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The role of the low affinity cyclic nucleotide-binding site B in the activation of cGMP-dependent protein kinase Iα

An Honors Thesis Presented

by

Jessica Wohlfahrt

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Abstract

Cyclic guanosine 3’,:5’-monophosphate (cGMP)-dependent protein kinase (PKG) activates a signaling pathway that leads to vascular smooth muscle cell relaxation, a process that reduces blood pressure. This enzyme consists of a dimerization domain, autoinhibitory domain, regulatory domain, and catalytic domain\textsuperscript{1}. PKG is activated by cGMP binding to two binding sites of the regulatory domain. In order to study how each of these two binding sites, A and B, contributes to PKG activation, a mutant that knocked out cGMP binding to the B site, PKG I\alpha E292A, was expressed in Sf9 cells and purified to apparent homogeneity. Despite the presence of this mutation, the affinity for cGMP determined by surface plasmon resonance (SPR) was unchanged. The mutant still displayed cGMP dependent activation. In addition to these cGMP-binding sites, the regulatory domain contains a switch helix motif that provides a place for crosstalk between the PKG protomers\textsuperscript{2}. It is not well known how this motif affects cyclic nucleotide binding. In order to determine this, cGMP binding was compared between a regulatory domain construct containing the switch helix and one without the switch helix, expressed and purified in \textit{E. coli}. The presence of the switch helix hindered cyclic nucleotide binding to the regulatory domain.
Acknowledgements

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**Introduction**

Blood vessels constrict and dilate in response to various stimuli, directing blood flow throughout the body. The protein cyclic guanosine 3',5'-monophosphate (cGMP)-dependent protein kinase (PKG), located in the smooth muscle layer of blood vessels, is an important regulator of vascular relaxation. There are two isoforms of PKG expressed in vascular smooth muscle from the *prkg1* gene: Iα and Iβ3. PKG I isoforms phosphorylate a number of intracellular proteins such as myosin/phosphatase-targeting subunit (MYPT1), calcium-dependent large conductance calcium-activated potassium channel (BKCa), vasodilator-stimulatory protein (VASP), and inositol 1,4,5-triphosphate receptor-associated PKG substrate (IRAG)4,5. Phosphorylation of these targets by PKG I culminating in dephosphorylation of myosin light chain (MLC) and cytoskeletal rearrangement results in vascular relaxation (Figure 1).

**Figure 1: Target substrates of PKG I in vascular smooth muscle cells**. PKG I activation by cGMP results in phosphorylation of target substrates. The culmination of this signaling cascade leads to a decrease in intracellular calcium, rearrangement of the actin/myosin cytoskeleton, resulting in vascular smooth muscle relaxation.
PKG I is a homodimer consisting of a dimerization/docking (DD) domain, an autoinhibitory domain (AI), a regulatory domain, and a catalytic domain. The dimerization domain is parallel leucine zipper (Figure 2). The regulatory domain contains two cGMP binding sites: the high-affinity A site and low-affinity B site. Cyclic GMP binds to the regulatory domain in a cooperative manner. The A site binds with equal affinities to cyclic adenosine monophosphate (cAMP) and cGMP, whereas the B-site has a higher affinity for cGMP. When PKG is fully occupied with cGMP, it results in a conformational change that exposes the catalytic domain, allowing PKG to phosphorylate target substrates. The two isoforms expressed in smooth muscle, Iα and Iβ, only differ in their dimerization and autoinhibitory domains. This research will specifically focus on the Iα isoform.

Figure 2: The structure of PKG I. The domains include the dimerization/docking domain (DD), autoinhibitory domain (AI), the regulatory domain consisting of A and B cGMP binding sites, the switch helix (SW), and the catalytic domain.

The FGE motif, which is found in the phosphate binding cassette (PBC) is highly conserved in cyclic nucleotide binding sites (Figure 3). The regulatory domain contains a highly conserved glutamate residue that binds to the 2’ hydroxyl group of the ribose sugar in cyclic nucleotides. The charged carboxylate group of this residue is thought to
be important in the PKG-cyclic nucleotide interactions by forming a hydrogen bond with the ribose\textsuperscript{8}. This motif is found both in the A and B site of PKG I\textsubscript{\alpha}.

![Figure 3: Structure of the cyclic nucleotide binding domains in PKG I\textsubscript{\alpha}.](chart)

A) Sequence alignment showing conservation of the FGE motif (in the red box) in the phosphate-binding cassette (PBC). The mutated residue is indicated with an arrow. B) Key residues that mediate cyclic nucleotide binding. Two of these residues are R176 bound to the phosphate group and E167 bound to ribose. The A site is pictured with cAMP, however, the same interaction occurs in the B site. The interaction that will be interrupted is indicated by the red box.

The first objective of this research was to determine the contribution of the A and B site on PKG I\textsubscript{\alpha} activation. To address this, we expressed the PKG I\textsubscript{\alpha} mutant E292A. Glutamate 292 is located in the B site within the FGE motif. We anticipated the PKG I\textsubscript{\alpha} E292A mutant would disrupt cyclic nucleotide binding to the B site. This allowed us to determine the contribution of the B site on PKG I\textsubscript{\alpha} activation. We assessed cGMP binding to the construct via surface plasmon resonance (SPR) and acetonitrile
precipitation. We also compared its activity to wild type PKG by in vitro phosphotransferase assays.

The second objective of this research was to determine the contribution of the switch helix on cyclic nucleotide binding to the regulatory domain. The regulatory domain of PKG Iα consists of the high affinity A site, low affinity B site, and the switch helix (Figure 4). The switch helix portion of the enzyme acts a second domain where crosstalk can occur between monomers. The switch helix of one monomer is able to interact with the other monomer to form a second area where dimerization can occur.

**Figure 4: Crystal structure of the regulatory domain of PKG I with switch helix interactions.** (A) Crystal structure of the regulatory domain of one PKG I monomer with the A and B cGMP binding sites as well as the switch helix motif. (B) The interaction between the switch helix motif of one monomer and the B site of the other monomer creates a second dimerization site.

The switch helix acts as an additional site where interaction between protomers is present, which could affect cyclic nucleotide binding to the B site. The switch helix motif acts as a hydrophobic “knob” that docks into the hydrophobic “nest” on the B site of the opposing protomer (Figure 5). The nest exists in the B site of each protomer. The proximity of this motif to the cGMP-binding site could affect cyclic nucleotide binding.
Understanding the interactions between cGMP and the regulatory domain with the switch helix would provide a clearer picture of how this feature affects the regulation of PKG Iα.

**Figure 5:** Structure of the knob-nest feature of PKG Iα². (A) The nest structure with cGMP binding site indicated. (B) The knob of one protomer bound in the nest of the opposing one.

In order to study the contribution of the switch helix, two constructs of the PKG Iα regulatory domain were expressed: 78-326 (-SW) and 78-356 (+SW) (Figure 6). Cyclic nucleotide binding to these constructs was assessed by SPR. Attempts were made to crystallize the regulatory domain without the switch helix (PKG Iα 78-326) in the presence of cGMP for x-ray crystallography. The goal of this was to compare the structure of PKG Iα 78-326 to the previously crystallized PKG Iα 78-356 in order to elucidate the affect of the switch domain on the cyclic nucleotide binding domain².

**Figure 6:** Structure of the regulatory domain constructs. The top construct, PKG Iα 78-326, contains the A and B sites. The bottom construct, PKG Iα 78-356, contains these sites as well as the switch helix domain.
Materials and Methods

Two types of cells were used for expression in these experiments. Rosetta (DE3)pLysS Escherichia coli (E. coli) will be used for expressing the segments of protein containing only the regulatory domain. Since these cells are already competent, they will more easily take up the plasmid DNA. This particular type of E. coli has more codons that can be used than typical bacterial cells (EMD Millipore). For the full length PKG expression, Sf9 cells will be used. These cells are derived from the Spodotera frugiperda cell line (Invitrogen).

Isolating Plasmid DNA Stocks

New plasmid DNA stocks were made from previously cloned plasmid stocks containing DNA segments for parts of the protein PKG α. The plasmids used were PKG α 78-356•pH6HTN and PKG α 78-326•pRSET A. They expressed protein segments bound to a hexahistidine tag (His-tag). These plasmids were transformed into TOP10 E. coli cells and colonies were selected with ampicillin (AMP) and chloramphenicol (CHL). The bacteria had to be restreaked onto new plates in order to get single colonies. Single colonies were suspended in Luria Broth (LB) with AMP and CHL. The plasmids were purified using a Mini Prep Kit (Qiagen).

Protein Expression and Extraction

The constructs PKG α 78-356•pH6HTN and PKG α 78-326•pRSET A were used for the expression. These protein segments include the regulatory domain of PKG 1α with and without the switch helix domain respectively. Bacterial growth was determined by OD_{600}. For the expression, Rosetta E. coli were transformed with the plasmid DNA stocks and selected with AMP and CHL. Single, transformed colonies
from these plates were transferred to liquid media to grow starter cultures. The liquid media used was LB with 25 µg/mL CHL and 200 µg/mL carbenicillin (CARB). CARB was used in place of AMP in the liquid media, as it does not break down as readily. The bacteria for the expressions were grown at 37°C and the induction phase was done at 25°C.

An expression trial of both PKG Iα 78-356 and PKG Iα 78-326 was started using 50 mL starter cultures with both constructs. The starter culture samples were diluted 3:40 into the four 40 mL refresh cultures, two for each construct. The PKG Iα 78-356•pH6HTN flasks were induced at an OD$_{600}$ of about 0.9 and the PKG Iα 78-326•pRSET A flasks were induced at an OD$_{600}$ of about 0.5. One of the PKG Iα 78-356•pH6HTN flasks and one of the PKG Iα 78-326•pRSET A flasks were induced with 0.5 mM IPTG. The other two flasks were induced with 1 mM IPTG. Samples (1mL) were taken at 0, 1, 2, and 3 hours as well as the next morning. Analysis with SDS-PAGE showed evidence of protein in the PKG Iα 78-356•pH6HTN samples, but not in the PKG Iα 78-326•pRSET A samples.

The first full expression was of the PKG Iα 78-356•pH6HTN construct based on the test expression. A 7 mL starter culture was used. At OD$_{600}$ 0.454, the starter culture was diluted 1:500 into 1 L of LB with CHL and CARB. The OD$_{600}$ of the 1 L of media was allowed to reach 0.771 before it was induced with 1 mM IPTG. The induction period was 3 hours at 25°C. The samples from before and after induction showed protein expression. After 3 hours, the cells were collected by centrifugation and resuspended in lysis buffer with 300 mM NaCl and 5 mM TES pH 7.4.
In order to extract protein from the PKG Iα 78-356 pellet cells were lysed using a French Press at over 1000 PSI. The cells were lysed in the presence of a protease inhibitor cocktail of 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride, phosphoramidon, bestatin, E-64, leupeptin, aprotinin, and pepstatin. The cell fragments were removed using centrifugation at 26000xg and filtration. The remaining fraction was applied to a Bio-Scale Mini Profinity Nickel IMAC cartridge (Bio-Rad). The column bound to the His-tags on the protein and isolated it from the rest of the cell material. The protein was eluted from the column using a Profinia Protein Purification System (Bio-Rad). A midwash buffer with 500 mM NaCl, 20 mM Imidazole, and 5 mM TES pH 7.4 was used to wash away anything not tightly bound to the column. An elution buffer with 300 mM NaCl, 250 mM Imidazole, and 5 mM TES pH 7.4 was used to release the protein from the column. The eluted protein was dialyzed for 2 hours and then overnight, each time at 4°C in 2 L dialysis buffer with 100 mM NaCl, 1 mM TCEP, and 50 mM MES pH 6.9 to remove the excess imidazole groups from the protein. A final dialysis was done in 1 L of dialysis buffer with 10% glycerol. The protein was concentrated to 6.59 mg/mL using a spin concentrator tube with a molecular weight cut off of 10 kDa. The concentrated protein was separated into aliquots, frozen in liquid nitrogen, and stored at -80°C.

The second full expression was of the PKG Iα 78-326•pRSET A construct. The 50 mL starter culture, which was grown up to OD_{600} 0.831, was diluted 1:500 into 1 L of LB with CHL and CARB. Once the OD_{600} of the 1 L of media had reached 0.771, the flask was induced with 0.1 mM IPTG. This was incubated at 25°C overnight. The cells were collected and frozen in liquid nitrogen as in the previous expression. This was
extracted using a French Press as it was done previously. The protein was at a concentration of 0.812 mg/mL in 15 mL. It was dialyzed twice in 2 L dialysis buffer, then in 1 L of dialysis buffer with 10% glycerol. The protein was concentrated to 4.99 mg/mL using a spin concentrator tube with a molecular weight cut off of 10 kDa. This was separated into aliquots, frozen in liquid nitrogen, and stored at -80˚C.

**SDS-PAGE and Western Blot**

The sample pellets from each expression were lysed using lysis buffer with 150 mM NaCl, 1 mM EDTA, 1% Igepal, and 50 mM Tris pH 8. The protein content from these samples was then analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to determine whether the desired segment of PKG-Iα was being produced. The analysis was done with 12% Tris-glycine polyacrylamide gels. In order to analyze the results of the electrophoresis, the gels were either analyzed with Western Blot or stained with Instant Blue (C.B.S. Scientific). For Western Blotting, the gels were transferred to a polyvinylidene fluoride (PVDF) membrane at 100V for 1 hour using a transfer buffer with 12.5 mM Tris, 96 mM Glycine, 0.05% SDS, and 20% methanol.

**Crystallography**

PKG Iα 78-326 was crystallized in the presence of cGMP. Four molar equivalents of cGMP were mixed with the protein. The sample was 715 µL of protein at a concentration of 4.24 mg/mL after filtration. This equated to 93.7 nmol of protein, so 3.74 µL of 100 mM cGMP was added to the protein. This mixture was then concentrated to 12.6 mg/mL, separated into aliquots, frozen in liquid nitrogen, and stored at -80˚C. A crystal screen was prepared with Hampton Research Index Kit HR2-144. A 1:1 drop
ratio and 200 µL wells were used. All of the trays were incubated at 20°C and observed periodically.

The plate was observed under a microscope and two conditions showed needles. These two conditions were seen as the most promising, so they were used as the basis for further optimization. They were condition 15 from the screen, 0.5 M magnesium formate dehydrate with 0.1 M HEPES pH 7.5, and condition 16, 0.3 M magnesium formate dehydrate with 0.1 M Tris pH 8.5. These same conditions were repeated in 5 wells each to ensure the results could be duplicated, and needles were again found in each of these conditions.

A screen was done with concentrations of magnesium formate dehydrate ranging from 0.1 M to 0.8 M with drop ratios of 1:1, 1:2, and 2:1 (Supplemental Figure 1). Concentrations of magnesium formate dehydrate ranging from 0.1 M to 0.4 M were tested with 5%, 15%, and 25% PEG 3350. The buffer used for this screen was 0.1 M Tris pH 8.

Another screen of the magnesium formate dehydrate concentrations ranging from 0.1 M to 0.8 M with drop ratios of 1:1, 1:2, and 2:1 was done (Supplemental Figure 2). This time, each was replicated with 0.1 M HEPES pH 7.5 and 0.1M Tris pH 8.5 as the buffers. The original conditions from the index screen, solutions 15 and 16, were plated as references.

The fourth plate done was a pH and magnesium formate screen (Supplemental Figure 3). Concentrations of magnesium formate dehydrate ranging from 0.1 M to 0.8 M were tested with pH ranging from 6.5 to 8.5. The buffers used, which were all at 0.1 M, were MES pH 6.5, MES pH 7, HEPES pH 7.5, Tris pH 8, and Tris pH 8.5.
**Sf9 Expression and Extraction**

Site directed mutagenesis was used to create a mutated PKG Iα gene. This was accomplished using primers: `5'-GGA AAA GGA GAT TGG TTT GGA GCG AAA GCC TTG-3’` and `5’-TTC CCC CTG CAA GGC TTT CGC TCC AAA CC-3’`. The Bac-to-Bac Baculovirus System was used for this process (Invitrogen). DH10Bac bacterial cells were transformed with the gene encoding for the entire PKG Iα protein with the glutamate amino acid at position 292 mutated to an alanine amino acid (PKG Iα E292A). The bacteria were grown on a plate with kanamycin, gentamycin, tetracylin, IPTG, and x-gal (KGTIX). This was allowed to incubate at 4°C until a clear difference could be seen between blue and white colonies.

Three white colonies and one blue colony were selected from the original plate and restreaked onto new KGTIX plates to confirm plasmid uptake. The white colonies each produced more white colonies and the blue colony produced more blue colonies. One colony from each of the white colony restreaks was added to 3 separate sterile tubes with 6 mL LB containing 50 µg/mL kanamycin and 7 µg/mL gentamycin. The bacmid DNA was collected using ethanol precipitation. Nanodrop was used to determine the concentrations of bacmid for each of the clonal isolate.

M13 polymerase chain reactions (PCR) were used to check for transposition of the PKG Iα E292A into the bacmid (Bac-to-Bac Baculovirus Expression System). The reactions were set up and incubated in a thermocycler (Table 1 and Table 2).
Table 1: PCR reactions mixtures to test for bacmid insertion. FP stands for forward primer and RP for reverse primer.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>E292A (1)</th>
<th>E292A (2)</th>
<th>E292A (3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Bacmid] (ng/µL)</td>
<td>430.7</td>
<td>445.8</td>
<td>448.3</td>
</tr>
<tr>
<td>Template (µL)</td>
<td>0.697</td>
<td>0.673</td>
<td>0.669</td>
</tr>
<tr>
<td>ddH2O (µL)</td>
<td>22.32</td>
<td>22.33</td>
<td>22.33</td>
</tr>
<tr>
<td>DreamTaq 2x (µL)</td>
<td>25</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>5µM M13 FP (µL)</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Total (µL)</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
</tbody>
</table>

Table 2: Thermocycler conditions for PCR to test for bacmid insertion.

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature (˚C)</th>
<th>Time (min)</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>95</td>
<td>1:30</td>
<td>Hot Start</td>
</tr>
<tr>
<td>2</td>
<td>95</td>
<td>0:45</td>
<td>Denaturation</td>
</tr>
<tr>
<td>3</td>
<td>50</td>
<td>0:45</td>
<td>Annealing</td>
</tr>
<tr>
<td>4</td>
<td>68</td>
<td>5:00</td>
<td>Extension</td>
</tr>
<tr>
<td>5</td>
<td>Go to step 2, 30x</td>
<td></td>
<td>Cycle</td>
</tr>
<tr>
<td>6</td>
<td>68</td>
<td>7:00</td>
<td>Final Extension</td>
</tr>
<tr>
<td>7</td>
<td>4</td>
<td></td>
<td>Forever</td>
</tr>
</tbody>
</table>

The first amplification of the virus was set up in a 6-well plate. Sf9 cells were added to the plate at 0.9x10^6 cells per well. The media was replaced with Graces and the cells were allowed to recover. 3 µg and 6 µg bacmid as well as a control containing no bacmid were added in duplicates to separate wells in the plate. 200 µL of a mixture of the appropriate bacmid and cellfectin were added to the wells. The plates were periodically checked until the cells had begun to lyse. At this point, the cells were scraped off the bottom of the well and the supernatant containing the amplified bacmid was collected.

For the second amplification, 15 mL of cells Sf9 at 1.0x10^6 cells/mL were added to a 75 cm^2 flask. 300 µL of the first amplification virus was added to the cells and this was incubated at 27˚C until the cells had begun to lyse. At that point, the cells were scraped off the bottom of the flask and the supernatant containing the amplified bacmid
was collected. For the third amplification, 2 mL of the second amplification virus was added to a flask containing 40 mL of Sf9 cells at 1.0x10^6 cells/mL. This was shaken at 27˚C until the cells had begun to lyse. At this point, the supernatant containing the amplified bacmid was collected.

The third amplification virus was used in expression trials. 40 mL of cells at 1.2x10^6 cells/mL were used for each ratio. The ratios of mL virus to mL cells tested were 1:100, 1:500, 1:1000, and 1:10000. 1mL samples were collected every 24 hours, starting at 0 hours, for 96 hours. The samples were analyzed with SDS-PAGE and western blot. This showed the highest protein production at 96 hours with a ratio of 1:500. These values were used to determine the ratio and time for the full expression.

For the PKG Iα E292A expression, 250 mL of cells were mixed with 750 mL of serum-free media with lipids, gentamycin, and pluronics. 2 mL of the third amplification virus was added to reach a ratio of 1:500. This was shaken at 27˚C for 96 hours. At this point, the cells were collected by centrifugation at 500xg for 20 minutes, resuspended in lysis buffer, frozen with liquid nitrogen, and stored at -80˚C.

The extraction of these cells was done in a similar way to the E. coli extractions. The only difference was the French Press step was done at a PSI of over 500. After the three dialysis steps, the protein was concentrated to 1.35 mg/mL. The concentrated protein was separated into aliquots, frozen in liquid nitrogen, and stored at -80˚C.

**Surface Plasmon Resonance (SPR)**

SPR experiments were carried out on each of the different protein segments, PKG Iα E292A, PKG Iα 78-326, and PKG Iα 78-356 to characterize the binding interactions with cGMP and cAMP. The buffer used contained 50 mM MES pH 6.9, 150 mM NaCl,
1 mM TCEP, and 0.05% Tween 20. The PKG was covalently bound with a heterobifunctional crosslinking to the gold-dextran chip using a 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) mixture. Ethanolamine was used to coat any of the binding sites that were not bound to PKG. The binding interactions were tracked between PKG and cGMP at concentrations from 10 nM to 500 µM, as well as between PKG and cAMP at concentrations from 500 nM to 10 mM. Each concentration was tested three times.

**Kinase Assay**

The phosphotransferase activity of PKG Iα E292A was determined using a radioactive phosphorous-32 kinase assay. The activity was measured by testing the counts per minute after the reaction using liquid scintillation. The 100 µL reactions were carried out at 30°C for 1.5 minutes. The reactions had 10 µL W-15 substrate, 50mM MES pH 6.9, 1 mg/mL BSA, and 10 mM DTT. W-15 is a synthetically developed target for phosphorylation by PKG⁹. The incorporation of radioactive phosphate onto W-15 was measured. In each of the reactions for PKG Iα E292A, 10.8 ng of protein was added. The control PKG data used for these experiments was wild type PKG Iα isolated in the same way as the PKG Iα E292A sample. The reactions were started with radioactively labeled ATP. After the incubation time, 15µL of the reactions were spotted onto 1 by 1 cm filter paper squares. These squares were washed with 0.2% phosphoric acid to remove any of the excess radiation and dried. The radioactivity of these squares was determined using liquid scintillation counting. Concentrations of cGMP ranging from 4 nM to 4 µM were tested. Each reaction was run in duplicate.
Acetonitrile Precipitation

An acetonitrile precipitation was used to determine the ratio of cGMP binding to PKG Iα E292A. A sample of PKG Iα Δ53 was used as a control for the experiment. PKG Iα Δ53 exists as a monomer, therefore each peptide only binds 2 molecules of cGMP, while wild type PKG Iα binds 4 molecules of cGMP for every enzyme. 2 nmol PKG Iα E292A was combined with 5 molar equivalents of cGMP, and 1nmol Δ53 was combined with 3 molar equivalents of cGMP. These were allowed to incubate on ice for 15 minutes. After this time, the samples were loaded onto two Zeba Spin Desalting Columns with a molecular weight cut off of 7 kDa (Thermo Fisher Scientific). The column was subjected to centrifugation at 1500xg for 2 minutes. At this point, the concentration of the flow through was determined using a Bradford assay. This, and the volume of the samples were used to determine the amount of protein present after the column.

An equal volume of acetonitrile was added to each sample. The samples were incubated on ice with periodic mixing by vortex for an hour. They were then subjected to centrifugation at 20,000xg for 30 minutes to remove the precipitated protein. The supernatant for each was transferred to a new tube and vacuum concentrated for 1.5 hours to increase the cGMP concentration. At this point, the absorbance at 254 nm was determined by Nano Drop. Beer’s law and the cGMP extinction coefficient of 16,900 were used to the concentration of cGMP and determine the ratio of protein to cGMP in each sample.
Results

Expression of PKG Iα E292A

Using the Bac-to-Bac Baculovirus Expression System (Invitrogen), the PKG Iα E292A construct was successfully expressed in Sf9 cells. This system required transposition of the PKG Iα E292A gene into bacmid. As determined by M13 PCR (Invitrogen), bands at approximately 4.5 kb indicated successful transposition of the PKG Iα E292A gene into all three clonal isolate bacmid (Figure 7).

![Figure 7: Agarose gel of PKG Iα E292A M13 PCR samples stained with SYBR safe viewed at 302 nm. All three samples indicated transposition of the gene into the bacmid.](image)

The third clonal isolate bacmid was used to produce baculovirus in Sf9 cells. The third amplification virus was used to infect Sf9 cells to express the PKG Iα E292A protein. Western blot analysis probing for the hexahistidine-tagged PKG Iα E292A protein indicated that 1:500 ratio produced the highest amount of protein at 96 hours, therefore this condition was used in all subsequent expressions (Figure 8).
Figure 8: Western Blot of PKG Iα E292A test expression samples with Rabbit anti-his primary antibody and Goat anti-rabbit 680 secondary antibody. The samples were reduced with TCEP. The darkest band appears in the 1:500 ratio of virus to cells at 96 hours.

SDS-PAGE analysis indicated that PKG Iα E292A, purified by Ni IMAC column, was fully reduced post dialysis due to the presence of TCEP (Figure 9). The column elution was not reduced, so it was run on the gel in the form of a dimer. A disulfide bond forms between the two protomers of the PKG Iα enzyme, keeping them bound even after boiling has removed the leucine zipper interaction\(^\text{10}\). The dimeric protein formed a bond that remained around 150 kDa. After dialysis with 1 mM TCEP, the protein had been reduced, now forming a band at around 75 kDa. The protein purity was determined to be 83.5% based on Image Lab lane analysis (Bio-Rad).

Figure 9: SDS-PAGE of extraction samples of PKG Iα E292A from Sf9 expression stained with Instant Blue. The protein had been successfully isolated and reduced after dialysis.
**SPR of PKG Iα E292A**

To gain insight about how the B site mutation of PKG Iα E292A affects the kinetics of cGMP binding, association, and dissociation, SPR experiments were carried out on this construct. This produced an association rate \( (k_a) \) value of \( 1.0 \times 10^5 \text{ M}^{-1} \text{s}^{-1} \) and a dissociation rate \( (k_d) \) of \( 0.10 \text{ s}^{-1} \) with residual standard deviation of 2.560 (Figure 10 A and B). The equilibrium binding constant \( (K_D) \) was determined to be 1.38 μM (Figure 10 C).
Figure 10: Kinetic analysis of PKG Iα E292A with cGMP. Response refers to SPR response in µRIU, the time is reported in seconds, and concentration is the cGMP concentration in M. (A) Association and dissociation of cGMP. (B) Residuals associated with the kinetics data. (C) Binding affinity isotherm.

SPR data for PKG Iα wild type (WT) from a previous lab experiment was used as a comparison. The $k_a$ was calculated at $5.3 \times 10^4$ M$^{-1}$s$^{-1}$, while a $k_d$ of 0.057 s$^{-1}$ was found with residual standard deviation of 0.977 (Figure 11 A and B). The $K_D$ was determined to be 1.35 µM (Figure 11 C).
Figure 11: Kinetic analysis of PKG Iα WT with cGMP. Response refers to SPR response in µRIU, the time is reported in seconds, and concentration is the cGMP concentration in M. (A) Association and dissociation of cGMP. (B) Residuals associated with the kinetics data. (C) Binding affinity isotherm.

Kinetics data for cGMP binding to PKG Iα E292A was compared to cGMP binding data for WT PKG Iα produced in previous lab experiments. The $K_D$ for PKG Iα, 1.38 µM, was very similar to the WT enzyme at 1.35 µM (Table 3). The mutation, despite interfering with B site cGMP binding, does not seem to have affected the affinity of the enzyme for cGMP. The $k_a$ and $k_d$ rates for E292A, $1.0 \times 10^5$ M$^{-1}$s$^{-1}$ and 0.10 s$^{-1}$, were higher than those for WT, $5.3 \times 10^4$ M$^{-1}$s$^{-1}$ and 0.057 s$^{-1}$. The mutant enzyme binds and dissociates with cGMP at a faster rate than the WT enzyme. The $K_D$ was calculated from the isotherm as well as using the kinetics rates. There was no significant difference between the values calculated by each method.
Table 3: Summary of surface plasmon resonance (SPR) data for wild type (WT) PKG Iα and the PKG Iα E292A mutant with cGMP. Rate of association ($k_a$), rate of dissociation ($k_d$), and equilibrium constant ($K_D$) for different segments of PKG with cGMP, as determined by SPR. The $K_D$ was calculated for the isotherm and using the rate constants.

<table>
<thead>
<tr>
<th>PKG Construct</th>
<th>$k_a$ (M$^{-1}$*s$^{-1}$)</th>
<th>$k_d$ (s$^{-1}$)</th>
<th>$K_D$ (Isotherm)</th>
<th>$K_d$ (Rate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PKG Iα E292A</td>
<td>1.0x10$^5$</td>
<td>0.10</td>
<td>1.38 µM</td>
<td>1.0 µM</td>
</tr>
<tr>
<td>PKG Iα WT</td>
<td>5.3x10$^4$</td>
<td>0.057</td>
<td>1.35 µM</td>
<td>1.1 µM</td>
</tr>
</tbody>
</table>

Kinase Assay of PKG Iα E292A

The effect of reduction in cGMP binding sites on the phosphotransferase activity of PKG Iα E292A mutant was also studied. A radioactive P-32 assay was used to determine how the mutant’s ability to phosphorylate target substrates differed from wild type PKG Iα. PKG Iα Δ53 was used as the control in this experiment. The logarithm of the cGMP concentration was graphed against the velocity of the reaction (Figure 12). A nonlinear fit was used to determine the kinetics of this reaction.

![PKG Iα E292A Kinase Assay](image)

Figure 12: Graph of the logarithm of substrate (cGMP) concentration versus the velocity of the reaction based on a measuring the phosphotransferase activity of PKG Iα with radioactive P-32 labeled ATP. Based on data from two PKG Iα E292A trials and seven PKG Iα wild type (WT) trials.
The nonlinear fit of the graph was used to determine the equilibrium association constant ($K_A$), Hill coefficient, and maximal velocity ($V_{\text{max}}$) for the two PKG Iα E292A samples and the seven PKG Iα WT control samples (Table 4). The Hill coefficient is a measure of the cooperativity between binding sites in the enzyme. The Hill coefficient of 1.57 is below the expected wild type value of 2$^{11}$. The Hill coefficient is less than 2 but not as low as 1, a value indicating no cooperativity$^{12}$. This indicates there was some, but not total loss of cooperativity with the mutation. PKG Iα E292A has a reduced equilibrium constant 112 nM instead of 243 nM for the control. This indicates that cGMP seems to bind more strongly with the mutant enzyme. Despite knocking out cGMP binding to the B site, the mutant enzyme is still able to carry out phosphotransferase reactions. The $V_{\text{max}}$ of the reaction, 3.31 μmol*mg$^{-1}$*min$^{-1}$, is only slightly less than the WT $V_{\text{max}}$, 4.63 μmol*mg$^{-1}$*min$^{-1}$.

Table 4: Summary of the kinetics data obtained from the kinase assay. This table gives the association constant ($K_A$) and Hill constant for the two samples and the wild type (WT).

<table>
<thead>
<tr>
<th>PKG Iα sample</th>
<th>$K_A$ (nM)</th>
<th>Hill coefficient</th>
<th>$V_{\text{max}}$ (μmol*mg$^{-1}$*min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PKG Iα WT</td>
<td>243</td>
<td>1.76</td>
<td>4.63</td>
</tr>
<tr>
<td>PKG Iα E292A</td>
<td>112</td>
<td>1.57</td>
<td>3.31</td>
</tr>
</tbody>
</table>

**Acetonitrile Precipitation of PKG Iα E292A with cGMP**

The result of the acetonitrile precipitation was heavily obscured by a high salt concentration. The cGMP concentration and stoichiometry could not be determined from the absorbance at 254 nm.
**Expression and Extraction of PKG Iα 78-356 and PKG Iα 78-326**

In order to determine the effect of the switch helix motif on cGMP binding to the regulatory domain of PKG Iα, two regulatory domain constructs, with and without the switch helix, were expressed. An expression trial of both PKG Iα 78-356 and PKG Iα 78-326 was done in Rosetta *E. coli* cells to choose the conditions that produced protein. The samples for PKG Iα 78-356 had the highest expression with 1 mM IPTG induction after 3 hours (Figure 13). The samples for PKG Iα 78-326 showed no protein expression in any of the time points.

![Figure 13: SDS-PAGE of PKG Iα 78-356 samples stained with Instant Blue.](image)

The highest level of protein appeared after 3 hours with 1 mM IPTG induction.

Based on the test expression data, a 1 L expression of PKG Iα 78-356 was done. Samples for this expression were only taken at the beginning and end of the expression, 0 hours and 3 hours. There was no band in the expected location at 0 hours, but by the end of the induction period, PKG Iα 78-356 was expressed (Figure 14).
An SDS-PAGE analysis on the extraction samples from the PKG Iα 78-356 expression pellet, purified with a Ni IMAC column, indicated that the protein was isolated in the extraction (Figure 15). After dialysis, the protein purity was calculated to be 79.3% using Image Lab Software (Bio-Rad).

A 1 L expression of PKG Iα 78-326 was also done in *E. coli*. Samples were taken at the beginning and end of the expression, 0 hours and overnight. There was no band in
the expected location at 0 hours, but by the end of the induction period, a band for PKG Iα 78-326 had appeared (Figure 16).

![Figure 16: SDS-PAGE of Expression 4 (PKG Iα 78-326) samples stained with Instant Blue. The protein was expressed by the overnight sample (ON).](image)

PKG Iα 78-326 was extracted from this pellet. Samples were taken throughout the extraction process and analyzed with SDS-PAGE (Figure 17). The protein had been isolated in the elution. However, there may have been some degradation beneath the main protein band.

![Figure 17: SDS-PAGE of extraction samples from Expression 4 (PKG Iα 78-326) stained with Instant Blue. The protein had been successfully isolated. The samples analyzed were post dialysis (PD), column elution (E), clarified lysate (CL), supernatant, and load flow through (FT).](image)
Crystallization of PKG Iα 78-326

The structure of the PKG Iα regulatory domain without the switch helix has not been determined in the presence of cGMP. This research attempted to crystallize this PKG Iα 78-326 and cyclic nucleotide interaction in order to reveal this unknown structure. Successfully crystallized protein from this experiment could have been used in x-ray diffraction studies to characterize the crystal structure. From there, the structure could be compared to the previously crystalized PKG Iα regulatory domain with the switch helix bound to cAMP$^2$. However, diffraction quality crystals were not produced in these experiments.

A Hampton Index (HR2-144) Screen was used to test for starting conditions on which to base further optimizations. The most promising conditions from this screen were condition 15, 0.5M magnesium formate dehydrate with 0.1M HEPES pH 7.5, and condition 16, 0.3M magnesium formate dehydrate with 0.1M Tris pH 8.5. These conditions both produced needles after two weeks. The conditions were repeated and photographed with visible and ultraviolet (UV) light after 4 weeks. The crystals could be seen in the visible light image (Figure 18). They could also be seen in the UV image, indicating that they were composed of protein (Figure 19). There was a bright ring around the well in the UV image. This suggests that there was a large amount of protein remaining in solution. Optimization could hopefully cause more of this protein to crystallize.
Figure 18: Visible light image of PKG Iα 78-326 with cGMP needles in the condition with 0.5M magnesium formate dehydrate and 0.1M HEPES pH 7.5. The thin, dark lines in the center of the well are the protein needles.
Figure 19: UV image of PKG Iα 78-326 with cGMP needles in the condition with 0.5M magnesium formate dehydrate and 0.1M HEPES pH 7.5. The light spots indicated that needles present in the condition were in fact made of protein.

Additional attempts at optimization did not produce crystals. Other optimization conditions produced more needles after two weeks. Some of these needles developed into “sea urchin” shapes. Low concentrations of magnesium formate, 0.1 to 0.2 M, more often lead to precipitation. Higher concentrations of magnesium formate, 0.7 to 0.8 M, produced needles that took around 3 weeks to appear as opposed to the 2 weeks in the middle concentrations. The needles grew at pH levels between 7.5 and 8.5. The theoretical pI of PKG Iα 78-326 is around 5.7; therefore the protein seems to prefer being in a negative charge state. Adding PEG 3350 caused the protein to precipitate.
The PKG Iα 78-326 protein sample used in the crystallization was only 81% pure, a fact that may have hindered crystallography efforts. High purity protein samples are needed to produce the regular crystal lattice necessary for x-ray diffraction. Ni IMAC columns do not produce completely pure protein samples; therefore there may have been contaminants that prevented the protein from forming single crystals. For further optimization of crystallography techniques, a cAMP column could be used in the extraction to isolate a higher purity protein sample. An increase in protein concentration could also lead to the formation of crystals. Other crystallization experiments in the lab produced diffraction quality crystals at high PKG concentrations, so increasing the concentration above the 12-17 mg/mL used in this thesis could create diffraction quality crystals of this interaction.

**SPR of PKG Iα 78-326 and PKG Iα 78-356**

The switch helix motif of the regulatory domain of PKG Iα exists directly next to the cGMP binding B site. It was hypothesized that this motif would play a role in the kinetics of cyclic nucleotide binding. In the interest of determining the effect of this interaction, SPR was used to analyze the two PKG Iα regulatory domain constructs, PKG Iα 78-326 and PKG Iα 78-356. The interactions with cGMP were studied for both of these protein segments. The interactions between PKG Iα 78-326 and cAMP were also observed to compare to cGMP binding. The rate of cyclic nucleotide association (kₐ), rate of dissociation (k₍), and equilibrium constant (Kₐ) were determined for each combination. The Kₐ values were calculated from the isotherm as well as using the
kinetics rates. There was no significant difference between the values calculated by each method.

The PKG Iα 78-356 and cGMP interactions were characterized using kinetics data. This data produced a $k_a$ value of $1.8 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ and a $k_d$ of 0.29 s$^{-1}$ with residual standard deviation of 1.492 (Figure 20 A and B). The $K_D$ was determined to be 16.6 µM (Figure 20 C).
Figure 20: Kinetic analysis of PKG Iα 78-356 with cGMP. Response refers to SPR response in µRIU, the time is reported in seconds, and concentration is the cGMP concentration in M. (A) Association and dissociation of cGMP. (B) Residuals associated with the kinetics data. (C) Binding affinity isotherm.

The kinetics of the associations between PKG Iα 78-326 and cGMP were determined by fitting the data from SPR. This provided a $k_{a}$ value of $6.5 \times 10^4$ M$^{-1}$s$^{-1}$ and a $k_d$ of 0.136 s$^{-1}$ with residual standard deviation of 9.434 (Figure 21 A and B). The $K_D$ was determined to be 2.54 µM (Figure 21 C).
Figure 21: Kinetic analysis of PKG Iα 78-326 with cGMP. Response refers to SPR response in μRIU, the time is reported in seconds, and concentration is the cGMP concentration in M. (A) Association and dissociation of cGMP. (B) Residuals associated with the kinetics data. (C) Binding affinity isotherm.

The associations between PKG Iα 78-326 and cAMP were characterized using the SPK kinetics data. A $k_a$ value of $9.8 \times 10^2$ M$^{-1}$s$^{-1}$ and a $k_d$ of 0.188 s$^{-1}$ with residual standard deviation of 12.950 were found (Figure 22 A and B). The $K_D$ was determined to be 200 μM (Figure 22 C).
Figure 22: Kinetic analysis of PKG Iα 78-326 with cAMP. Response refers to SPR response in µRIU, the time is reported in seconds, and concentration is the cAMP concentration in M. (A) Association and dissociation of cAMP. (B) Residuals associated with the kinetics data. (C) Binding affinity isotherm.

SPR data was used to compare the kinetics of cGMP binding between the regulatory domain constructs, PKG Iα 78-326 to PKG Iα 78-356. The $K_D$ for PKG Iα 78-356 at 16.6 µM was slightly higher than that of the other construct at 2.54 µM (Table
5). The switch helix motif does not seem to significantly increase the affinity of the regulatory domain for cGMP, and in fact seems to lower it. The \( k_a \) for PKG I\( \alpha \) 78-326 at \( 6.5 \times 10^4 \text{ M}^{-1}\text{s}^{-1} \) is higher than for PKG I\( \alpha \) 78-356 at \( 1.8 \times 10^5 \text{ M}^{-1}\text{s}^{-1} \). However, the \( k_d \) for PKG I\( \alpha \) 78-326 of 0.136 s\(^{-1}\) is lower than 0.29 s\(^{-1}\) for the other construct. This indicates that cGMP binds to the regulatory domain and dissociates at a faster rate when the switch helix is not present.

The kinetics of PKG I\( \alpha \) 78-326 binding with cGMP was also compared with the binding of the construct to cAMP. The \( K_D \) for cGMP, 2.54 \( \mu \text{M} \), for this protein segment was much lower than that of cAMP, 200 \( \mu \text{M} \), indicating a much higher cAMP affinity (Table 5). Full length PKG I\( \alpha \) also has a much higher affinity for cGMP than cAMP. Therefore the switch helix may be not needed for cyclic nucleotide selectivity. Cyclic AMP has a slightly higher \( k_d \), 0.188 s\(^{-1}\) versus 0.136 s\(^{-1}\) for cGMP, however the \( k_a \), 9.8 \( \times 10^2 \text{ M}^{-1}\text{s}^{-1} \) is much lower than cGMP with \( 6.5 \times 10^4 \text{ M}^{-1}\text{s}^{-1} \). It takes a much longer time for the association between PKG I\( \alpha \) 78-326 and cAMP to form than between PKG I\( \alpha \) 78-326 and cGMP. Due to experimental error, the kinetics for the association between PKG I\( \alpha \) 78-356 and cAMP could not be determined. PKG I\( \alpha \) 78-326 has kinetics data that is closer to wild type than PKG I\( \alpha \) 78-356.

**Table 5: Summary of surface plasmon resonance (SPR) data for the regulatory domain constructs.**

Rate of association (\( k_a \)), rate of dissociation (\( k_d \)), and equilibrium constant (\( K_D \)) for different segments of PKG with cyclic nucleotides, as determined by SPR. The \( K_D \) was calculated for the isotherm and using the rate constants. Kinetics values for PKG I\( \alpha \) 78-356 with cAMP were not determined (ND).

<table>
<thead>
<tr>
<th>PKG Construct</th>
<th>Cyclic nucleotide</th>
<th>( k_a ) (M(^{-1})s(^{-1}))</th>
<th>( k_d ) (s(^{-1}))</th>
<th>( K_D ) (Isotherm)</th>
<th>( K_D ) (Rate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PKG I( \alpha ) 78-326</td>
<td>cGMP</td>
<td>( 6.5 \times 10^4 )</td>
<td>0.136</td>
<td>2.54 ( \mu \text{M} )</td>
<td>2.1 ( \mu \text{M} )</td>
</tr>
<tr>
<td>PKG I( \alpha ) 78-326</td>
<td>cAMP</td>
<td>( 9.8 \times 10^2 )</td>
<td>0.188</td>
<td>200 ( \mu \text{M} )</td>
<td>192 ( \mu \text{M} )</td>
</tr>
<tr>
<td>PKG I( \alpha ) 78-356</td>
<td>cGMP</td>
<td>( 1.8 \times 10^3 )</td>
<td>0.29</td>
<td>16.6 ( \mu \text{M} )</td>
<td>16.1 ( \mu \text{M} )</td>
</tr>
<tr>
<td>PKG I( \alpha ) 78-356</td>
<td>cAMP</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>PKG I( \alpha ) WT</td>
<td>cGMP</td>
<td>( 5.3 \times 10^4 )</td>
<td>0.057</td>
<td>1.35 ( \mu \text{M} )</td>
<td>1.1 ( \mu \text{M} )</td>
</tr>
</tbody>
</table>
Discussion

It is well known that the regulatory domain of PKG Iα consists of two cGMP binding sites, however the contribution of each binding site to overall PKG Iα activation has not been clearly characterized. We expressed the mutant construct, PKG Iα E292A, to help address this gap in knowledge. Each of the two binding sites contains a conserved FGE motif for cyclic nucleotide binding\(^1\). The glutamate residue of this motif binds to the 2'-hydroxyl group on the ribose sugar of cyclic nucleotides\(^7\). Mutating this residue in the B site interfered with this binding interaction. The charged glutamate residue was no longer present in this binding site to allow interactions with cGMP. Despite this partial loss of cGMP binding, PKG Iα E292A had a cGMP affinity that was not significantly different from wild type PKG. Previous lab research determined that mutations of key residues in the A site that blocked cGMP lead to PKG Iα mutants that displayed only basal activity with cGMP\(^14\). This current research shows that the B site mutation PKG Iα E292A maintains the ability for cGMP-dependent activation, with a \(V_{\text{max}}\) slightly less than that of the wild type. The Hill coefficient for the mutant is reduced from wild type but not reduced as low as one, indicating partial loss of cooperativity with this mutant. The difference in activation between the A site mutant and B site mutant suggests that the enzyme only requires a functional A site for activation. Since the B site is not required for PKG activation, the role of the B site seems to instead be regulation of cGMP selectivity for the enzyme\(^15\). This is supported by the fact that the B site is significantly more selective for cGMP over cAMP than the A site\(^16\).

In furtherance of the goal of understanding the A and B site contributions to PKG Iα activation additional regulatory domain mutants could be expressed. The same
glutamate to alanine mutation could be incorporated into the FGE motif of the A site. This would allow for a direct comparison of the effect of loss of cyclic nucleotide binding to each of the cGMP binding sites.

The result of the acetonitrile precipitation with cGMP was obscured by excess salt in the sample. This experiment could be repeated, in hopes of determining the molar equivalents of cGMP bound to protein and revealing the number of sites where cGMP is able to bind. Due to the reduced Hill coefficient seen in the mutant, it is expected that the mutant will bind only two molar equivalents of cGMP, indicating that binding to the B site has been interrupted. This partial loss of cooperativity seems to indicate that there is a loss in cGMP binding sites.

In addition to the two cGMP binding sites, the regulatory domain contains a switch helix motif that may influence cyclic nucleotide binding\(^1\). This domain sits adjacent to the B site of the regulatory domain, possibly allowing it to affect binding of cyclic nucleotides. Two regulatory domain constructs, with (PKG I\(\alpha\) 78-356) and without the switch helix (PKG I\(\alpha\) 78-326), allowed for study of the effect of this motif on cyclic nucleotide binding kinetics. This research determined that the presence switch helix domain decreased the affinity of the regulatory domain for cGMP. Previous SPR research from this lab found the opposite, that the regulatory domain had a higher affinity with the switch helix\(^17\). The similar constructs used in the previous research were dialyzed without a reducing agent, which may have affected cGMP binding. This could be confirmed by preparing two samples using the same method with presence of reducing agent being the only difference. The construct without the switch helix has kinetic properties that are much closer to the wild type enzyme than the construct with the switch
helix. For the regulatory domain segment, the switch helix seems to have a detrimental effect on cGMP binding, decreasing the binding affinity. The PKG Iα 78-326 construct showed a significant increase in $K_D$ for cGMP over cAMP binding. Wild type PKG Iα has a much higher affinity for cGMP over cAMP, therefore it would appear the enzyme has high selectivity regardless of the presence of the switch helix.$^{18}$

In order to provide a clearer picture of the effect the switch helix has on cyclic nucleotide binding, further attempts could be made at crystallizing the regulatory domain without the switch helix in the presence of cGMP. Increasing the purity and concentration, as well continuing optimization around the initial needle-producing conditions could eventually produce diffraction quality crystals. Once a crystal structure has been revealed, it can be compared to the previously crystallized domain with the switch helix in the presence of cGMP, to possibly provide an explanation for the differences in kinetics between the two constructs.$^2$

The expression of constructs for each objective was done in different types of expression systems. The full-length mutated protein PKG Iα E292A was isolated using an Sf9 expression system. Eukaryotic cells are needed to phosphorylate a key tyrosine residue in order for the full-length construct to have phosphotransferase activity. The two regulatory domain constructs, PKG Iα 78-356 and PKG Iα 78-326, did not contain this residue; therefore they could be expressed in E. coli. Since these constructs do not contain the catalytic domain, they cannot be active, even when purified in Sf9 cells.
Funding Acknowledgements

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References


17. Menke, A. A functional analysis of the switch helix interchain communication domain of the cyclic GMP dependent protein kinase. (University of Vermont, 2013).

Supplemental Figure 1: Magnesium formate dehydrate and PEG 3350 optimization screen for crystallization of PKG Iα 78-326 with cGMP. All of these conditions had 0.1 M Tris pH 8.0 buffer. Needles grew under the 1:1 ratio with 0.3 M to 0.5 M magnesium formate dehydrate, and 2:1 with 0.3 to 0.6 M magnesium formate dehydrate, and 1:2 with 0.3 M magnesium formate dehydrate. Most of the PEG 3350 wells had precipitation and the one that did not, 5% PEG 3350 and 0.4 M magnesium formate dehydrate, was clear.
Supplemental Figure 2: Optimization screen for crystallization of PKG Iα 78-326 with cGMP. This plate template was used for two different plates, one with 0.1 M HEPES pH 7.5 as buffer and the other with 0.1 M Tris pH 8.5 as buffer. Magnesium formate dehydrate was varied with the ratio of protein to well solution. The Hampton Research (HR) solution was reagent #15 for the first plate and #16 for the second plate. Plate 1: Needles grew under the conditions 0.1 M HEPES pH 7.5 with 0.5 M magnesium formate dehydrate and a 1:1 ratio. They also appeared with 0.1 M HEPES pH 7.5, 0.7 M and 0.8 M magnesium formate dehydrate, and a 2:1 ratio. All of the controls produced needles. Plate 2: Needles grew with a 2:1 ratio containing 0.1 M Tris pH 8.5 and 0.5 M, 0.7 M, or 0.8 M magnesium formate dehydrate. All of the controls on this plate produced precipitation.
Supplemental Figure 3: pH optimization screen for crystallization of PKG Iα 78-326 with cGMP. Needles grew under the conditions 0.1 M HEPES pH 7.5 with 0.3 M, 0.4 M, and 0.5 M magnesium formate dehydrate.