Supplemental Information

Neutron crystallography detects differences in protein dynamics: Structure of PKG II cyclic nucleotide binding domain in complex with an activator

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MATERIALS AND METHODS

Protein Expression and Purification. The truncated CNB-B domains of human PKG I and II were expressed and purified as described previously [1]. Briefly, the CNB-B domains of PKG I and II were cloned into the pQTEV vector and overexpressed in the TP2000 cell lines [2, 3]. Tobacco Etch Virus (TEV) protease cleavable, 7 x His-tagged, CNB-Bs were first purified by using nickel affinity chromatography. Elution fractions were dialyzed overnight with TEV and then passed over a second nickel affinity column. The flow though was concentrated and injected on a Superdex S75 size exclusion chromatography column. The protein sample was flash frozen and stored at -80C, sample purity >95% was confirmed by SDS-PAGE.

Crystallization. For crystallization trials the complex PKG II:8-pCPT-cGMP (with the final concentration of ~35 mg/mL for PKG II and 5 mM for the analog), and the reservoir (30% MPD, 20 mM $SrCl_2$, 100 mM NaOAc pH 4.6 in H_2O) solutions were combined at a 1:1 ratio in the 30

μL sitting drops and incubated at 4°C. Over a period of three weeks some drops yielded showers of small crystals, while others resulted in precipitate formation. A recrystallization procedure was then applied as follows: sitting drops in Hampton microbridges were held over water for a few days at 10°C until some of them became clear, indicating that the protein complex had re-dissolved. The drops then were returned to the original reservoir solution and placed back to 4°C. A few drops produced large crystals; one crystal that appeared suitable for neutron diffraction was mounted in a quartz capillary containing the reservoir solution made with 100% D_2O and per-deuterated MPD. The labile H atoms were allowed to exchange with deuterium by vapour for several weeks before starting neutron data collection. This crystal was also used for room-temperature X-ray data collection that was done after the neutron diffraction data had been collected.

Data collection. The crystal diffraction quality was tested and preliminary data were collected on the IMAGINE [4] instrument located at the High Flux Isotope Reactor (Oak Ridge National Laboratory) from a 0.6 mm^3 PKGII:8-pCPT-cGMP crystal at room temperature. The crystal demonstrated good diffraction pattern and was then considered for full data collection. The beamtime was awarded and the complete dataset was collected at room temperature on the LADI-III beamline at the Institut Laue-Langevin [5]. Quasi-Laue neutron data were collected to 2.2 Å resolution. As is usual for a Laue experiment, the crystal was held stationary at a different φ setting for each exposure. In total 17 images were collected (with an average exposure time of 24 h per image) from 3 different crystal orientations. The neutron data were processed using the Daresbury Laboratory *LAUE* suite program *LAUEGEN* [6] modified to account for the cylindrical geometry of the detector [7]. The program *LSCALE* [8] was used to determine the wavelength-normalization curve using the intensities of symmetry-equivalent reflections

measured at different wavelengths. No explicit absorption corrections were applied. These data were then merged in *SCALA* [9]. Monochromatic X-ray diffraction data were collected from the same crystal used for the neutron diffraction data collection using an in-house Rigaku HomeFlux system, equipped with a MicroMax-007 HF generator and Osmic VariMax optics. The diffraction images were obtained using an RAXIS-IV++ image-plate detector. Diffraction data were collected, integrated and scaled using HKL3000 software suite [10]. The structure was solved by molecular replacement using Phaser from CCP4 suite [11] and the PKG II CNB-B:cGMP binary complex (PDB ID 5BV6) [1] was used as a starting model. The room-temperature X-ray structure of the PKGII:8-pCPT-cGMP complex was refined using *SHELX-97* [12] at the resolution of 2.0 Å before using it as a starting model in the joint XN refinement. A summary of the experimental and refinement statistics is given in Table S1.

Joint XN structure refinement. The joint XN structure of the PKGII:8-pCPT-cGMP complex was determined using *nCNS* [13] and manipulated in *Coot* [14]. After initial rigid-body refinement, several macrocycles of positional, atomic displacement parameter, and occupancy refinement followed. Between each macrocycle the structure was checked, side-chain conformations were altered and water molecule orientations were built based on the F_O-F_C difference neutron scattering density map. The $2F_O$-F_C and F_O-F_C neutron scattering density maps were then examined to determine the correct orientation of hydroxyl (Ser, Thr, Tyr) and ammonium (Lys) groups, and protonation states of His and Lys residues. The protonation states of some disordered side chains could not be obtained directly, and remained ambiguous. All water molecules were refined as D_2O. Initially, water oxygen atoms were positioned according to their electron density peaks, and then were shifted slightly in accordance with the neutron scattering density maps. The level of H/D exchange at OH, NH and SH sites was refined. All

labile H positions in PKG II and the ligand were modelled as D, and then the occupancy of D was allowed to refine within the range of -0.56 to 1.00 (the scattering length of H is -0.56 times the scattering length of D). Before depositing the final structure to the PDB, a script was run that converts a record for the coordinate of D atom into two records corresponding to an H and a D atom partially occupying the same site, both with positive partial occupancies that add up to unity. The joint XN structure of PKGII-8-pCPT-cGMP has been deposited to the Protein Data Bank (code 6BQ8).

Fluorescence Polarization Assay. Fluorescence polarization (FP) was used to measure the binding affinity of 8-fluo-cGMP to the CNB domain constructs. The assay was conducted in 150 mM Nacl, 20 mM MOPS, 0.005% (v/v) Tween-20, pH 7.0, 1nM 8-fluo-cGMP (Biolog Life Science Institute, Bremen, Germany) in a black, flat-bottomed 384-well plate (Perkin Elmer, Optiplate). The FP signal was read for 2 seconds per well at an excitation wavelength of 485 nm and an emission wavelength of 535 nm, on BioTek Synergy2 equipped with a FITC optics cube. Data were analyzed and fit to a sigmoidal dose response curve using GraphPad Prism 5.03 (GraphPad Software, San Diego, CA) to generate K_D values. Competition experiments were performed at concentration approximately equal to the K_D of the direct binding assay and 8-fluo-cGMP was added to a final concentration of 1 nM. The 8-pCPT-cGMP was titrated via a serial dilution. Polarization data were measured and analyzed as described above to generate EC_{50} values.

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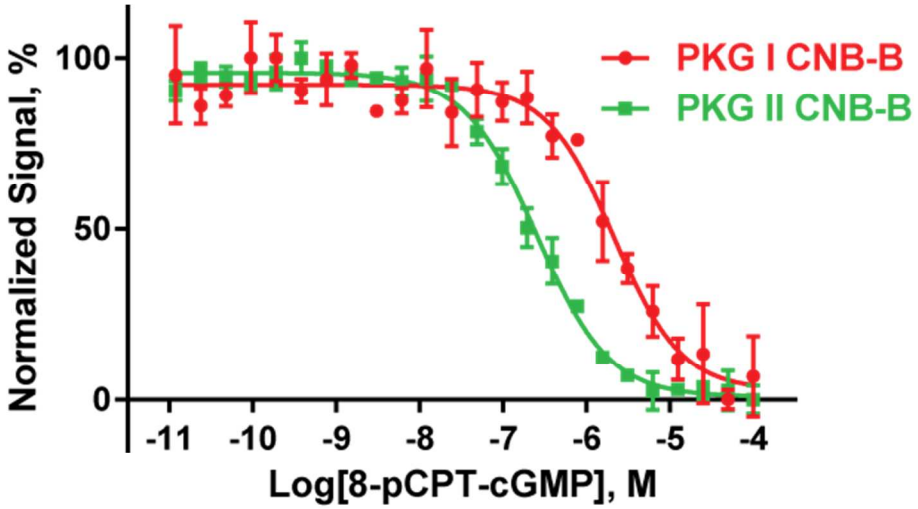
Figure S1.



Protein	EC_{50} ± SD[a] (nM), 8-pCPT-cGMP
PKG Iβ CNB-B	1.627 ± 0.264 uM
PKG II CNB-B	0.259 ± 0.009 uM

[a] mean values with standard deviation (SD) of at least four replicates

Table S1. Room-temperature crystallographic data collection and joint XN refinement statistics.

	PKG II:8-pCPT-cGMP PDB ID 6BQ8	
Data collection:	**Neutron**	**X-ray**
Beamline/Facility	LADI-III/ILL	Rigaku HighFlux HomeLab
Space group	$P2_12_12_1$	
Cell dimensions:		
a, *b*, *c* (Å)	43.65, 51.30, 68.20	
α, β, γ (°)	90, 90, 90	
Resolution (Å)	40.00-2.20 (2.32-2.20)*	10.00-2.00 (2.06-2.00)
No. reflections measured	21665 (1420)	34375 (2454)
No. reflections unique	5966 (589)	10492 (770)
R_{merge}	0.141 (0.190)	0.064 (0.535)
$I / \sigma I$	7.5 (4.5)	12.5 (2.8)
Completeness (%)	74.7 (51.9)	97.3 (97.6)
Redundancy	3.6 (2.4)	3.3 (3.2)
Joint XN Refinement:		
Resolution (neutron, Å)	40-2.20	
Resolution (X-ray, Å)	40-2.00	
Data rejection criteria	no observation & |F|=0	
Sigma cut-off	2.0	
No. reflections (neutron)	5949	
No. reflections (X-ray)	10401	
R_{work} / R_{free} (neutron)	0.232 / 0.288	
R_{work} / R_{free} (X-ray)	0.199 / 0.245	
No. atoms		
Protein including H and D	2410	
Ligand	45	
Metal	1	
MPD	22	
Water	120 (40 D_2Os)	
B-factors		
Protein	37.7	
Ligand	26.7	
Water	46.3	
R.m.s. deviations		
Bond lengths (Å)	0.007	
Bond angles (°)	0.931	

* Values in parentheses are for highest-resolution shell. Data were collected from 1 crystal for each structure.

Table S2. Comparison of the backbone amide H/D exchange in PKG II and PKG I.

'Non-exchanged' corresponds to the positions with the D atom occupancy in the range –0.56 ÷ 0, i.e. between 0 and 36% (~1/3) of D.

'Partially' is for partially exchanged positions; D atom occupancy of 0.01 ÷ 0.49, i.e. between 36% (~1/3) and 68% (~2/3) of D.

'Fully' is for fully exchanged positions; D occupancy of 0.50 ÷ 1, i.e. between 68% (~2/3) and 100% (1.0) of D.

Residues are arranged side-by-side in the columns according to the sequence alignment published in reference 1 listed above.

Disordered residues that are not visible in the electron density are labeled 'Not visible in the structure' and are not numbered.

PBC residues are colored blue; additional residues that make hydrogen bonds with 8-pCPT-cGMP are colored green.

PKG II			PKG I		
Residue	D atom occupancy	H/D status	Residue	D atom occupancy	H/D status
269 Thr	-0.28	Non-exchanged	Thr	Not visible in the structure	
270 Ala	1.0	Fully	Gly	Not visible in the structure	
271 Gln	1.0	Fully	Leu	Not visible in the structure	
272 Ala	0.99	Fully	Ile	Not visible in the structure	
273 Arg	1.0	Fully	223 Lys	0.58	Fully
274 Asp	1.0	Fully	224 His	1.0	Fully
275 Glu	0.57	Fully	225 Thr	1.0	Fully
276 Glu	1.0	Fully	226 Glu	1.0	Fully
277 Tyr	1.0	Fully	227 Tyr	1.0	Fully
278 Arg	-0.15	Non-exchanged	228 Met	1.0	Fully
279 Asn	1.0	Fully	229 Glu	1.0	Fully
280 Phe	0.45	Partially	230 Phe	1.0	Fully
281 Leu	-0.56	Non-exchanged	231 Leu	0.24	Partially
282 Arg	-0.34	Non-exchanged	232 Lys	0.41	Partially
283 Ser	0.23	Partially	233 Ser	1.0	Fully
284 Val	0.36	Partially	234 Val	-0.19	Non-exchanged
285 Ser	0.32	Partially	235 Pro		
286 Leu	0.57	Fully	236 Thr	0.85	Fully
287 Leu	0.86	Fully	237 Phe	0.0	Non-exchanged
288 Lys	0.53	Fully	238 Gln	0.51	Fully
289 Asn	0.37	Partially	239 Ser	1.0	Fully
290 Leu	0.49	Partially	240 Leu	0.83	Fully
291 Pro			241 Pro		

292 Glu	1.0	Fully	242 Glu	0.4	Partially
293 Asp	0.62	Fully	243 Glu	1.0	Fully
294 Lys	1.0	Fully	244 Ile	0.46	
295 Leu	-0.12	Non-exchanged	245 Leu	-0.55	Non-exchanged
296 Thr	0.65	Fully	246 Ser	-0.56	Non-exchanged
297 Lys	0.38	Partially	247 Lys	0.52	Fully
298 Ile	-0.56	Non-exchanged	248 Leu	-0.29	Non-exchanged
299 Ile	-0.56	Non-exchanged	249 Ala	-0.06	Non-exchanged
300 Asp	-0.24	Non-exchanged	250 Asp	1.0	Fully
301 Cys	-0.56	Non-exchanged	251 Val	0.67	Fully
302 Leu	0.3	Partially	252 Leu	1.0	Fully
303 Glu	-0.56	Non-exchanged	253 Glu	-0.49	Non-exchanged
304 Val	-0.56	Non-exchanged	254 Glu	0.83	Fully
305 Glu	-0.56	Non-exchanged	255 Thr	-0.56	Non-exchanged
306 Tyr	1.0	Fully	256 His	0.86	Fully
307 Tyr	-0.32	Non-exchanged	257 Tyr	-0.44	Non-exchanged
308 Asp	1.0	Fully	258 Glu	0.19	Partially
309 Lys	0.46	Partially	259 Asn	0.19	Partially
310 Gly	0.73	Fully	260 Gly	1.0	Fully
311 Asp	0.19	Partially	261 Glu	-0.56	Non-exchanged
312 Tyr	0.39	Partially	262 Tyr	0.40	Partially
313 Ile	-0.14	Non-exchanged	263 Ile	-0.27	Non-exchanged
314 Ile	-0.42	Non-exchanged	264 Ile	-0.16	Non-exchanged
315 Arg	0.84	Fully	265 Arg	1.0	Fully
316 Glu	-0.56	Non-exchanged	266 Gln	-0.56	Non-exchanged
317 Gly	0.11	Partially	267 Gly	1.0	Fully
318 Glu	0.59	Fully	268 Ala	0.93	Fully
319 Glu	0.82	Fully	269 Arg	0.41	Partially
320 Gly	0.95	Fully	270 Gly	0.74	Fully
321 Ser	1.0	Fully	271 Asp	1.0	Fully
322 Thr	0.21	Partially	272 Thr	1.0	Fully
323 Phe	-0.56	Non-exchanged	273 Phe	0.0	Non-exchanged
324 Phe	-0.11	Non-exchanged	274 Phe	-0.5	Non-exchanged
325 Ile	-0.56	Non-exchanged	275 Ile	-0.31	Non-exchanged
326 Leu	-0.16	Non-exchanged	276 Ile	-0.56	Non-exchanged
327 Ala	-0.43	Non-exchanged	277 Ser	-0.56	Non-exchanged
328 Lys	-0.56	Non-exchanged	278 Lys	0.19	Partially
329 Gly	0.94	Fully	279 Gly	1.0	Fully
330 Lys	0.87	Fully	280 Thr	0.41	Partially
331 Val	-0.54	Non-exchanged	281 Val	0.17	Partially

332 Lys	-0.56	Non-exchanged	282 Asn	-0.35	Non-exchanged
333 Val	-0.56	Non-exchanged	283 Val	-0.31	Non-exchanged
334 Thr	-0.56	Non-exchanged	284 Thr	-0.56	Non-exchanged
335 Gln	0.74	Fully	285 Arg	0.05	Partially
336 Ser	0.76	Fully	286 Glu	0.24	Partially
337 Thr	0.82	Fully	287 Asp	0.83	Fully
338 Glu	1.0	Fully	288 Ser	-0.39	Non-exchanged
339 Gly	0.27	Partially	289 Pro		
340 His	1.0	Fully	290	Not visible in the structure	
341 Asp	1.0	Fully	291	Not visible in the structure	
342 Gln	-0.41	Non-exchanged	292 Asp	0.56	Fully
343 Pro			293 Pro		
344 Gln	1.0	Fully	294 Val	0.77	Fully
345 leu	1.0	Fully	295 Phe	0.9	Fully
346 Ile	-0.56	Non-exchanged	296 Leu	0.94	Fully
347 Lys	-0.43	Non-exchanged	297 Arg	0.94	Fully
348 Thr	-0.14	Non-exchanged	298 Thr	0.78	Fully
349 Leu	-0.56	Non-exchanged	299 Leu	0.49	Partially
350 Gln	1.0	Fully	300 Gly	0.18	Partially
351 Lys	1.0	Fully	301 Lys	0.18	Partially
352 Gly	1.0	Fully	302 Gly	1.0	Fully
353 Glu	-0.56	Non-exchanged	303 Asp	-0.10	Non-exchanged
354 Tyr	-0.56	Non-exchanged	304 Trp	0.47	Partially
355 Phe	-0.26	Non-exchanged	305 Phe	-0.56	Non-exchanged
356 Gly	-0.56	Non-exchanged	306 Gly	-0.56	Non-exchanged
357 Glu	0.73	Fully	307 Glu	-0.56	Non-exchanged
358 Lys	1.0	Fully	308 Lys	0.0	Non-exchanged
359 Ala	-0.34	Non-exchanged	309 Ala	-0.56	Non-exchanged
360 Leu	-0.16	Non-exchanged	310 Leu	-0.56	Non-exchanged
361 Ile	-0.21	Non-exchanged	311 Gln	0.79	Fully
362 Ser	0.49	Partially	312 Gly	0.7	Fully
363 Asp	0.65	Fully	313 Glu	1.0	Fully
364 Asp	1.0	Fully	314 Asp	0.67	Fully
365 Val	0.75	Fully	315 Val	0.13	Partially
366 Arg	-0.49	Non-exchanged	316 Arg	-0.50	Non-exchanged
367 Ser	-0.01	Non-exchanged	317 Thr	0.23	Partially
368 Ala	-0.24	Non-exchanged	318 Ala	-0.44	Non-exchanged
369 Asn	-0.56	Non-exchanged	319 Asn	0.07	Partially
370 Ile	-0.56	Non-exchanged	320 Val	-0.50	Non-exchanged
371 Ile	-0.56	Non-exchanged	321 Ile	-0.21	Non-exchanged

372 Ala	0.18	Partially	322 Ala	-0.56	Non-exchanged
373 Glu	1.0	Fully	323 Ala	1.0	Fully
374 Glu	0.11	Partially	324 Glu	1.0	Fully
375 Asn	0.83	Fully	325 Ala	0.38	Partially
376 Asp	0.89	Fully			
377 Val	0.93	Fully	326 Val	0.16	Partially
378 Ala	-0.56	Non-exchanged	327 Thr	0.25	Partially
379 Cys	-0.56	Non-exchanged	328 Cys	-0.56	Non-exchanged
380 Leu	-0.29	Non-exchanged	329 Leu	0.0	Non-exchanged
381 Val	-0.56	Non-exchanged	330 Val	-0.56	Non-exchanged
382 Ile	-0.56	Non-exchanged	331 Ile	-0.56	Non-exchanged
383 Asp	0.45	Partially	332 Asp	1.0	Fully
384 Arg	0.69	Fully	333 Arg	0.69	Fully
385 Glu	-0.09	Non-exchanged	334 Asp	0.81	Fully
386 Thr	0.40	Partially	335 Ser	1.0	Fully
387 Phe	-0.56	Non-exchanged	336 Phe	-0.16	Non-exchanged
388 Asn	1.0	Fully	337 Lys	0.75	Fully
389 Gln	0.68	Fully	338 His	1.0	Fully
390 Thr	1.0	Fully	339 Leu	0.87	Fully
391 Val	0.64	Fully	340 Ile	1.0	Fully
392 Gly	0.6	Fully	341 Gly	0.44	Partially
393 Thr	-0.44	Non-exchanged	342 Gly	0.72	Fully
394 Phe	1.0	Fully	343 leu	1.0	Fully
395 Glu	1.0	Fully	344 Asp	1.0	Fully
396 Glu	1.0	Fully	345 Asp	1.0	Fully
397 Leu	0.66	Fully	346 Val	1.0	Fully
398 Gln	0.6	Fully	347 ser	1.0	Fully
			348 Asn	0.60	Fully
399 Lys	0.55	Fully	349 Lys	0.64	Fully
400 Tyr	0.42	Partially	350 Ala	-0.19	Non-exchanged
401 Leu	0.90	Fully	351 Tyr	0.65	Fully
402 Glu	0.90	Fully	Glu	Not visible in the structure	
403 Gly	1.0	Fully	Asp	Not visible in the structure	
404 Tyr	1.0	Fully	Ala	Not visible in the structure	
405 Val	-0.56	Non-exchanged	Glu	Not visible in the structure	
406 Ala	0.54	Fully	Ala	Not visible in the structure	
407 Asn	1.0	Fully	Lys	Not visible in the structure	
408 Leu	-0.56	Non-exchanged	Ala	Not visible in the structure	
409 Asn	1.0	Fully	Lys	Not visible in the structure	
410 Arg	1.0	Fully	Tyr	Not visible in the structure	

411 Asp	1.0	Fully	Glu	Not visible in the structure
412 Asp	0.63	Fully	Ala	Not visible in the structure
413 Glu	0.50	Fully	Glu	Not visible in the structure
414 Lys	0.58	Fully	Ala	Not visible in the structure
415 Arg	1.0	Fully	Ala	Not visible in the structure
416 His	0.69	Fully	Phe	Not visible in the structure
417 Ala	1.0	Fully	Phe	Not visible in the structure
418 Lys	1.0	Fully	Ala	Not visible in the structure
			Asn	Not visible in the structure