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The amino terminus of cGMP-dependent protein kinase Iβ 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 Iα and PKG Iβ 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 Iα has a >10-fold higher affinity for cGMP than PKG Iβ, and PKG Iβ that is missing its leucine zipper has a 3-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 Iβ'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.

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 Iα and PKG Iβ) 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 Iα and PKG Iβ are splice variants that have identical catalytic and regulatory domains but differ in their first ~100 amino acids, and thus contain isoform specific leucine/isoleucine zippers and autoinhibitory domains [2].

The leucine/isoleucine zippers of PKG Iα and PKG Iβ 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 Iα and PKG Iβ 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 5–10-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 Iα and PKG Iβ 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 Iβ lacking its N-terminal leucine/isoleucine zipper has a 2–3-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 Iα and PKG Iβ 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 hydro-

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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gen 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 Iα 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 Iβ affects the conformational dynamics of PKG Iβ'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 Iβ consisting of residues 4–352 and 98–352 were generated using polymerase chain reaction (PCR) and the following primers: 5′-GATGGATCCTTG-CGGGATTTACAGTAC-3′ (4–352 forward); 5′-GATGGATCCTTCTACCCCAAGAGCCCAC-3′ (98–352 forward); 5′-GATGAATTCTTATTATTCATATGCTTTATTAGAAAC-3′ (common reverse). The PCR products were digested with BamHI/EcoRI and ligated into BamHI/EcoRI digested pRSET-Xa. Vector pRSET-Xa was created by digesting pRSET B (Invitrogen) with NheI/HindIII and ligating a linker composed of the primers: 5′-CTAGCATTGAGGGACGCGGATCCGCACTCGAGGCAGAAT-TCGA-3′ (sense) and 5′-AGCTTCGAATTCTGCCTCGAGTGCGGATCCGCGTCCCTCAATG-3′ (antisense). In mammalian cells, PKG Iβ is processed and lacks an N-terminal methionine and begins with the amino acid sequence GTLRDL-. Since the extreme N-terminus of PKG Iβ has no effect of cGMP-affinity [3] and to facilitate the introduction of a BamHI fusion site, our construct begins GSLRDL, with the BamHI 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 A_{280}. All post-lysis purification steps were performed at 4 °C. Proteins were stored on ice until DXMS analysis (less than 24 h).

2.3. Peptide fragment optimization

The optimum buffer conditions for DXMS analysis were determined by performing test digests of 50 μg 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 μl 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 μg recombinant protein in a total volume of 20 μl (all manipulations were done on wet ice). Then, 120 μl ice-cold quench buffer was added and the sample was split into two 100 μl 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 μl buffered D_2O [8.3 mM Tris (pH 7.4) and 150 mM NaCl] to 20 μl purified PKG. At the appropriate time points exchange was quenched by adding 120 μl 1.6 M GuHCl/0.8% formic acid. The samples were split into two 100 μl 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 Iβ 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 Iβ 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 RIα 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 Iβ sequence into solved structures for cAMP-dependent protein kinase. The final model was validated using ProCheck [25], and we found that 87.2% of

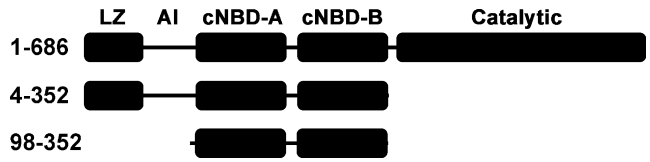

Fig. 1. Domain organization of PKG Iβ. The domain organization of PKG Iβ is shown with the constructs used in this study indicated below. PKG Iβ has an N-terminal regulatory domain and a C-terminal catalytic domain. The regulatory domain contains functional sub-domains, including a leucine/isoleucine 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).

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 Iβ domain organization and regulatory domain constructs used for DXMS analysis

PKG Iβ contains an N-terminal regulatory domain and a C-terminal catalytic domain, and each domain can be broken down to functional sub-domains as shown in Fig. 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 pseudosubstrate sequence that inhibits kinase activity by binding within the substrate recognition cleft of the catalytic domain [17]. Autophosphorylation of PKG Iβ 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 Mg^{2+}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 activation loop phosphorylation [29]. Even though it lacks kinase activity, the soluble fraction of full-length PKG Iα 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 Iα and PKG Iβ 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 Iβ regulatory domain (residues 4–352 and 98–352, respectively, see Fig. 1) were expressed in *E. coli* and purified as described in Section 2.

3.2. Tuning of PKG Iβ 4–352 and PKG Iβ 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 Iβ 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 Iβ 4–352 and 196 peptides for PKG Iβ 98–352. Within the cGMP-binding pockets, there were 71 peptides in common between the two proteins; these are shown in Fig. 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 Iα is stable over a large range of temperatures and pH [31], and we expect that the longer leucine/isoleucine zipper in PKG Iβ to be even more stable. In fact, when a protein comprising the PKG Iβ 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 1 M 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 Iβ 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 Section 2. A color bar representation showing percentage deuteration of selected peptides is shown in Fig. 3. Predicted secondary structural elements, based on alignment to PKA RIα 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 s), 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 Iα [17]. In full-length PKG Iα, 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 β5–β8 in domain A and β6–β8 in domain B; they include the P-helices that lie between β6 and β7 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 RIα and RIIβ, 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.

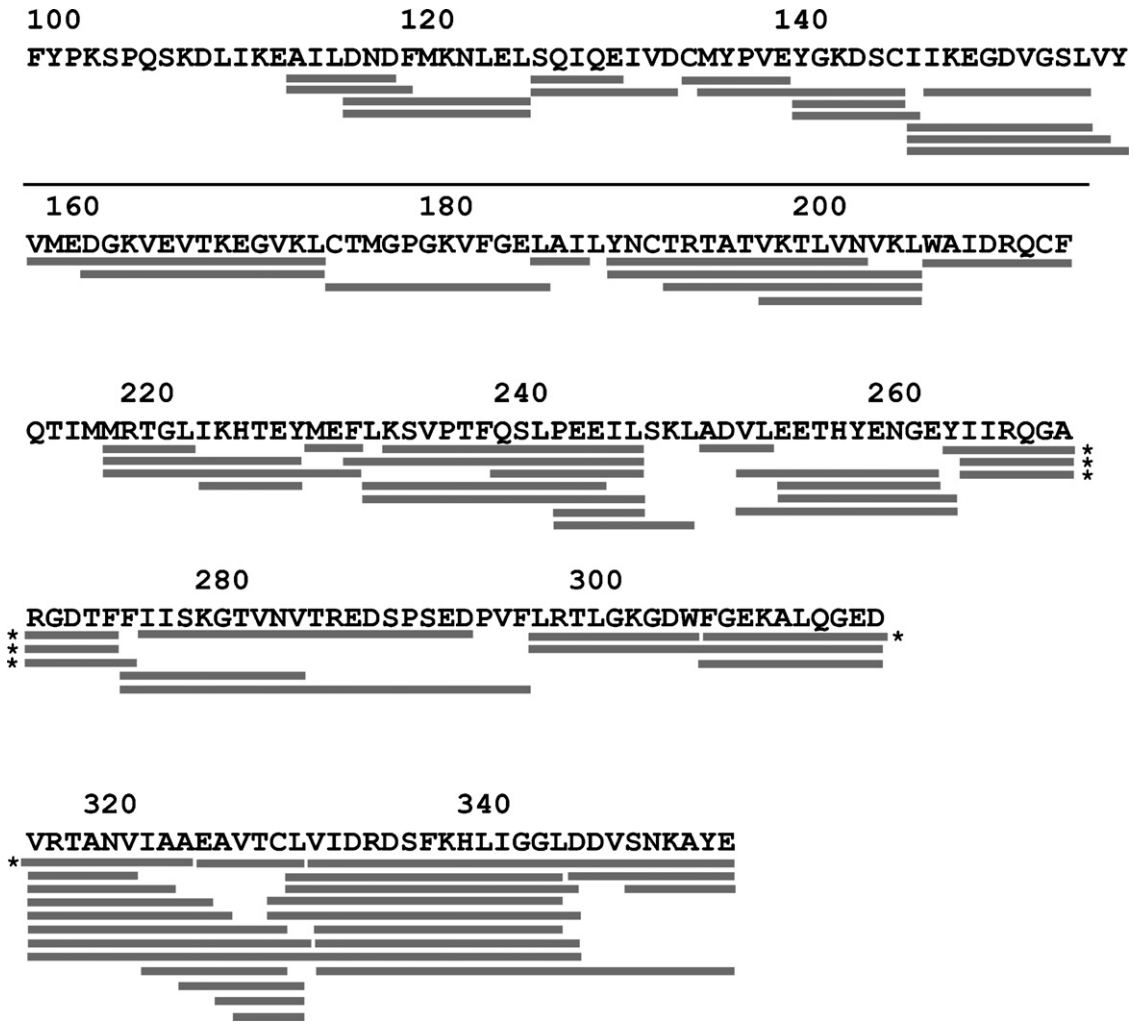

Fig. 2. Pepsin digest map of the cGMP-binding region of PKG Iβ. Pepsin digest map showing identified peptides that are in common between PKG Iβ 4–352 and 98–352. The peptides are shown as grey bars below the amino acid sequence.

3.4. Deuterium on exchange PKG Iβ 98–352

PKG Iβ 98–352 (±) cGMP was subjected DXMS analysis as described above for PKG Iβ 4–352. As expected, and like PKG Iβ 4–352, deuterium incorporation into the nucleotide binding pockets was reduced in the presence of cGMP (see below). Interestingly, when deuterium incorporation into PKG Iβ 98–352 was compared to deuterium incorporation obtained for PKG Iβ 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 Iβ 4–352 and PKG 98–352, in the absence of cGMP, at three different time points, are shown in Fig. 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}-helicies 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 predicated 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 β7 and β8 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 and 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 Iβ's three-dimensional structure. There are no crystal structures of PKG Iβ's cGMP-binding pockets, but there exist multiple structures for the homologues protein PKA. Residues 103–352 of PKG Iβ and residues 122–371 of PKA RIα have 34.5% sequence identity (59.2% similar-

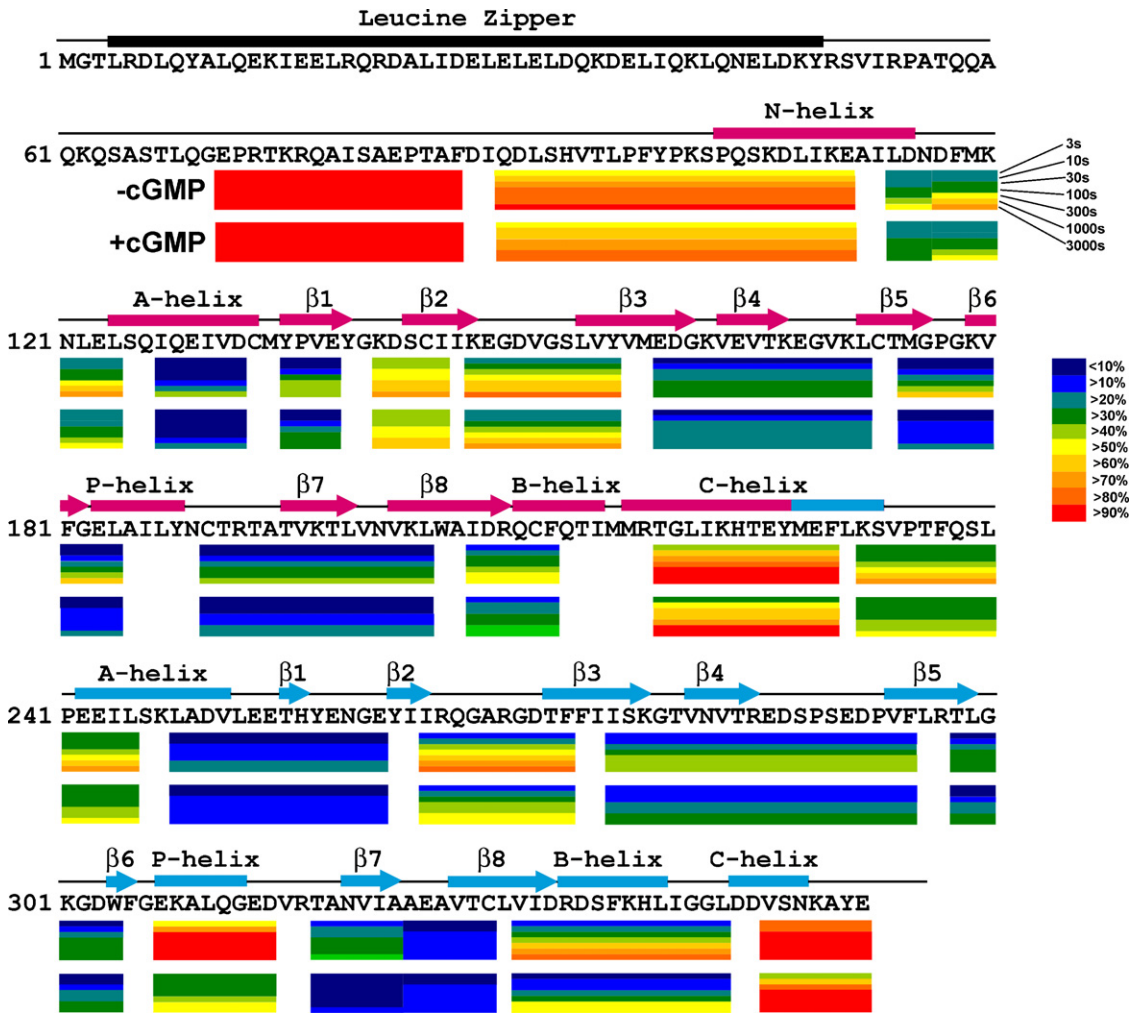

Fig. 3. Hydrogen/deuterium exchange of PKG Iβ 4–352 in the presence and absence of cGMP. The primary sequence of PKG Iβ is shown with predicted secondary structural elements based on homology to PKA RIα diagramed directly above. Secondary structural elements in cNBD-A and cNBD-B are colored magenta and cyan, respectively. Color bars indicating percent deuteration at various time points (3–3000 s) 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.

ity when conserved substitutions are considered). Therefore, we built models of PKG Iβ using the PKA RIα structure as a template (PDB: 1RGS); this model, with exchange graphs from four interesting peptides, is shown in Fig. 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. There 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 s 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 RIα and RIIβ demonstrated isoform specific differences in H/D exchange within this region; cAMP-binding led to decreased exchange in RIα but had no effect on RIIβ. 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 RIα and not RIIβ.

4. Discussion

We have used DXMS to study how the N-terminus of PKG Iβ modulates the conformation of the nucleotide binding pockets. In experiments examining PKG Iβ 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 Iα and PKG Iβ [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 RIα:C-subunit and RIIβ:C-subunit complexes persist even in

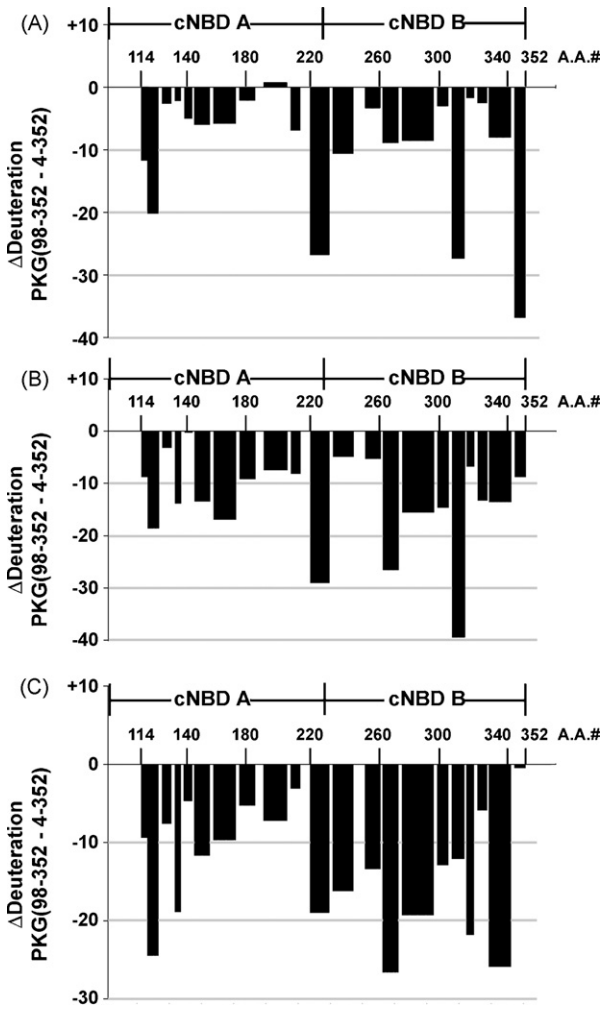

Fig. 4. The leucine/isoleucine zipper/autoinhibitory region increases H/D exchange throughout the cGMP-binding domain. Average difference in deuteration between PKG Iβ 4–352 and PKG Iβ 98–352 at 3, 30, and 300 s are shown (labeled A, B and C, respectively.) Negative values represent a decrease in deuteration in the absence of the leucine/isoleucine zipper/autoinhibitory domain.

the presence of saturating concentrations of cAMP [37]. While addition of a substrate peptide causes complete disassociation of the RIα:C-subunit complex, the RIIβ: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 Iα and PKG Iβ 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 Iβ, 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 Iβ 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 Iβ 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 Iβ 4–352 when compared to PKG Iβ 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 Iβ'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 RIα and RIIβ revealed that residues within the B/C helix underwent isoform specific responses in response to cAMP-binding [19,38]. In RIα residues 232–247 showed a decreased rate of H/D exchange in the presence of cAMP, an analogous peptide in RIIβ showed no change. Interestingly, a peptide within the C-helix of PKG Iβ (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 Iβ may be similar to those in PKA RIα.

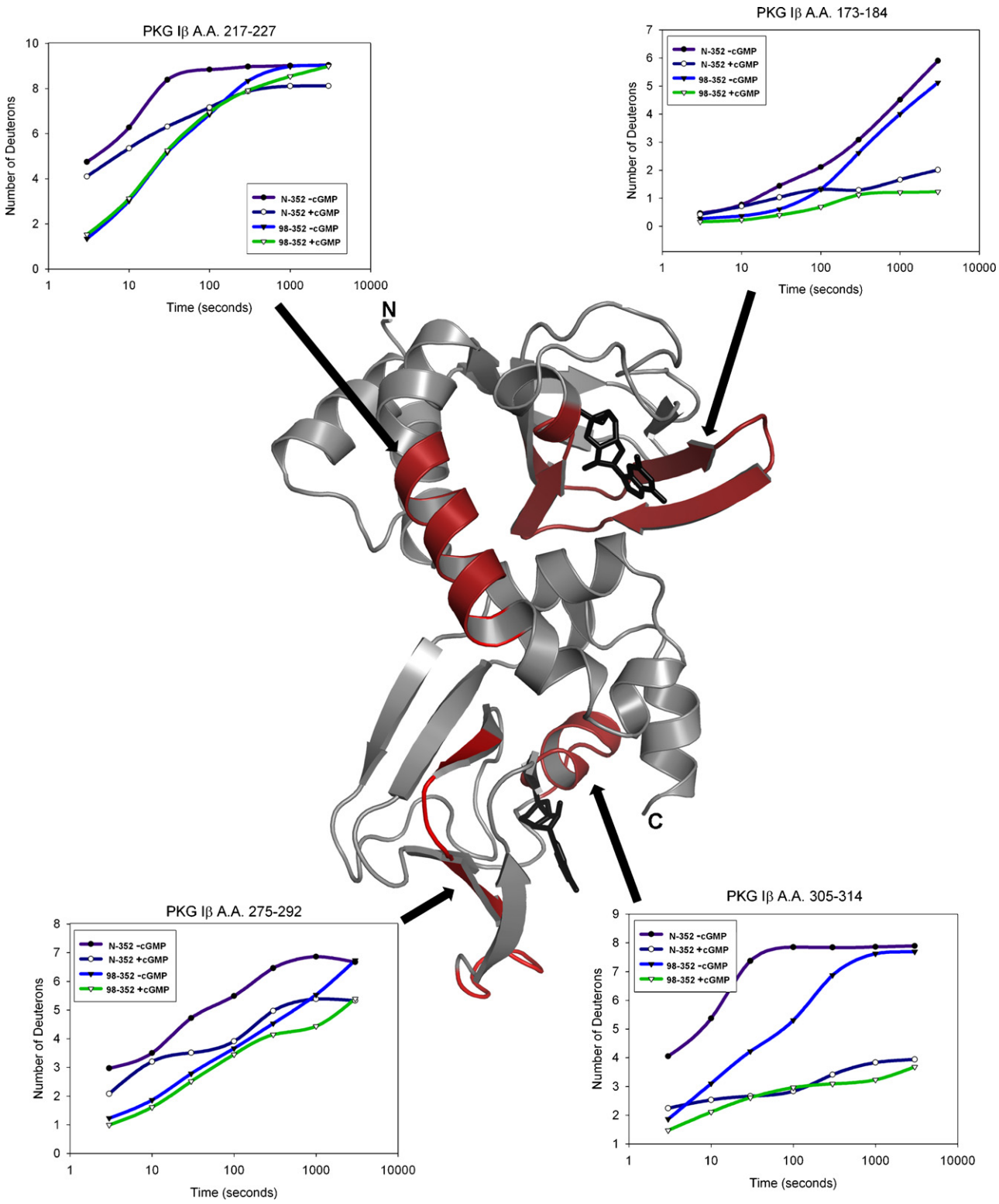

Fig. 5. Structural/functional interpretation of the exchange data. Molecular models of PKG Iβ (residues 98–352) were built using PKA RIα (PDB: 1RGS) as a template. cGMP molecules are shown in black. Graphs showing deuterium incorporation of selected peptides are shown, with corresponding regions colored red in the model. (For interpretation of the references to color in the figure caption, the reader is referred to the web version of the article.)

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 Iβ'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 the how the N-terminus affects cGMP-affinity within the regulatory domain of PKG Iβ. PKG Iα has its own distinct N-terminus and a higher affinity for cGMP than PKG Iβ [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 Iα increases the dynamics dynamics of its cGMP-binding pockets in a similar manner as PKG Iβ. To gain insight into PKG I isoform specific cGMP-affinity, we are currently performing DXMS studies on the isolated regulatory domain of PKG Iα.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijms.2010.07.021.

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