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The catalytic domain of the cGMP-dependent protein kinase Iα modulates the cGMP-binding characteristics of its regulatory domain

Wolfgang R.G. Dostmann*, Nicole Koep, Robert Endres

Institut für Pharmakologie und Toxikologie, Technische Universität München, Biedersteiner Straße 29, 80802 München, Germany

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Abstract The cGMP-dependent protein kinase Iα (PKG Iα) possesses two functional moieties, the regulatory and catalytic domains, which reside on a single polypeptide chain. Here we report on the influence of the catalytic domain on the binding of cGMP to the regulatory domain. A deletion mutant, Δ352–670 of PKG Iα, lacking the catalytic domain, was constructed and expressed in *E. coli*. The purified 38 kDa mutant protein showed strong reactivity toward tryptic proteolysis at residue Arg^{77}. Thus, a double deletion fragment Δ1–77/352–670 PKG Iα, lacking the N-terminus, was also purified. Both proteins had functional cGMP binding, but differed kinetically from the wild-type protein. First the affinity constants for cGMP were modulated, second the constructs showed no signs of cooperative cGMP binding and third dimerization of the Δ352–670 mutant was abolished. Our results provide evidence that the catalytic domain forms an intimate interaction with the regulatory domain and modulates the kinetics of cGMP binding.

Key words: cGMP-dependent protein kinase; Protein expression; Protein domain interaction

1. Introduction

The domain structure of the cGMP-dependent protein kinase type Iα (PKG Iα) consists of two independent functional elements. The N-terminal regulatory domain contains the dimerisation region followed by an auto-inhibitory sequence and two in-tandem cGMP-binding sites, whereas the ATP- and peptide substrate binding sites reside within the C-terminal catalytic domain of the enzyme [1–3]. The crystal structures of the regulatory subunit [4,5] and the catalytic subunit [6–8] of the closest relative, the cAMP-dependent protein kinase type Iα (PKA Iα), have served as valuable structural models for the regulatory and catalytic domains of PKG I_α ([2] and Tsigelny and Dostmann, manuscript in preparation). However, the stereochemical relationship between the two domains remains unknown. Binding of cGMP to both cGMP-binding sites modulates the interaction between the domains which, in turn, leads to the activation of the enzyme. In contrast to PKA I_α, the activation of PKG I_α does not require the dissociation of the domains [9].

Although very similar in primary sequence, the cGMP-binding sites differ with respect to their binding kinetics and analog specificity [10–12]. Each PKG monomer consists of a low and a high affinity cGMP-binding site, referred to as site A (or site 2) and B (or site 1) [1,2]. cGMP site A is characterized by the rapid dissociation of bound cGMP and an apparent K_D of 110–150 nM, whereas cGMP site B shows a slow cGMP off-rate paired with an apparent K_D of 14–17 nM [13,14]. In addition, the cGMP-binding sites, as well as the cAMP-binding sites of PKA, show positive cooperativity for cyclic nucleotide binding. This kinetic fidelity of the cyclic nucleotide binding sites is dependent on an intact N-terminus [15]. Removal of the N-terminal 77 amino acids of PKG I_α generated a constitutively active enzyme with altered cGMP-binding characteristics [15,16]. In analogy, deletion of the first 91 amino acids of PKA I_α abolished the cooperative binding of cAMP and modified the cAMP-binding constants [17]. The cGMP-binding characteristics in PKG I_α also depend on the state of phosphorylation [14,18] and oxidization [19]. However, the possible modulatory role of the catalytic domain on the cGMP-binding properties has not been addressed. Therefore, we expressed the regulatory domain of PKG I_α in *E. coli*, purified the recombinant mutant protein and studied its cGMP-binding kinetics in order to identify the functional role of the catalytic domain more precisely.

2. Materials and methods

2.1. Construction of δ352–670 PKG Iα

A pUC18 derivative containing the full-length cDNA of PKG Iα (pUC-PKG) was constructed as described previously [13]. The regulatory domain of PKG was amplified using specific PCR primers: the forward primer 5'ATGTCACTCGTGAAGACT3' (bp 800–817) and the reverse primer 5'GCCGGGATCCTTAGGCGAAGAAAGCAGCTTC3' (bp 1042–1059). This latter primer also contained a non-annealing sequence which carried a STOP signal followed by a *Bam*HI restriction site. The amplified DNA product was then digested with *Bsu*36I and *Bam*HI to yield a fragment for ligation into *Bsu*36I/*Bam*HI cut pUC-PKG. Transformation of this ligation mixyure in *E. coli* JM101 and subsequent screening of positive transformants with specific PCR primers (above) yielded the plasmid pUC-Δ352–670 PKG. After sequencing the amplified region [20], several colonies were picked and screened for high yields of Δ352–670 PKG Iα expression, by scanning the total cell extracts with SDS-PAGE and immunoblotting.

2.2. Expression and purification of δ352–670 and δ1–77/352–670 PKG Iα

4 l of LB-ampicillin were inoculated with *E. coli* JM101/pUC-Δ352–670 PKG and grown at 37°C for 24–48 h. The cell pellet was resuspended in buffer A (25 mM KPO_4 pH 6.6, 10 mM DTT, 5 mM EDTA, 5 mM EGTA, 10 mM benzamidine, 1 mM PMSF, 10 μg/ml SBTI, 284 μM TPCK, 135 μM TLCK) and passed twice through a French pressure cell at 4°C. All subsequent steps were performed at 4°C. The homogenate was centrifuged for 30 min at 10000 rpm and the supernatant diluted with distilled water to the conductivity of buffer A (0.3 mS/cm) before application to a Whatman-DE52 ion-exchange column. Δ352–670 PKG and Δ1–77/352–670 PKG were eluted with a 0–250 mM NaCl gradient in buffer A. Peak fractions of both proteins were pooled and applied to freshly synthesized cAMP-agarose column. Following an isocratic wash protocol with buffer A+1 M NaCl, the proteins were eluted at room temperature in buffer A+1 mM cAMP. The peak fractions were dialyzed against buffer B (10 mM KPO_4 pH 6.6, 2 mM EDTA), concentrated to a

*Corresponding author. Fax: (49) (89) 3849-3261.
E-mail: dostmann@ipt.med.tu-muenchen.de

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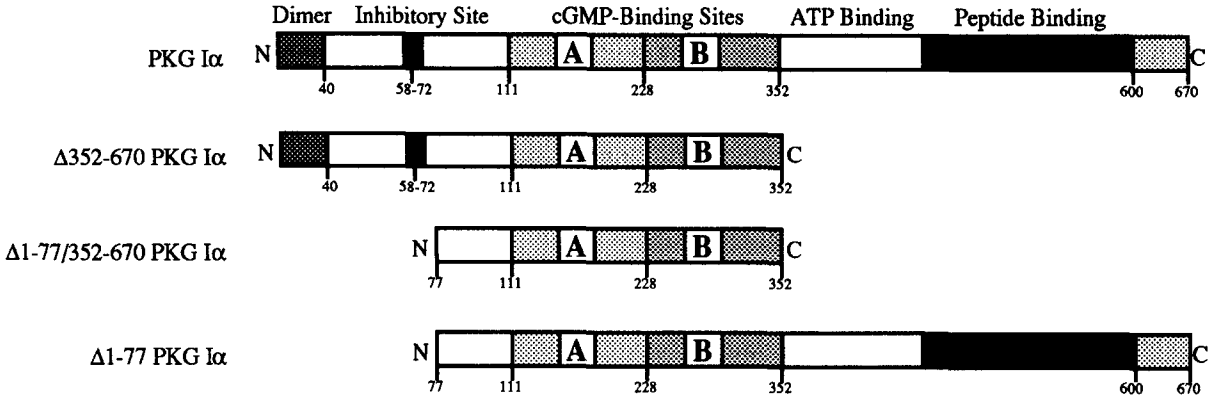

Fig. 1. Domain structure of PKG type Iα and several deletion mutants. The C-terminal deletion Δ352–670 PKG Iα was generated using PCR-overlap-extension and purified from *E. coli* E222 cells. The double deletion protein Δ1–77/352–670 PKG Iα was co-purified as a proteolytic fragment. The N-terminal deletion Δ1–77 PKG Iα is shown for comparison and was characterized earlier [15,16]. The designation A and B for the slow and fast cGMP-binding sites is in accordance with previously published data [26] and our own work (Dostmann et al., unpublished results).

volume of approx. 1.5 ml, and applied on a 5.5×7 cm 7.5% non-denaturing preparative polyacrylamide gel column (BioRad Prep cell model 491). Electrophoresis was carried out at 4°C and 300 V and 4-ml fractions were collected at 0.25 ml/min. Δ1–77/352–670 PKG was found in fractions 5–7 and Δ352–670 PKG eluted with fractions 8–11.

2.3. Equilibrium cGMP-binding and cGMP exchange rates

Equilibrium cGMP-binding constants (K_D) for wild-type PKG and mutants Δ352–670 PKG and Δ1–77/352–670 PKG were determined by modification of a previously described method [21]. Proteins were incubated in buffer C (50 mM MES, pH 6.9, 0.4 mM EGTA, 1 mM Mg-acetate, 10 mM NaCl, 10 mM DTT and 0.5 mg/ml BSA) with varying concentrations of [^{3}H]cGMP (30 Ci/mmol, Amersham). After 90 min incubation at 0°C, aliquots were precipitated in 3 ml of ice-cold 95% ammonium sulfate (containing 10 mM HEPES-NaOH pH 7.0, 2 mM EDTA) and filtered over 0.45 μm pore size nitrocellulose filters (Schleicher and Schuell). To distinguish the two kinetically different cGMP-binding sites, [^{3}H]cGMP bound to site A was selectively displaced by an excess of unlabeled cGMP as described previously [21].

cGMP exchange rates (k_d) were measured by incubating the proteins with excess [^{3}H]cGMP for 90 min at 0°C in buffer D (50 mM MES pH 6.9, 0.5 mM EGTA, 1 mM Mg-acetate, 10 mM NaCl, 10 mM DTT, 1 μM [^{3}H]cGMP, 0.5 mg/ml BSA) [13]. For exchange rates with $\tau_{1/2} \geq 30$ s, cold cGMP was added to a final concentration of 100 μM in buffer E (15 mM HEPES-NaOH pH 7.0, 5 mM EDTA, 0.3 mM EGTA, 20 mM DTT, 0.5 mg/ml BSA, 150 mM NaCl and 1 mM cAMP). Aliquots were taken at various time points, precipitated in 3 ml of ice-cold 95% ammonium sulfate and filtered over 0.45 μm nitrocellulose filters. For exchange rates with $\tau_{1/2} \leq 30$ s the incubation mixture was aliquoted in tubes, cold cGMP added and the reaction stopped by the addition of 3 ml of ice-cold 95% ammonium sulfate. In addition, 8-Br-cGMP which selectively binds to the slow dissociating site [11] was added to a final concentration of 10 μM in experiments designated to measure selectively the fast dissociating binding site.

The filters were washed twice with 3 ml of ice-cold 70% ammonium sulfate (10 mM HEPES pH 7.0, 2 mM EDTA), and dissolved in 10 ml scintillation fluid prior to counting.

2.4. Sucrose density gradient ultracentrifugation

5–15% sucrose density gradients were used to determine the Svedberg constants ($s_{w,20}$). Centrifugation was carried out at 65 000 rpm for 108 min. Proteins (45–300 μg) were dissolved in buffer F (20 mM KPO_4 pH 7.0, 2 mM benzamidine, 2 mM EGTA). The gradients were divided into 23 aliquots and protein concentrations were measured by either Bradford assay [22] or [^{3}H]cGMP binding. The following internal marker proteins were used: phosphorylase *b* ($s_{w,20} = 8.2$), γ-globulin (rabbit, $s_{w,20} = 6.36$), horseradish peroxidase ($s_{w,20} = 3.67$) and lysozyme ($s_{w,20} = 1.9$). PKA regulatory subunit I was run as a control ($s_{w,20} = 4.7$) [23,24].

2.5. Miscellaneous methods

cAMP-agarose was synthesized by coupling 25 ml *N*-hydroxysuccinimide-activated agarose (Affigel, Bio-Rad) with 50 μmol 8-(2-aminoethyl)-amino-cAMP (BioLog) in 100 mM HEPES pH 7.0 at room temperature under continuous shaking. The reaction was complete after 60 min as was deduced from the absorbance of the supernatant at 273 nm. Unreacted groups were blocked with 100 μl 1 M ethanolamine, pH 8.0 for 90 min. The resin was stored in buffer A+0.05% Na-azide.

Immunoblotting was performed by using specific antibodies raised against the N-terminus of the PKG Iα [25]. Purified or crude extract proteins were separated on 10–12% SDS-PAGE and blotted on PVDF membranes under semi-dry conditions (1 h, 0.8 mA/cm^2). The membranes were treated as described previously [25] and the specifically bound antibodies visualized by an anti-rabbit IgG antibody conjugated to alkaline phosphatase.

For peptide sequencing the purified proteins were separated on 12% SDS-PAGE and transferred to PVDF membranes. The specific bands (approx. 1–3 μg protein/band) were analyzed on a gas-phase sequencer.

Non-denaturing PAGE was performed on 6% gels with the following buffer conditions: stacking gel: 62.5 mM Tris-HCl, pH 6.8; separating gel: 375 mM Tris-HCl, pH 8.9; running buffer: 25 mM Tris-HCl, pH 8.8, 52 mM glycine; 5×sample buffer: 312 mM Tris-HCl, pH 6.8, 50% glycerol, 0.05% bromophenol blue. The gels were run at 8–10 mA for 12–16 h and subsequently stained with Coomassie blue R250.

3. Results

3.1. Construction and purification of the PKG Iα regulatory subunits

The truncated form Δ352–670 PKG Iα (Fig. 1) of the wild-type kinase coding for the N-terminal 352 amino acids was constructed using overlap-extension-PCR as outlined in Section 2. Selected transformants were screened for expression by screening aliquots of the total cell extract by SDS-PAGE. Several colonies expressed large amounts of protein that migrated with a molecular mass of approx. 38 kDa (data not shown). Expressed Δ352–670 PKG Iα was found in the supernatant fraction as well as in the particulate fraction at a ratio of approx. 1:1 (data not shown). The supernatant fraction was applied to a DEAE ion-exchange column and those protein fractions showing cGMP-binding capacity were pooled and applied on a cAMP-affinity resin. Elution with 1 mM cAMP yielded two proteins with molecular masses of 38 and 30 kDa, respectively, as deduced from SDS-PAGE (Fig.

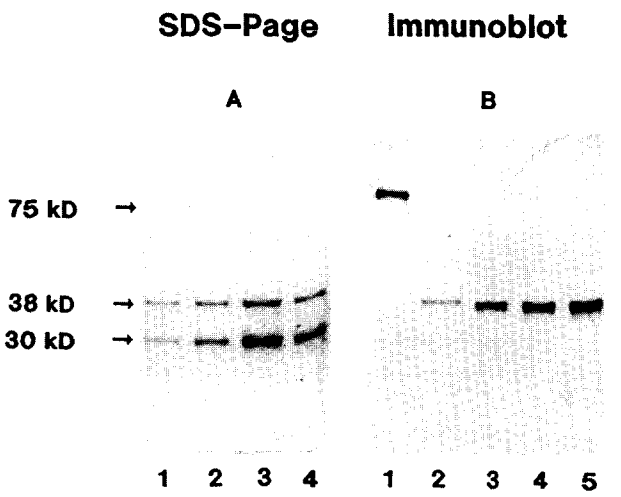

Fig. 2. cAMP-agarose affinity chromatography of *E. coli* E222 extracts. *E. coli* E222 was transformed with the vector coding for Δ352–670 PKG Iα, lysed, and the supernatant chromatographed on a DEAE-ion-exchange column (data not shown). Fractions which specifically bound [^{3}H]cGMP were pooled and loaded on a freshly synthesized 8-AEA-cAMP-agarose column as outlined in Section 2. Cyclic nucleotide binding proteins were eluted with 1 mM cAMP and fractions 1–4 monitored by (A) SDS-PAGE, lanes 1–4 and (B) immuno-blotting with a specific antibody raised against the N-terminus of PKG type Iα [25]. Lane 1 shows native PKG Iα and lanes 2–5, fractions 1–4. The proteins at 38 and 30 kDa were transferred onto PVDF membranes, subjected to gas-phase sequencing and identified as Δ352–670 PKG Iα (38 kDa) and Δ1–77/352–670 PKG Iα (30 kDa).

2A). Immuno-blot analysis of the cAMP-affinity column fractions revealed that only the 38 kDa band was detectable by a PKG Iα specific antibody. Since the antibody is directed against the N-terminal residues 13–35 of PKG Iα [25], the 30 kDa band most likely reflected a proteolytic fragment of Δ352–670 PKG Iα, rather than protein contamination (Fig. 2B). Gas-phase protein sequencing of the 38 kDa and 30 kDa proteins confirmed that the lower molecular mass protein was a tryptic degradation product of Δ352–670 PKG Iα (data not shown). The cleavage site was identified in the hinge region at Arg^{77}, an identical position to that described previously for native PKG Iα [15]. Complete suppression of degradation could not be achieved even when using efficient trypsin protease inhibitors, partly because proteolysis already occurred during protein biosynthesis in the bacterium (data not shown). Apparently, the catalytic domain of PKG Iα protects the hinge region of the enzyme at Arg^{77} from proteolysis. Preparative non-denaturing gel electrophoresis was applied to separate effectively Δ352–670 PKG Iα from its Δ1–77 truncation fragment (Fig. 3). A 10–12 l preparation yielded 6–10 mg each of Δ352–670 PKG Iα and Δ1–77/352–670 PKG Iα purified to apparent homogeneity.

3.2. Characterisation of δ352–670 and δ1–77/352–670 PKG Iα

We next examined the structural features of the two recombinant proteins. The native PKG Iα consisted of two protomers linked probably by a leucine zipper type hydrophobic interaction located at the N-terminus of each protomer [27]. In order to identify dimer formation of the deletion mutants we employed 5–15% sucrose density gradient ultracentrifugation. Native PKG Iα showed a Svedberg constant of 7.6 S which was in accordance with earlier experiments [14]. Similarly, the regulatory subunit of PKA had the characteristics of a dimer with $s_{20,w} = 6.1$ S [17,25]. However, Δ352–670 PKG Iα did not dimerize, as was indicated by a Svedberg constant of 2.5 (Table 1). The monomeric state of Δ352–670 PKG Iα was further observed with non-denaturing PAGE (data not shown). The double deletion fragment Δ1–77/352–670 PKG Iα, which lacked the dimerisation site, had a Svedberg constant of 2.2, supporting the monomeric structure of Δ352–670 PKG Iα. Thus, the catalytic domain influences not only the dimerization at N-terminus, but protects the hinge region from tryptic cleavage as well.

To determine whether the recombinant PKG regulatory subunit possessed the ability to interact with the catalytic subunit of PKA, Δ352–670 PKG Iα was dialyzed in the presence of a 10% excess of recombinant Cα under conditions that would typically lead to the formation of holoenzyme [28]. After 48 h no holoenzyme formation was detectable as demonstrated by non-denaturing gel electrophoresis and cAMP-dependent activation assays (data not shown). Additional attempts to express the catalytic domain of PKG Iα in *E. coli* in an active conformation were unsuccessful (Dostmann and Endres, unpublished results).

The kinetic analysis of the deletion mutants is summarized in Table 1. Both Δ352–670 PKG Iα and Δ1–77/352–670 PKG Iα contained two functional cGMP-binding sites. However, their binding and dissociation characteristics differed clearly from those of the wild-type enzyme and another previously characterized N-terminal deletion mutant Δ1–77 PKG Iα [15,16]. While the exchange rates for the 'fast' site B differed only by a factor of 2 between wild-type and mutant Δ1–77/

Table 1
Kinetic and hydrodynamic constants for wild-type and deletion mutants of PKG Iα

	PKG type			
	Iα[a]	Δ352–670	Δ1–77/352–670	Δ1–77[b]
cGMP equilibrium binding, K_D (nM)				
Site A (slow)	17.6	79	218	8.8
Site B (fast)	109	≥500	218	370
Hill coefficient (n)	1.4–1.6		0.95	0.88 1
.0				
cGMP exchange rate, k_d (min^{-1})				
Site A (slow)	0.0044	0.22	0.83	0.067
Site B (fast)	3.46	6.52	6.60	6.06
Molecular mass (kDa, SDS-PAGE)	75	38	30	65
Svedberg constant, $s_{20,w}$ (S)	7.6 dimer	2.5 monomer	2.2 monomer	4.6 monomer

Purified mutants Δ352–670 PKG Iα and Δ1–77/352–670 PKG Iα were characterised as described under Section 2.

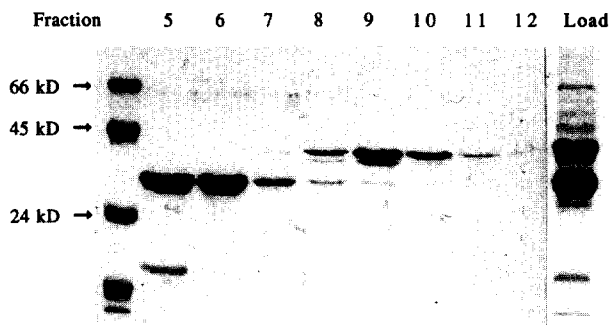

Fig. 3. Preparative non-denaturing polyacrylamide gel electrophoresis was employed to separate Δ352–670 PKG Iα from the double deletion fragment Δ1–77/352–670 PKG Iα. Pooled fractions from the cAMP-agarose column (Fig. 2) were applied to the gel following dialysis (Section 2). Typically, when running a 7.5% gel (5.5 cm+1.5 cm stacking gel) at 300 V and 4°C the 30 kDa protein Δ1–77/352–670 PKG Iα eluted with fractions 5 and 6 and Δ352–670 PKG Iα (38 kDa) with fractions 9–12.

352–670 PKG Iα, we saw a far more dramatic effect on the 'slow' site A. Deletion of the N-terminus (Δ1–77 PKG Iα) increased the k_d by a factor of 15, whereas deletion of the catalytic domain (Δ352–670 PKG Iα) shifted the cGMP exchange rate 50-fold. More drastic was the effect on Δ1–77/352–670 PKG Iα, the k_d was shifted by a factor of 180. These findings demonstrate that the catalytic domain has a more pronounced effect on cGMP dissociation than the N-terminus does. This effect was also apparent for cGMP equilibrium binding to the proteins. While Δ1–77 PKG Iα had a 3-fold decreased cGMP affinity to site B, the constructs with the deleted catalytic subunit showed a similar effect on both sites A and B. For the mutant Δ352–670 PKG Iα, the low affinity site could not be measured exactly with the conventional ammonium sulfate filter assay, as has been noted previously [29]. However, by comparing the experimental results for Δ352–670 PKG Iα and Δ1–77/352–670 PKG Iα with estimated equilibrium binding constants, consistently lower values for site A and B were found (data not shown). The stoichiometry of cGMP binding was 1.89 mol cGMP/mol protein for the deletion mutant Δ1–77/352–670 PKG and 0.5–1.3 mol cGMP/mol protein for Δ352–670 PKG Iα, again demonstrating our inability to detect this low affinity site adequately with the standard ammonium sulfate assay. In addition, we observed a loss of cooperativity for cGMP binding with all deletion mutants. This result is in accordance with observations from an N-terminal deletion mutant of the regulatory subunit of the PKA [17]. Apparently, even subtle changes on the domain structure flanking the cGMP-binding sites abolish completely the crosstalk between the sites [30].

4. Discussion

The results presented in this work demonstrate that the catalytic domain of the PKG Iα exerts an impact on three distinct regions of the N-terminal regulatory domain of the enzyme: (i) the dimerization region; (ii) the hinge region; and (iii) the cGMP-binding sites.

At first, since dimerisation was abolished when the catalytic domain was deleted, a stabilizing influence on dimer formation must be postulated for the catalytic domain. In contrast to PKA, dimer formation in PKG Iα occurs presumably via the hydrophobic forces of two N-terminal α-helices (amino acids 1–40) [27]. However, factors that could interfere with this interaction have not been identified. Since the catalytic domain of the enzyme is inhibited by an auto-inhibitory domain (hinge region) [31] C-terminal of the α-helix (amino acids 58–72) a steric connection between the dimerisation domain and the catalytic domain appears possible.

Secondly, the strong tendency for proteolytic cleavage at residue Arg^{77} indicates that, in fact, this stabilizing influence of the catalytic domain extends beyond the immediate N-terminus to the hinge region. Interestingly, auto-phosphorylation of PKG Iα occurs at multiple positions [31], indicating that the hinge region is far more flexible in nature than the type II regulatory subunit of PKA [32]. Therefore, cleavage of the catalytic domain may leave the hinge region exposed and prone to proteolysis.

Thirdly, the catalytic domain influences the cGMP-binding affinity of the cGMP-binding sites A and B. Our results show that both deletion mutants Δ352–670 PKG Iα and Δ1–77/352–670 PKG Iα, which lack the catalytic domain, possess two cGMP-binding sites which both showed reduced ligand binding affinities and a loss of cooperative cGMP binding. Taken together, the data from this study and previously published results from N-terminal deletion mutants of PKA and PKG [15–17] suggest that the cGMP-binding affinity for each binding site is regulated from regions outside the actual cGMP-binding domains. Therefore, the observed K_D values in those mutants reflect the 'true' affinities for each site. These additional regions are part of the N-and C-terminus; they complement the cyclic nucleotide binding sites as well as influence cooperativity between the sites.

In conclusion, the catalytic domain appears to affect all known distinct functional regions of the regulatory domain. Our results show that the catalytic domain of PKG Iα forms an intimate contact with its own regulatory domain demonstrating the compactness of the enzyme. Elucidation of the structural nature of this interaction, however, must wait until a crystal structure of PKG Iα becomes available.

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