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The amino terminus of cGMP-dependent protein kinase I β increases the dynamics of the protein's cGMP-binding pockets

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Abstract

The type I cGMP-dependent protein kinases play critical roles in regulating vascular tone, platelet activation and synaptic plasticity. PKG I α and PKG I β differ in their first ~100 amino acids giving each isoform unique dimerization and autoinhibitory domains with identical cGMP-binding pockets and catalytic domains. The N-terminal leucine zipper and autoinhibitory domains have been shown to mediate isoform specific affinity for cGMP. PKG I α has a >10 fold higher affinity for cGMP than PKG I β , and PKG I β that is missing its leucine zipper has a three-fold decreased affinity for cGMP. The exact mechanism through which the N-terminus of PKG alters cGMP-affinity is unknown. In the present study, we have used deuterium exchange mass spectrometry to study how PKG I β 's N-terminus affects the conformation and dynamics of its cGMP-binding pockets. We found that the N-terminus increases the rate of deuterium exchange throughout the cGMP-binding domain. Our results suggest that the N-terminus shifts the conformational dynamics of the binding pockets, leading to an "open" conformation that has an increased affinity for cGMP.

Keywords

PKG; cGMP-affinity; leucine zipper; hydrogen exchange; mass spectrometry

1. INTRODUCTION

The cGMP-dependent protein kinases (PKGs) are involved in a variety of cellular processes including regulation of vascular tone, platelet activation, nociception and synaptic transmission [1,2]. Mammalian cells express three different PKGs from two separate genes. There are two type I PKGs (PKG I α and PKG I β) and one type II (PKG II). Each family member has an N-terminal regulatory domain and a C-terminal catalytic domain, with the regulatory domain containing conserved sub-domains including a leucine/isoleucine zipper, followed by an autoinhibitory loop and two tandem cGMP-binding pockets [2]. PKG I α and PKG I β are splice variants that have identical catalytic and regulatory domains but differ in

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their first ~100 amino acids, and thus contain isoform specific leucine/isoleucine zippers and autoinhibitory domains [2].

The leucine/isoleucine zippers of PKG I α and PKG I β have at least three functions: they mediate isoform-specific homodimer formation; they mediate specific protein-protein interactions and they are at least partly responsible for isoform-specific cGMP affinity [3–10]. While the molecular details of dimer formation and protein-protein interactions have been reported [4,11], it is unclear how the leucine/isoleucine zipper affects cGMP-binding to the kinases.

PKG I α and I β differ only in their N-terminal dimerization and autoinhibitory regions, and even though they have identical amino acid sequences in their cyclic nucleotide binding pockets, they differ 5- to 10-fold in their affinity for cGMP [12,13]. Studies by Ruth *et al.* demonstrated that isoform-specific kinase activation constants (K_a) could be localized to specific amino acid sequences in the leucine/isoleucine zipper and autoinhibitory domains [3]. Later, Richie-Jannetta *et al.* found that isolated PKG I α and PKG I β regulatory domains (i.e. PKG lacking the catalytic domain) retained isoform-specific cGMP-disassociation constants (K_D) and underwent distinct conformational changes, as determined by Stokes radius [12]. Other studies have shown that PKG I β lacking its N-terminal leucine/isoleucine zipper has a two-three fold lower affinity for cGMP [14]. From these studies it is clear that the isoform-specific affinities for cGMP reside entirely within the regulatory domains, and that the leucine/isoleucine zipper and/or autoinhibitory domain(s) somehow affect the conformation of the cGMP-binding pockets. How the N-terminal ~100 amino acids of PKG I α and PKG I β affect the cGMP-binding pockets is unknown.

Deuterium exchange mass spectrometry (DXMS) is an ideal method for probing the structural/conformational dynamics of proteins [15,16]. The technique uses mass spectrometry to measure time dependent deuterium incorporation into the amide hydrogens of the peptide backbone. Since exchange rates are determined by solvent accessibility and the stability of secondary structure hydrogen bonds, DXMS provides a sensitive way to measure changes in protein structure and dynamics in response to ligand binding, protein-protein interactions, and interdomain interactions [15]. Previous deuterium exchange experiments on full-length PKG I α have demonstrated that cGMP binding leads to increased deuterium incorporation within residues of the autoinhibitory loop and catalytic cleft of the kinase and supported a model of PKG activation in which, in the absence of cGMP, the autoinhibitory loop binds within the catalytic cleft and maintains the kinase in an inactive state [17]. How the N-terminus of PKG affects cGMP affinity was not addressed in this prior study.

In the study presented here, we used DXMS to examine how the N-terminus of PKG I β affects the conformational dynamics of PKG I β 's cGMP-binding pockets. Proteins consisting of the full-length regulatory domain (residues 4–352) or just the two cGMP-binding pockets (residues 98–352) were examined. We found that in the presence of the N-terminus the rate of H/D exchange is increased throughout the cGMP-binding pockets, indicating an increased dynamic state. We infer that increased dynamic state causes the binding pockets to adopt an “open” state, allowing access for cGMP.

2. METHODS

2.1 DNA Constructs

N-terminal fragments of PKG I β consisting of residues 4–352 and 98–352 were generated using polymerase chain reaction (PCR) and the following primers: 5'-GATGGATCCTTG-CGGGATTACAGTAC-3' (4–352 forward); 5'-

GATGGATCCTTCTACCCCAAGAGCCCAC -3' (98–352 forward); 5'-GATGAATTCTTATTATTCATATGCTTTATTAGAAAC-3' (common reverse). The PCR products were digested with *Bam*HI/*Eco*RI and ligated into *Bam*HI/*Eco*RI digested pRSET-Xa. Vector pRSET-Xa was created by digesting pRSET B (Invitrogen) with *Nhe*I/*Hind*III and ligating a linker composed of the primers: 5'-CTAGCATTGAGGGACGCGGATCCGCACTCGAGGCAGAAT-TCGA-3' (sense) and 5'-AGCTTCGAATTCTGCCTCGAGTGCGGATCCGCGTCCCTCAATG-3' (antisense). In mammalian cells, PKG I β is processed and lacks an N-terminal methionine and begins with the amino acid sequence GTLRDL-. Since the extreme N-terminus of PKG I β has no effect of cGMP affinity [3] and to facilitate the introduction of a *Bam*HI fusion site, our construct begins GSLRDL, with the *Bam*HI site coding for the GS residues and the fourth amino acid (L) being the first conserved amino acid within our construct. All vectors were sequenced to insure the absence of PCR induced mutations.

2.2 Protein Purification

Recombinant proteins were expressed in BL21 *E. coli* at 30°C using LB Media. Bacteria were harvested by centrifugation, resuspended in ice cold 50 mM potassium phosphate and 500 mM NaCl (pH 8.0) and lysed by sonication. The lysate was cleared by centrifugation and recombinant proteins were purified by nickel affinity chromatography using Profinia resin (BioRad). Eluted proteins were concentrated and further purified over a Sepharose 200 HR column equilibrated in running buffer (20 mM Tris (pH 7.4), 150 mM NaCl and 5% Glycerol). Fractions containing the recombinant proteins were pooled and concentrated to 5–10 mg/ml. Protein concentrations were determined by A₂₈₀. All post lysis purification steps were performed at 4°C. Proteins were stored on ice until DXMS analysis (less than 24 hours).

2.3 Peptide Fragment Optimization

The optimum buffer conditions for DXMS analysis were determined by performing test digests of 50 μ g recombinant protein with 0.5, 1.0 and 2.0 M guanidium-hydrochloride quench buffers (all quench buffers contained 0.8% formic acid and 16.6 % glycerol). Specifically, 60 μ l buffered water [8.3 mM Tris (pH 7.4) and 150 mM NaCl], which mimics the deuterated buffer used for exchange reactions, was added to 100 μ g recombinant protein in a total volume of 20 μ l (all manipulations were done on wet ice). Then, 120 μ l ice-cold quench buffer was added and the sample was split into two 100 μ l aliquots. The samples were frozen on dry ice and stored at -80°C until analysis by mass spectrometry.

2.4 Deuterium on Exchange

All deuterium exchange reactions were performed on ice in a cold room at 4°C. Exchange reactions were initiated by adding 60 μ l buffered D₂O [8.3 mM Tris (pH 7.4) and 150 mM NaCl] to 20 μ l purified PKG. At the appropriate time points exchange was quenched by adding 120 μ l 1.6M GuHCl/0.8% Formic Acid. The samples were split into two 100 μ l aliquots and frozen on dry ice. Frozen samples were stored at -80°C until analysis by mass spectrometry. To analyze deuterium exchange profiles in the presence of cGMP, aliquots of purified PKG I β proteins were incubated with 1 mM cGMP on ice for 3 h before performing exchange reactions.

2.5 Data Analysis

Samples were analyzed by way of an automatic process that first thawed the frozen samples, which were then immediately proteolyzed over a solid-state pepsin column (~2 mg pepsin), and then the resulting peptides were subjected to LC-MS analysis. Procedures for pepsin digestion for DXMS have been described previously [18–21]. Briefly, the samples were

passed through an immobilized pepsin column and the protease-generated fragments were collected on a C18 HPLC column. The effluent was then directed to a Thermo Finnigan LCQ Classic electrospray ion trap mass spectrometer with data acquisition in either MS1 profile mode or data-dependent MS2 mode. The pepsin-generated peptides from the MS/MS data sets were identified using SEQUEST (Thermo Finnigan Inc.). This set of peptides was then further analyzed using specialized DXMS data reduction software (Sierra Analytics Inc., Modesto, CA). Corrections for back exchange were made through measurement of loss of deuterium from reference protein samples that had been equilibrium-exchange-deuterated under denaturing conditions. Deuterium incorporation was calculated via the methods of Zhang and Smith [22]:

$$\text{deuteration level (\%)} = \frac{m(P) - m(N)}{m(F) - m(N)} \times 100$$

Where $m(P)$, $m(N)$, and $m(F)$ are the centroid value of the partially deuterated, nondeuterated, and fully deuterated peptide, respectively. The experiments were performed twice using independent protein preparations, and the reported results are the average of these two experiments.

2.6 Model Building

A PKG I β molecular model was built using the automated Swiss Model homology-modeling server at the Swiss Institute of Bioinformatics (University of Basel, Switzerland) [23]. The model was constructed using a cAMP-bound PKA RI α structure (PDB 1RGS) as a template. Sequence alignments were performed using clustalW [24] (see Supplemental Figure 1), and the cGMP-binding pockets were modeled by threading the PKG I β sequence into solved structures for cAMP-dependent protein kinase. The final model was validated using ProCheck [25], and we found that 87.2% of the residues were in the most favored region of a Ramachandran Plot, with only three residues (K101, D142, and E324) in disallowed regions, and these are all in surface loops. Figures were rendered using PyMol.

3. RESULTS

3.1 PKG I β Domain Organization and Regulatory Domain Constructs Used for DXMS Analysis

PKG I β contains an N-terminal regulatory domain and a C-terminal catalytic domain, and each domain can be broken down to functional subdomains as shown in Figure 1. At the extreme N-terminus, a leucine/isoleucine zipper mediates homo-dimerization and targets the kinase to isoform-specific interacting proteins [4,5,10]. The leucine/isoleucine zipper is followed by an autoinhibitory region, which contains autophosphorylation sites and a pseudo-substrate sequence that inhibits kinase activity by binding within the substrate recognition cleft of the catalytic domain [17]. Autophosphorylation of PKG I β increases basal kinase activity, without affecting cGMP affinity, presumably by decreasing the interaction between the autoinhibitory loop and the catalytic subunit [26]. Next, two-tandem cGMP-binding pockets mediate kinase activation in response to increased cellular cGMP levels. The more N-terminal binding pocket (cNBD-A) has a higher affinity for cGMP than the C-terminal pocket (cNBD-B); this order is the reverse from what is seen for the cyclic nucleotide binding pockets in PKA [27]. The cGMP-binding pockets are followed by the catalytic domain which can be divided into a small lobe that binds Mg²⁺ATP and a large lobe that mediates substrate recognition [28].

Full-length PKG cannot be functionally expressed in *E.coli*, presumably due to the lack of phosphorylation [29]. Even though it lacks kinase activity, the soluble fraction of full-length PKG I α expressed in *E.coli* still binds cGMP with high affinity, indicating that functional regulatory domains can be expressed in bacteria; this finding has been supported by others [30]. Previous studies have shown that the isolated regulatory domains of PKG I α and PKG I β retain cGMP-binding characteristics of the full-length proteins [12]. Therefore, to produce proteins in sufficient quantity for DXMS analysis, full-length and truncated versions of the PKG I β regulatory domain (residues 4–352 and 98–352, respectively, see Fig. 1) were expressed in *E.coli* and purified as described in Materials and Methods.

3.2 Tuning of PKG I β 4–352 and PKG I β 98–352 Proteolytic Fragmentation

DXMS analysis involves incubating proteins in deuterated buffer and stopping the exchange by adding ice cold “quench buffer” at specific time points. The quench buffer contains 0.8% formic acid, which quickly lowers the pH and stops the exchange reaction, and guanidium hydrochloride to denature the protein, rendering it more susceptible to pepsin proteolysis. We began our analysis of PKG I β by determining the conditions that produced the optimal number of peptides during pepsin proteolysis. To accomplish this, we tested various concentrations of guanidium hydrochloride in the quench buffer (0.5, 1.0 and 2.0 M) and found that a final concentration of 1 M was optimal. Under these conditions we obtained 117 peptides for PKG I β 4–352 and 196 peptides for PKG I β 98–352. Within the cGMP-binding pockets, there were 71 peptides in common between the two proteins; these are shown in Figure 2. Unfortunately, peptides within the leucine/isoleucine zipper region of PKG 4–352 were not identified. Others have shown that the N-terminal leucine/isoleucine zipper of PKG I α is stable over a large range of temperatures and pH [31], and we expect that the longer leucine/isoleucine zipper in PKG I β to be even more stable. In fact, when a protein comprising the PKG I β leucine/isoleucine zipper (residues 4–55) was subjected to DXMS analysis we found that the leucine/isoleucine zipper domain was not proteolyzed under the conditions typically used in DXMS (data not shown), indicating that the protein is not denatured in 1M GuHCl, or quickly refolds once injected into the HPLC. Either way, the folded protein is resistant to proteolysis. For the studies presented here, identification of residues within the leucine/isoleucine zipper was not required.

3.3 Deuterium on Exchange of PKG I β 4- 352

PKG I β 4–352 (+/- cGMP) was incubated in deuterated buffer, on ice, for various time points and quenched with 1.5 volumes 1.6 M GuHCl/0.8% formic acid. The amount of deuterium incorporation was determined by mass spectrometry, and percent deuterium incorporation was determined as described in Materials and Methods. A color bar representation showing percentage deuteration of selected peptides is shown in Figure 3. Predicted secondary structural elements, based on alignment to PKA RI α are shown above the sequence, with structural elements in cNBD-A and cNBD-B colored magenta and cyan, respectively. As described above, peptides within the leucine/isoleucine zipper domain could not be detected. We found that residues 71–86, in the autoinhibitory loop, were fully deuterated at the shortest time point examined (3 seconds), even in the absence of cGMP, and showed no change in deuterium incorporation in the presence of cGMP. As expected, our results differ from those previously seen with full-length PKG I α [17]. In full-length PKG I α , in the absence of cGMP, the autoinhibitory loop is protected from H/D exchange, and this protection is most likely the result of the autoinhibitory loop binding within the substrate binding cleft of the catalytic subunit. Therefore, our data are consistent with the predicted model for PKG inhibition/activation.

In the presence of cGMP, deuterium incorporation was reduced throughout the rest of the protein (residues 89–352) with regions at the C-terminus of each binding pocket being most

affected (residues 172–210 and 304–331). These residues span β 5– β 8 in domain A and β 6– β 8 in domain B; they include the P-helices that lie between β 6 and β 7 in each pocket and encompass the phosphate binding cassettes (PBCs), which are expected to coordinate binding of the cyclic nucleotide phosphates. In crystal structures of cAMP-bound PKA RI α and RII β , the amide hydrogens in this region are involved in multiple hydrogen bonding events that are stabilized by nucleotide binding. These hydrogen bonds include those that form the short P-helix [32,33]. We also observe that peptides at the extreme C-terminus of this construct (residues 346–352) have decreased deuterium incorporation in the presence of cGMP; these residues are predicted to form the C-helix at the end of cNBD-B, which in PKA undergoes a dramatic rearrangement upon binding to the C-subunit [34]. Our data suggests that this region contributes to the cGMP-induced conformational changes that activate PKG.

3.4 Deuterium on Exchange PKG I β 98–352

PKG I β 98–352 (+/–) cGMP was subjected DXMS analysis as described above for PKG I β 4–352. As expected, and like PKG I β 4–352, deuterium incorporation into the nucleotide binding pockets was reduced in the presence of cGMP (see below). Interestingly, when deuterium incorporation into PKG I β 98–352 was compared to deuterium incorporation obtained for PKG I β 4–352, an overall decrease in deuterium incorporation in peptides throughout the cyclic nucleotide pockets was evident, with certain peptides being more affected than others. Differences in exchange behavior between identical peptides from cGMP PKG I β 4–352 and PKG 98–352, in the absence of cGMP, at three different time points, are shown in Figure 4. At the shortest time point examined, there are four regions that are specifically affected (Fig. 4A). These regions span amino acids 114–124, 219–230, 307–314 and 346–352. Based on homology to PKA, residues 114–124 are predicted to form part of the N- and 3_{10} -helices that are part of an N-3-A motif, which undergoes major conformational changes during kinase activation [35]. Residues 219–230 are within the C-Helix that connects the two cyclic nucleotide binding pockets, and residues 346–352 are predicted to be part of the C-helix in domain B; these regions are also predicted to undergo major conformational shifts during kinase activation. Finally, residues 307–314 make up the P-helix in the second cGMP-binding pocket, which is stabilized by cGMP-binding. It is interesting that the regions that show the greatest increase in H/D exchange, in the presence of the N-terminus, are located in helical regions which, in analogy to PKA, are predicted undergo dramatic conformational changes during kinase activation. This suggests that the N-terminus may increase cGMP affinity by positioning these helices in a conformation that mimics the active, cGMP-bound, conformation (discussed below). While PKA structures have shown dramatic changes in the relative conformation of the helical domains as the R-subunits shift from cAMP to C-subunit bound forms, the conformation of the β -strands barely change [32–34,36]. Along these lines, the area with the least difference in H/D exchange in the absence of the N-terminus appears to be within the β -strands, with β 7 and β 8 in domain A being the least affected. At later time points, the differences in H/D exchange are localized to different areas within the cGMP-binding pockets, but the general trend of an overall decrease in H/D exchange in the absence of the N-terminus remains constant (Fig. 4B,C).

3.5 Modeled cGMP Binding Pockets

In order to thoroughly interpret our DXMS data, we next examined selected peptides within the context of PKG I β 's three dimensional structure. There are no crystal structures of PKG I β 's cGMP-binding pockets, but there exist multiple structures for the homologues protein PKA. Residues 103–352 of PKG I β and residues 122–371 of PKA RI α have 34.5% sequence identity (59.2% similarity when conserved substitutions are considered). Therefore, we built models of PKG I β using the PKA RI α structure as a template (PDB:

IRGS); this model, with exchange graphs from four interesting peptides, is shown in Figure 5. As can be seen, the regions that become most protected upon cGMP binding (residues 173–184 in the A-domain and residues 305–314 in the B-domain) are located in the PBCs at the base of the cGMP-binding pocket. A different pattern of protection is evident residues 275–292. These residues are located in β -strands and loop regions in the cNBD-B and flank the cGMP-binding site. In the presence of the N-terminus, there is a higher exchange rate than in the truncated protein, and there is a pronounced decrease in deuterium incorporation in the presence of cGMP. In the truncated protein, cGMP does not cause a noticeable decrease in H/D exchange until the 300 second time point. The peptide consisting of residues 217–227, which lies within the C-helix and connects the two cNBDs, has a dramatically different H/D exchange profile in the presence and absence of the N-terminus and in the presence and absence of cGMP. One salient difference is that in PKG containing the N-terminus the C-helix responds to cGMP binding whereas in the 98–352 construct this response is severely attenuated. Interestingly, DXMS analysis of PKA RI α and RII β demonstrated isoform specific differences in H/D exchange within this region; cAMP-binding led to decreased exchange in RI α but had no effect on RII β . Our results show that in the presence of the N-terminus, residues within the C-helix have a very pronounced cGMP-dependent decrease in H/D exchange which suggests that it undergoes conformational changes that mirror those in RI α and not RII β .

4. DISCUSSION

We have used DXMS to study how the N-terminus of PKG I β modulates the conformation of the nucleotide binding pockets. In experiments examining PKG I β 4–352, we found that the autoinhibitory domain has an extremely fast exchange rate that was not altered by cGMP binding; indicating that this region is unstructured, and as such probably not involved in modulating cGMP-affinity within the isolated regulatory domains. Thus, cGMP-affinity is most likely modulated by the leucine/isoleucine zipper domain alone. In fact, residues within the leucine/isoleucine zipper have been shown to modulate the isoform specific *K_a* values in full-length PKG I α and PKG I β [3], but these studies also demonstrated that residues within the autoinhibitory region were also involved in modulating isoform specific *K_a* values. This discrepancy can be reconciled by analogy to studies on PKA which suggest that cGMP-binding and PKG activation may not be completely correlated. Small angle X-ray scattering experiments on PKA have shown that RI α :C-subunit and RII β :C-subunit complexes persist even in the presence of saturating concentrations of cAMP [37]. While addition of a substrate peptide causes complete disassociation of the RI α :C-subunit complex, the RII β :C-subunit complex is partially retained [37]. In PKG, the regulatory and catalytic subunits reside on the same peptide chain, therefore this phenomenon, in which the cyclic-nucleotide bound regulatory domain continues to bind and inhibit the catalytic domain, may be even more prevalent. Another complicating factor in relating *K_a* values to cGMP-binding affinity is the fact that both PKG I α and PKG I β are activated by autophosphorylation within their autoinhibitory domains [26]. Thus in the full-length kinases, the role of the autoinhibitory regions in altering isoform specific kinase activation may be due to their different levels of autophosphorylation, rather than in their ability to modulate cGMP-affinity.

Comparing cGMP-bound and unbound PKG I β , we found that deuterium incorporation is reduced throughout the cGMP-binding pockets; with regions most affected being residues within and flanking the phosphate binding cassette. This result is similar to what was found in DXMS analysis of PKA [19,38], and can be explained by the stabilization of inherent secondary structure elements in the cNBD and the large number of hydrogen bonds that form between the cyclic nucleotide's phosphate and the protein's main chain amide groups [32,33]. Thus, our results strongly suggest that the molecular interactions found to be

involved in binding the cyclic nucleotide phosphate in PKA, especially the stabilization of the P-helix, are conserved in PKG.

We found that the N-terminus of PKG I β caused an increased rate of deuterium incorporation throughout the cGMP-binding domain. This result was unexpected, in that we anticipated that the N-terminus would increase cGMP affinity by stabilizing the folded state of the binding pockets. Based on our results we now propose that the N-terminus increases cGMP-affinity within the binding pockets by inducing a conformational change that produces an “open” conformation allowing cGMP access. The basis of this open conformation can be understood in the context of protein dynamics, in which proteins exist in multiple conformational states and ligand binding simply stabilizes a preexisting conformation [39,40]. Based on this model we predict that the increased dynamics induced by the N-terminus of PKG I β increases cGMP-affinity by causing the cGMP-binding pockets to spend relatively more time in a conformation that mirrors the cGMP-bound form.

We also expected to identify the residues where the N-terminus contacts the cGMP-pockets, which would have appeared as a region that had decreased deuterium incorporation in PKG I β 4–352 when compared to PKG I β 98–352. We were unable to identify such a region. The reason for this may be due to the very dynamic and unstructured nature of the autoinhibitory loop that connects the leucine/isoleucine zipper to the cNBDs, thus the interaction between the binding pockets and the leucine/isoleucine zipper may be very dynamic and undetectable using our experimental protocol. Interestingly, while there are many solved structures for the various isoforms of PKA regulatory subunits, encompassing either the cAMP-binding pockets [32–34,36] or the N-terminal dimerization/docking domain [41–43], as yet there are no structures for a full-length PKA regulatory domain. It has been proposed that since the autoinhibitory loop between the two domains is very dynamic, complexes containing the two domains may be very difficult to crystallize [44]. Likewise, our inability to detect an interaction interface between PKG I β 's N-terminus and the cGMP-binding pockets, suggest that it may be difficult to obtain crystal structures of full-length PKG. While structures of PKGs nucleotide binding pockets, in both cGMP-bound and unbound forms, would be extremely valuable for designing isoform specific PKG activators and inhibitors, the fact that the N-terminus affects the conformation and/or dynamics of the binding pockets means that crystal structures lacking the N-terminus will not provide isoform-specific information.

Some of the residues that have the largest increase in H/D exchange rates in the presence of the N-terminus are within the C-helix, which connects the two binding pockets. In PKA, the B- and C-helices between cNBD-A and cNBD-B undergo dramatic conformational changes between C-subunit bound and unbound states [34]. In fact, when bound to the C-subunit the B and C helices form one long B/C helix [34]. Since the B/C helix connects the two cNBDs its conformation likely plays a role in controlling communication between the two binding pockets. In PKA, the B/C helix also makes direct contact with the C-subunit, and plays a critical role in mediating kinase inhibition [34]. DXMS analysis of PKA RI α and RII β revealed that residues within the B/C helix underwent isoform specific responses in response to cAMP binding [19,38]. In RI α residues 232–247 showed a decreased rate of H/D exchange in the presence of cAMP, an analogous peptide in RII β showed no change. Interestingly, a peptide within the C-helix of PKG I β (residues 217–227) showed a cGMP-induced decrease in H/D exchange in the presence of N-terminus, and this decrease was markedly reduced when the N-terminus was absent. This result suggests that cGMP-induced conformational changes within the C-helix of PKG I β may be similar to those in PKA RI α .

Cyclic nucleotide binding domains in PKG and PKA are thought to share an evolutionally conserved set of secondary structural elements, including an N-terminal helical N-3-A motif, an eight stranded β -barrel and a C-terminal helical domain. Data from X-ray

crystallography, NMR, and deuterium exchange experiments, comparing the cyclic nucleotide bound and unbound forms of PKA, have allowed the elucidation of a general model for cyclic nucleotide induced changes in the binding domains [35]. The cyclic nucleotide binds within the stably folded β -barrel which induces global movements of the N- and C-terminal helical domains, leading to kinase activation. Our data strongly supports a model in which the conformation of PKG I β 's helical domains is sensitive to the presence of both cGMP and the N-terminus.

5. CONCLUSION

In this study we used DXMS to examine how the N-terminus affects cGMP-affinity within the regulatory domain of PKG I β . PKG I α has its own distinct N-terminus and a higher affinity for cGMP than PKG I β [3,12], and this isoform difference in cGMP affinity indicates the dimerization itself is not responsible for modulating cGMP affinity. At this time it is unknown if the N-terminus of PKG I α increases the dynamics of its cGMP-binding pockets in a similar manner as PKG I β . To gain insight into PKG I isoform specific cGMP-affinity, we are currently performing DXMS studies on the isolated regulatory domain of PKG I α .

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

PKG	cGMP-dependent protein kinase
PKA	cAMP-dependent protein kinase
DXMS	deuterium exchange mass spectrometry
H/D	hydrogen/deuterium
cNBD	cyclic-nucleotide binding domain

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Figure 1. Domain organization of PKG Iβ

The domain organization of PKG Iβ is shown with the constructs used in this study indicated below. PKG Iβ has an N-terminal regulatory domain and a C-terminal catalytic domain. The regulatory domain contains functional sub-domains, including a leucine/isoleucine zipper/dimerization domain (LZ), an autoinhibitory domain (AI) and two tandem cGMP-binding pockets. The catalytic domain is positioned at the C-terminus. The constructs used in this study consist of the complete regulatory domain (residues 4–352) or isolated cGMP-binding pockets (residues 98–352).

Figure 2. Pepsin digest map of the cGMP-binding region of PKG I β

Pepsin digest map showing identified peptides that are in common between PKG I β 4–352 and 98–352. The peptides are shown as grey bars below the amino acid sequence.

Figure 3. Hydrogen/Deuterium exchange of PKG I β 4–352 in the presence and absence of cGMP
 The primary sequence of PKG I β is shown with predicted secondary structural elements based on homology to PKA RI α diagramed directly above. Secondary structural elements in cNBD-A and cNBD-B are colored magenta and cyan, respectively. Color bars indicating percent deuteration at various time points (3–3000s) are shown below the sequence. Exchange experiments were performed with and without bound cGMP, as indicated. The results are the average of two independent experiments performed on two protein preparations.

Figure 4. The leucine/isoleucine zipper/autoinhibitory region increases H/D exchange throughout the cGMP-binding domain

Average difference in deuteration between PKG I β 4-352 and PKG I β 98-352 at 3, 30, and 300 seconds are shown (labeled A, B and C, respectively.) Negative values represent a decrease in deuteration in the absence of the leucine/isoleucine zipper/autoinhibitory domain.

Figure 5. Structural/functional interpretation of the exchange data

Molecular models of PKG I β (residues 98–352) were built using PKA RI α (PDB: 1RGS) as a template. cGMP molecules are shown in blue. Graphs showing deuterium incorporation of selected peptides are shown, with corresponding regions colored red in the model.