosphorylation but had very low levels of radioactive phosphoryl transfer (~6%).

Next we wanted to enhance the catalytic activity by simultaneously introducing the R-spine stabilizing mutation (RS3L/F) as well as removing the rate limiting step (H87/A). These mutations did not yield an increase in catalytic activity for any of the mutants. This led us to believe that β3K had more functional roles than just participating in the formation of a salt bridge with αCE. To elucidate these roles we ran MD simulations of all the mutants with the AL phosphorylated (pT197). The MD simulations were started with the closed (ATP+substrate bound) structure (PDB ID:3FJQ) (Thompson et al., 2009). We then removed the substrate to simulate the intermediate ATP bound conformation for WT-C, β3K/A, β3K/M, β3K/H and β3K/R to search for any conformational changes that could explain the inactivation of these mutants.

Is β3K required for orienting the N-lobe for catalysis?

The WT-C MD simulation mimicked an intermediate structure, with an average distance of 22.2Å between the G-loop and αF-helix and 20.6Å between the αC-helix and αF-helix (Figure 3.1d, 3.5a, Figure 3.6 and Table 3.4). The β3K/A simulation also showed a similar intermediate conformation with an average distance
Figure 3.5. Global intermediate conformation of the WT-C and β3K mutants. A) Average distance from the center of the αF-helix (A223-α) to the G-loop (S53-α) and αC-Helix (H87-α). B) Structural representation of the unique G-loop confirmation observed in β3K/M compared to the WT-C (top) and the specific interactions of the hydrophobic clamp unique to the β3K/M mutant (bottom). C) Structural representation of twisted conformation of the β3K/H mutant (red and teal) in comparison to the WT-C closed confirmation (grey and olive). D) Structural representation of the more open intermediate conformation of β3K/R (pink and teal) in comparison to the WT-C open confirmation (grey and olive).
Figure 3.6. Trajectory distance calculations elucidating the global profile. Distance calculations from the center of the αF-helix (A223-ca) to the G-loop (S53-ca) and αC-Helix (H87-ca).
**Table 3.4.** Measurements capturing the global profile of the intermediate confirmation of the WT-C and the different β3K mutants. Average distances were calculated from the αF-helix (A223) to the G-loop (S53) and αC-helix (H87).

<table>
<thead>
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<th>Distance Å</th>
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<tbody>
<tr>
<td><strong>WT-Open (G-loop-αF-helix)</strong></td>
<td>26.2</td>
</tr>
<tr>
<td><strong>WT-Open (αC-helix-αF-helix)</strong></td>
<td>21.6</td>
</tr>
<tr>
<td><strong>WT-Intermediate (G-loop-αF-helix)</strong></td>
<td>22.8</td>
</tr>
<tr>
<td><strong>WT-Intermediate (αC-helix-αF-helix)</strong></td>
<td>20.7</td>
</tr>
<tr>
<td><strong>WT-Closed (G-loop-αF-helix)</strong></td>
<td>19.3</td>
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<tr>
<td><strong>WT-Closed (αC-helix-αF-helix)</strong></td>
<td>19.7</td>
</tr>
<tr>
<td><strong>WT (G-loop-αF-helix)</strong></td>
<td>22.2 1.4</td>
</tr>
<tr>
<td><strong>WT (αC-helix-αF-helix)</strong></td>
<td>20.6 0.6</td>
</tr>
<tr>
<td><strong>β3K/A (G-loop-αF-helix)</strong></td>
<td>22.3 1.3</td>
</tr>
<tr>
<td><strong>β3K/A (αC-helix-αF-helix)</strong></td>
<td>21.2 0.6</td>
</tr>
<tr>
<td><strong>β3K/M (G-loop-αF-helix)</strong></td>
<td>21.1 0.6</td>
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<tr>
<td><strong>β3K/M (αC-helix-αF-helix)</strong></td>
<td>21.1 0.5</td>
</tr>
<tr>
<td><strong>β3K/H (G-loop-αF-helix)</strong></td>
<td>19.2 0.5</td>
</tr>
<tr>
<td><strong>β3K/H (αC-helix-αF-helix)</strong></td>
<td>20.2 0.5</td>
</tr>
<tr>
<td><strong>β3K/R (G-loop-αF-helix)</strong></td>
<td>25.8 1.4</td>
</tr>
<tr>
<td><strong>β3K/R (αC-helix-αF-helix)</strong></td>
<td>20.4 0.4</td>
</tr>
</tbody>
</table>
of 22.3Å between the G-loop and αF-helix and 21.2Å between the αC-helix and αF-helix. The MD simulation for the β3K/M mutant has an average distance of 21.1Å between the αC-helix and αF-helix whereas the average distance between the G-loop and αF-helix was slightly more closed with an average distance of 21.1Å as compared to the WT-C. The hydrophobic nature of β3K/M induces a conformational change in the side chain of β3K/M through a hydrophobic interaction with Sh3 (Figure 3.5b). This conformational change induces a side chain flip of the neighboring leucine (L74) which results in the formation of a hydrophobic clamp with the aid of two hydrophobic residues from the αB-helix (V79 and L82). A conserved aromatic residue from the G-loop (F54) (Bossemeyer, 1994) docks into this pocket and attains gauche\(^+\) chi1 and chi2 torsion angles as compared to gauche\(^-\) and gauche\(^+\) (chi1 and chi2 torsion angles respectively) in the WT-C (Figure 3.7). On the other hand, the β3K/H simulation shows a more closed conformation with an average distance of 19.2Å between the G-loop and αF-helix but a greater average distance of 20.2Å between the αC-helix and αF-helix. This is due to the twisting of the N-lobe by ~19° counterclockwise from the C-lobe (Figure 3.5c). This twisted conformation resembles the dephosphorylated conformation of PKA but β3K/H is more closed and maintains an assembled R-spine. Finally, the β3K/R simulation shows a more open conformation with an average distance of 25.8Å between the G-loop and αF-helix with an intermediate like average distance of 20.4Å between the αC-helix and αF-helix due to a clockwise rotation (~9°) between the N- and C-lobes (Figure 3.5d).
Figure 3.7. Dihedral angle of F54 from the G-loop in WT-C and the different β3K mutants.
Previous structural studies of the β3K/R mutation of the extracellular signal-regulated protein kinase 2 (ERK2) show a similar ATP-bound intermediate conformation with a distance of 26Å from the G-loop to the αF-helix and 20.4Å between the αC-helix and αF-helix (Robinson et al., 1996). Thus, alternative side chains at β3K tend to yield an altered relative orientation of the N-lobe and C-lobe, while removal of this sidechain β3K/A retains a WT-C interlobal orientation.

**Is β3K required for positioning ATP?**

The MD simulation of the WT-C (WT-MD) shows that the intermediate conformation maintains all the required interactions of the active site observed in the WT-C structure (WT-S) (Figure 3.1f, Fig3.8a, Figure 3.9, Table 3.5). In the β3K/A and β3K/M mutants which lack the positive charge of β3K, there is an unoccupied negative charge on αCE that interacts with Mg^{2+} which is constitutively bound to ATP. This interaction shifts ATP towards the αC-helix introducing an interaction between the γ-phosphate of ATP and H87 that stabilizes ATP in a nonproductive binding conformation (Figure 3.8a, b, c and Figure 3.9). The anchoring of the γ-phosphate results in the dissociation of Mg^{2+} from N171 with an average distance of 3.7Å and 3.6Å in β3K/A and β3K/M respectively (cutoff distance ~2.3Å) (Cates et al., 2002). The β3K/H mutant maintains all the required interactions in the active site but the twisting of the lobes induces a nonproductive conformation (Figure 3.8a, d). The β3K/R mutant simulation shows ATP is dislodged from the hinge (E121) and assumes multiple conformations within the active site (Fig 3.8a, e and Figure 3.9).
Figure 3.8. Active site conformation of the WT-C and β3K mutants. A) Average distance measurements capturing the essential interactions required for catalytic activity. Structural representation of the active site interactions of B) β3K/A mutant, C) β3K/M mutant, D) β3K/H mutant and E) β3K/R mutant aligned to the ATP of the WT-C (grey) intermediate confirmation aligned by the αF-helix.
Figure 3.9. Trajectory distance calculations elucidating the active site profile. Distances were calculated between the highly conserved interactions of the active site for the WT-C and the different mutants.
Table 3.5. Measurements capturing the active site profile of the intermediate confirmation of the WT-C and the different β3K mutants. Average distances were calculated between the highly conserved interactions of the active site.

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<th>WT-S</th>
<th>WT-MD</th>
<th>β3K/A</th>
<th>β3K/M</th>
<th>β3K/H</th>
<th>β3K/R</th>
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<td>1.9</td>
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<td>3.1</td>
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</tr>
<tr>
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<td>D184-Mg^{2+}_{(2)}</td>
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</tbody>
</table>
We observe that in the first 250ns of the trajectory the average distance between β3K/R and the ribose/α-phosphate oxygen is 2.8Å whereas in the WT-C the average distance is 4.7Å. The interaction of the β3K/R to the ribose/α-phosphate oxygen results in the unhinging and flexibility of ATP from its catalytically competent conformation (Figure 3.9). This unhinging shifts ATP towards the αC-helix promoting the interaction of H87 with the β-phosphate of ATP (3.9Å) and as a result the interaction between Mg$^{2+}$ and N171 is distant (3.3Å). Previous structural studies of the Kinase Suppressor of Ras (KSR) (a pseudokinase with a β3K/R mutation) as well as the ERK2-β3K/R mutant have shown defects in the binding of ATP (Brennan et al., 2011; Robinson et al., 1996). In the β3K/R simulation, ATP is oscillating between the hinged and unhinged conformations which could explain the ~6% and ~5% basal catalytic activity observed in β3K/R(PDK1) and ERK2-β3K/R mutants respectively (Robinson et al., 1996).

**Is it possible to rescue catalytic activity of the (β3)K mutants?**

To address this question we introduced a lysine on the last glycine of the G-loop (G55), which is a natural variant first identified in WNK (with no lysine) kinase (Min et al., 2004; Xu et al., 2000). We first introduced the G-loop glycine to lysine [(GL)G/K] mutation into β3K/A [β3K/A+(GL)G/K]. This mutant had ~400% the catalytic activity of WT-C and was independent of PDK1 (Figure 3.10a-c). We then introduced the (GL)G/K mutation into β3K/M (β3K/M+(GL)G/K) and β3K/H (β3K/H+(GL)G/K) to see if this mutation can unclamp the G-loop and untwist the
Figure 3.10. Active site conformation of the WT-C and β3K mutants. A) Molecular modeling of β3K/A+(GL)G-K mutant. B) Level of radioactive phosphoryl transfer of the different mutants as compared to the WT-C. C) Western blot of the different mutants showing the expression (PKA-C) and autophosphorylation state of the AL and C-tail (pT197 and pS338 respectively). D) Thermal shift assay capturing the conformational changes depicting the transition from the open to the intermediate and from the intermediate to the closed conformation through the addition of ATP and ATP+substrate.
Table 3.6. Radioactive phosphoryl transfer assay (GL)G/K rescue of the β3K mutants. The activity is represented by the percent of catalytic activity of each mutant relative to the wild type for triplicate experiments using a radioactive phosphoryl transfer assay.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Activity (%WT)</th>
<th>Relative Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>100.00</td>
<td>7.36</td>
</tr>
<tr>
<td>β3K/A+(GL)G/K</td>
<td>424.92</td>
<td>19.58</td>
</tr>
<tr>
<td>β3K/M+(GL)G/K(PDK1)</td>
<td>1.84</td>
<td>0.12</td>
</tr>
<tr>
<td>β3K/H+(GL)G/K(PDK1)</td>
<td>2.53</td>
<td>0.04</td>
</tr>
<tr>
<td>β3K/R(PDK1)</td>
<td>6.51</td>
<td>0.46</td>
</tr>
</tbody>
</table>
lobes respectively. Both of these mutants had no catalytic activity when expressed independently. When co-expressed with PDK1 both $\beta_3K/M+(GL)G/K$ and $\beta_3K/H+(GL)G/K$ were able to undergo cis-autophosphorylation (pS338) but had very low levels of steady state catalytic activity (1.84% and 2.5% respectively).

**Are the $\beta_3K$ mutants deficient in closing?**

We hypothesized that the $\beta_3K/M+(GL)G-K(PDK1)$, $\beta_3K/H+(GL)G-K(PDK1)$ and $\beta_3K/R(PDK1)$ were catalytically inefficient because of their inability to attain a perfectly closed conformation due to the hydrophobic clamp in $\beta_3K/M$, the twisting of the lobes in $\beta_3K/H$ and the misalignment of ATP in $\beta_3K/R$. Previous studies of PKA showed that the conformational changes induced by the binding of ATP and ATP+substrate induce a shift in thermal stability (Herberg et al., 1999). We thus utilized a thermal shift assay to capture these conformational changes for the various $\beta_3K$ mutants. The addition of ATP induced a change in the melting temperature ($\Delta Tm$) of 2.5°C, 3.9°C, 1.9°C, 1.4°C and 1.4°C in the WT-C, $\beta_3K/A+(GL)G/K$, $\beta_3K/M+(GL)G/K(PDK1)$, $\beta_3K/H+(GL)G/K(PDK1)$ and $\beta_3K/R(PDK1)$ respectively (Figure 3.10d and Table 3.6). This correlates with previous studies that have shown that mutations of $\beta_3K$ did not impact ATP binding (Carrera et al., 1993; Iyer et al., 2005a). To capture the ATP+substrate bound closed conformation we used a pseudo-substrate inhibitor (IP20, which has an alanine instead of a serine or threonine at the P-site) to inhibit phosphoryl transfer and capture a stable closed conformation. The addition of ATP+substrate to the WT-C and $\beta_3K/A+(GL)G/K$ mutant yields a $\Delta Tm$ of 3.4°C and 5.8°C respectively, whereas the addition of ATP+pseudo-substrate to
Table 3.7. Differential Scanning Fluorimetry. **a.** Melting temperature and standard deviation of the different \( \beta 3K \) mutants and WT-C in the Apo, ATP bound, ATP+substrate bound and substrate bound confirmations. **b.** \( \Delta T_m \) and relative standard deviation of the different \( \beta 3K \) mutants and WT-C.

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>( \beta 3K/A +G55K )</th>
<th>( \beta 3K/M+G55K+PDK1 )</th>
<th>( \beta 3KM/H+G55K+PDK1 )</th>
<th>( \beta 3KM/R+PDK1 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tm(ATP) - Tm(Apo)</td>
<td>2.5</td>
<td>3.9</td>
<td>1.9</td>
<td>1.4</td>
<td>1.4</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Standard deviation</td>
<td>0.1</td>
<td>0.1</td>
<td>0.2</td>
<td>0.3</td>
<td>0.4</td>
</tr>
<tr>
<td>Tm(ATP+Substrate) - Tm(Apo)</td>
<td>3.4</td>
<td>5.8</td>
<td>1.9</td>
<td>1.2</td>
<td>1.2</td>
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<td></td>
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<tr>
<td>Standard deviation</td>
<td>0.1</td>
<td>0.2</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>Tm(ATP+Substrate) - Tm(ATP)</td>
<td>0.9</td>
<td>1.9</td>
<td>-0.1</td>
<td>-0.2</td>
<td>-0.3</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Standard deviation</td>
<td>0.1</td>
<td>0.2</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>Tm(Substrate) - Tm(Apo)</td>
<td>-0.1</td>
<td>-2.7</td>
<td>0.0</td>
<td>-0.5</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Standard deviation</td>
<td>0.5</td>
<td>0.8</td>
<td>0.3</td>
<td>0.3</td>
<td>0.2</td>
</tr>
</tbody>
</table>
\( \beta 3K/M^+(GL)G/K(\text{PDK1}) \), \( \beta 3K/H^+(GL)G/K(\text{PDK1}) \) and \( \beta 3K/R(\text{PDK1}) \) had no significant \( \Delta Tm \) as compared to the ATP bound state. This indicates that these three mutants are unable to achieve a perfectly closed conformation. Finally, pseudo-substrate binding to the Apo conformation had no conformational change to the WT-C, \( \beta 3K/M^+(GL)G/K(\text{PDK1}) \), \( \beta 3K/H^+(GL)G/K(\text{PDK1}) \) and \( \beta 3K/R(\text{PDK1}) \) with the exception \( \beta 3K/A^+(GL)G/K \) which had a \( \Delta Tm \) of \(-2.5^\circ C\). These results indicate that the \( \beta 3K/A^+(GL)G/K \) is 4-fold more active than the WT-C because it undergoes a more stable transition in response to the binding of ATP and ATP+pseudo-substrate, while the binding of pseudo-substrate in the absence of ATP destabilizes the protein, making this mutant highly efficient.

### 3.4 Discussion

In summary, this study has revealed a critical function of the salt bridge which is separate from its previously assumed role of stabilizing \( \beta 3K \). We have shown that the salt bridge between \( \beta 3K \) and \( \alpha CE \) is required for orienting and anchoring the \( \alpha C \)-helix as well as synchronizing the \( \alpha C \)-helix during the transition from the closed to the open conformation. We also show that an alternate mechanism that utilizes the R-spine and Shell can synchronize the \( \alpha C \)-helix in the process of activation (inactive to active) as well as the closing and opening in the active state. Our studies show that \( \beta 3K \) is the catalytic synchronization hub that is required for orienting the N-lobe, positioning ATP and synchronizing the \( \alpha C \)-helix. Different mutations of \( \beta 3K \) induce different conformations as shown by the clamped G-loop in \( \beta 3K/M \), the inactive-like
clockwise twisting of the lobes in the shorter β3K/H mutant and the counter-clockwise twisting of the N-lobe in the bulkier β3K/R mutant. Furthermore, based on the MD simulations we observe that all β3K mutations lead to different nonproductive orientations of ATP. Finally, we have shown that only the β3K/A mutant can be rescued through the introduction of a G-loop WNK kinase-like lysine and that β3K/M, β3K/H and β3K/R are catalytically inactive due to the inability to transition from the intermediate to the closed conformation.

A large number of EPK mutations have been identified in numerous types of diseases but the lack of complete understanding of the biophysical interactions that regulate the functional dynamics has made it difficult to define these mutations as potentially causative or non-causative without exhaustive experimental testing. This study opens the possibility of easily identifying causative mutations that favor the active or inactive states as well as an understanding into the mechanism of already identified driver mutations. Moreover, most researchers utilize a mutation of the conserved lysine to generate an inactive EPK to decipher the non-canonical functions, but our findings suggest that these mutations should be designed with caution due to the conformational variations induced by specific mutations (Rauch et al., 2011). The results presented in this manuscript provide the tools required to differentiate and elucidate the specific conformations required for these non-canonical functions and could serve as a gateway for the design of non-canonical function inhibitors or enhancers.
Chapter 3 in its entirety is pending publication as the Intramolecular Interactions Regulating the Mechanics of Eukaryotic Protein Kinases. Hiruy S. Mehearena, Xiaorui Fan, Malik Keshwani, Christopher L. McClendon, Lalima G. Ahuja, Angela Chen, Joseph A. Adams, Susan S. Taylor. The dissertation author was the primary investigator and author of this work.
Chapter 4

Conclusions
Achieving catalytic activity in EPKs is a highly synchronized multi-faceted process. Initially, the EPK core needs to transition from the inactive to the active state. Ones in the active state the EPK core toggles between the open and the closed conformations. The results discussed in this thesis decipher the blueprint of the intramolecular interactions regulating and facilitating the activation and activity of EPKs. The combination of hydrophobic and electrostatic interactions position and integrate the dynamic components with extreme precision to position and allow the transfer of the $\gamma$-phosphate.

4.1 EPK Activation

Currently, more than 2,000 EPK structures have been deposited in the Protein Data Bank (PDB). 172 of these structures are solved in the apo conformation and analysis of these structures revealed that there are at least four possible inactive conformations that we confirmed using mutagenesis based biochemical studies in PKA.

I. The DFG-out (RS2-out) conformation (Inactive I), is where the phenylalanine from the DFG motif of the activation loop is dislodged from the hydrophobic pocket between RS1 and RS3. The members of this group vary in the extent of how far the phenylalanine is dislodged from the active conformation. Certain EPKs such as ABL kinase only have the side chain flipped out but the rest of the activation loop remains intact, whereas
kinases such as CDK6 have the entire activation loop shifted into the active site chamber and the phenylalanine lodges into the adenosine binding pocket completing the C-spine. Mutations of RS2 to any residue abolished catalytic activity in PKA.

II. The \( \alpha \)C-helix out (RS3-out) conformation (Inactive II) is where the \( \alpha \)C-helix is twisted anti-clockwise and shifts away from the active site. This class is represented by Src kinase. It is hypothesized this causes inactivation due to the inability to seal the active site chamber allowing water to enter the chamber and compete with the protein substrate for the \( \gamma \)-phosphate. This inactive conformation also disassembles the R-spine by dislodging RS3 as well dissociating the \( \beta \)3K-\( \alpha \)CE salt bridge as result of the twisting of the entire \( \alpha \)C-helix. Since the N-lobe region of the R-spine is further stabilized by the Shell residues, mutation of either Sh2 or Sh3 was required to mimic the RS3-out inactive conformation in PKA. The RS3-out inactivation was only observed in the EPKs that did not have a bulky hydrophobic residue at Sh2 or Sh3.

III. The HRD out (RS1-out) conformation (Inactive III) is a rare inactive conformation that was only observed in only two kinases, AMPK and MARK. Here the catalytic loop is dislodged from its active conformation and results in the disassembly of the R-spine by releasing RS1 from its anchor on the \( \alpha \)F-helix. Mutations of RS1 to any residue abolished catalytic activity in PKA.
IV. The twisted lobe conformation (Inactive IV) is where the two lobes are twisted away from each other. This inactive conformation disassembles the R-spine by splitting the N- and C-lobe parts. This inactive conformation also disorients the active site cleft.

Of the 172 structures only ~30% were captured in their inactive state. Even though certain EPK structures are in a biochemically inactive state, structurally these EPKs were captured in their active state. Unlike other enzymes EPKs in their inactive state retain basal levels of catalytic activity. This indicates that EPKs are naturally oscillating between the inactive and active states when inactive. Hence, the activation process is increasing the rate of achieving the active state and thus the biological process of activation might not achieve the full potential of activity. We have shown this by achieving up to 4-fold catalytic activity through the K72A+G55K PKA mutant as compared to the WT. This opens the possibility of EPK mutations to achieve higher levels of catalytic activity as compared to the WT.

Posttranslational modifications and protein-protein interactions are a few the well-studied mechanisms regulating EPK activation and inactivation. These mechanisms help transition EPKs from the four inactive conformations and stabilize a more active conformation. For example in several EPKs the phosphorylation of the activation loop transitions the EPK from the DFG-out inactive state to the R-spine assembled active state. On the other hand, CDKs form a hetrodimer with cyclin and Rafs form a homodimers to align their R-spine from their αC-helix out inactive conformation.
4.2 EPK Active Site Synchronization

After the assembly of the R-spine, the EPK core is poised to undergo catalysis. Ones in the active conformation the EPK core toggles between the open, intermediate and closed conformations. The binding of ATP and specifically the interaction of the adenosine portion of ATP with the C-spine and the hinge region transitions the EPK core from the open to the intermediate conformation. The closed conformation is achieved through the binding of substrate to the ATP or adenosine bound intermediate conformation. Our studies indicate that the EPK core can be divided into three “Dynamic Subdomains (DSds)” (Figure 4.1). The first DSd (DSd-I) is the $\beta$-sheet region of the N-lobe. DSd-I transitions from the open to the intermediate conformation in a unified manner after the binding of the adenosine portion of ATP with the $\beta 1/\beta 2$ residues of the C-spine (V57 and A70 in PKA) as well as the N-lobe residue (E121) of the hinge region and G-loop. The second DSd (DSd-II) consists of the $\alpha$C-helix from the N-lobe. DSd-II is synchronized with DSd-I through the $\beta 3K-\alpha$CE salt bridge which forms as the EPK core transitions from the open to the closed conformation and the hydrophobic interaction of the RS4, Sh2 and Sh3 from DSd-I and RS3 and Sh1 from DSd-II. The third DSd (DSd-III) consists of the C-lobe and substrate. DSd-III is synchronized with the N-lobe through two mechanisms. First, DSd-III is harmonized with DSd-I through the interaction of the C-lobe portion of the C-spine. This integration is mediated through the binding of ATP. Second, DSd-III is synchronized with DSd-II through the R-spine and shell as well as the interaction between the phosphorylated activation loop (pT197) with the H87 from the $\alpha$C-helix.
Figure 4.1. Dynamic Subdomains of the EPK core. The EPK core can be divided into three “Dynamic Subdomains (DSds)”. DSd-I (red) is the β-strand region of the N-lobe, DSd-II (grey) is mainly the αC-helix as αB-helix is not conserved in all EPKs and DSd-III (teal) constitutes of the C-lobe. These three DSds coordinate ATP, substrate and the dynamic catalytic residues through electrostatic and hydrophobic interactions to achieve phosphoryl transfer.
In chapter 3 we utilize mutagenesis based on the highly conserved residues from each DSd to understand how this dynamic synchronized. β3K from DSd-I is required for positioning the phosphates as well as the precisely locking the N- and C-lobes in the perfect orientation. Mutagenesis studies have shown that different mutations of β3K indicate that the size and biochemical property of the mutants result in varying biophysical and biochemical phenotypes. Our MD simulation data shows that replacing β3K with a shorter positively charged residue (β3K/H) results in the twisting of the N-lobe by ~19° counterclockwise with respect to the C-lobe. Whereas a bulkier mutation (β3K/R) induces a more open and slightly twisted (~8° clockwise). A hydrophobic mutation of β3K (β3K/M) induces the glycine-rich loop in a unique non-productive conformation on the other hand a mutation of β3K to an alanine (β3K/A) shows a global conformation similar to the WT-PKA. Trajectory of all the mutations shows that alteration of β3K induces a non-productive orientation of the γ-phosphate.

We hypothesized that DSd-I and DSd-II are synchronized through the β3K-αCE salt bridge and the removal of this salt bridge through mutations of these residues abolished catalytic activity. Next we asked if it would be possible to synchronize DSd-II with DSd-I in the absence of the salt bridge (αCE/A). Here we utilized the R-spine and Shell residues to attempt to re-synchronization of the αCE/A mutant. The N-lobe region of the R-spine has a residue from DSd-II (RS3) and another from DSd-I (RS4). Our previous studies from chapter 2 indicate that RS4 does not directly participate in catalytic activity, whereas the Shell has two residues (Sh2 and Sh3) from DSd-I that are crucial for catalysis. Sh2 and Sh3 are tightly packed on RS3 in the
closed conformation and further away in the open conformation. Using this property we synchronized the $\alpha$C-helix by introducing a bulkier hydrophobic residue (RS3L/F) to make it more receptive to the transitions of DSd-I.

### 4.3 Future Directions

Currently more than 30% of EPKs have been associated with human diseases, either as causative agents or as therapeutic intervention points. Thus, deciphering the biochemical, biophysical and biological regulation of EPKs is of high priority to the academic and pharmaceutical communities. One of the major challenges has been the successful design of specific EPK inhibitors targeting the EPK core as it is conserved in the entire kinome and the off-target effects have been detrimental and unsuccessful in therapeutics. These studies suggest that an in-depth study of the process of activation and activity simultaneously might allow insight for the design of a new class of therapeutic inhibitors.

Numerous EPK mutations have been reported but one of the major challenges is being able to differentiate between causative and non-causative mutations without extensive experimental analysis (Lahiry et al., 2010). These studies open an avenue of predicting mutations that could favor the active or inactive states as well as possible mutations that could increase the absolute catalytic activity. Here we have shown alterations that abolish as well as enhance the catalytic activity of PKA up to ~4-fold as compared to the fully active WT. Even though further investigation is required to decipher the specific reason for why these alterations enhance catalytic activity, these
results suggest that disease causing mutations could exist that do not alter the transition from inactive to active or vice versa but enhance the rate of catalytic activity ones in the active state.
References


