Mechanism of allostERIC inhibition in the Plasmodium falciparum cGMP-dependent protein kinase

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Most malaria deaths are caused by the protozoan parasite Plasmodium falciparum. Its life cycle is regulated by a cGMP-dependent protein kinase (PKG), whose inhibition is a promising antimalarial strategy. Allosteric kinase inhibitors, such as cGMP analogs, offer enhanced selectivity relative to competitive kinase inhibitors. However, the mechanisms underlying allosteric PKG inhibition are incompletely understood. Here, we show that 8-NBD-cGMP is an effective PKG antagonist. Using comparative NMR analyses of a key regulatory domain, PfPKG-A, in its apo, cGMP-bound, and cGMP analog–bound states, we elucidated its inhibition mechanism of action. Using NMR chemical shift analyses, molecular dynamics simulations, and site-directed mutagenesis, we show that 8-NBD-cGMP inhibits PKG not simply by reverting a two-state active versus inactive equilibrium, but by sampling also a distinct inactive “mixed” intermediate. Surface plasmon resonance indicates that the ability to stabilize a mixed intermediate provides a means to effectively inhibit PKG, without losing affinity for the cGMP analog. Our proposed model may facilitate the rational design of PKG-selective inhibitors for improved management of malaria.

Malaria is a serious health risk for about 40% of the world’s population, with more than 200 million diagnoses and 400,000 deaths globally estimated in 2018 (1). Most malarial deaths are caused by Plasmodium falciparum with life cycles in both the human and the mosquito hosts (2, 3). The regulation of such cycles relies on cyclic nucleotide signaling pathways (4). Specifically, the P. falciparum guanosine-3’,5’-cyclic monophos-
**Results and discussion**

**The apo versus cGMP-bound comparative NMR analyses of PfD reveal pervasive allosteric perturbations**

As a first step to probe the allosteric effects of cGMP on the PfD construct (residues 401–542) used in our NMR studies, we acquired NH HSQC spectra of apo and cGMP-bound PfD (Fig. 2A). The cGMP-bound spectrum was assigned through triple-resonance NMR experiments and selective amino acid labeling. However, the apo construct was prone to precipitation during acquisition of 3D NMR data sets. Hence, the apo assignments were obtained by transferring the holo assignments to the apo spectrum through 2D Nε-exchange (Fig. 2, B and D) and 2D difference Nε-exchange experiments acquired in the presence of substoichiometric amounts of cGMP (Fig. 2, C and E). Based on these assignments, the cGMP-induced residue-specific compounded 13N–1H chemical shift changes (ΔCCS) were measured (Fig. 2F).

The majority of the residues exhibit significant ΔCCS values upon cGMP binding (Fig. 2F). As expected, significant ppm shifts are observed in the PBC and BBR regions, which directly interact with cGMP, as well as the β2–3 loop, which is adjacent to the PBC (Fig. 2F). Furthermore, major ppm changes are also detected for several α-helical regions, especially the αA, αB, and αC helices (Fig. 2F). The ppm shifts in αB–αC are consistent with the cGMP-dependent structural changes for these regions, as shown by the local root mean square deviation between the apo and cGMP-bound structures (Fig. 2G). The αB–αC structural changes also affect adjacent regions, such as the αA helix and the β8 strand (Fig. 2F), although such regions are not subject to major structural changes upon cGMP binding (Fig. 2G). Hence, the cGMP-dependent ΔCCS values (Fig. 2F) are in full agreement with the structures of apo- and holo-PfD, providing an initial validation of our NMR assignments. Our NMR assignments were further validated by the agreement between the measured and the computed Ca and CB chemical shift values based on the apo- and holo-PfD structures (Fig. 2, H and L). Once the NMR assignments were validated, we extended our analyses to evaluate the effects of cGMP analogs.

**cGMP analog versus cGMP chemical shift differences report on inhibitory conformational changes of PfD**

We focused on three cGMP base analogs with distinct base modifications: 8-NBD-cGMP, 8-pCPT-cGMP, and PET-cGMP (Fig. 1, I–K), as they act as PfPKG activators to different degrees. Whereas PET-cGMP acts as an agonist, as shown by kinase assays (Fig. 1L), 8-pCPT- and 8-NBD-cGMP elicit progressively increasing potential as antagonists. 8-NBD-cGMP is the most potent inhibitor of PfPKG (Fig. S1), followed by 8-pCPT-cGMP. This range of kinase responses (Fig. 1L) makes this group of cGMP analogs an excellent toolset to investigate the mechanisms underlying the allosteric inhibition of PfPKG. For this purpose, we first measured the cGMP analog versus cGMP differences in compounded chemical shifts (Fig. 3A). The overall distributions of such ppm differences (Fig. 3B) rank in full agreement with the inhibitory potencies of the cGMP analogs (Fig. 1L). Furthermore, the
pervasiveness of the effects caused by the cGMP modification (Fig. 3, A and C–E) also reflects the extent of inhibition. The perturbations resulting from the replacement of cGMP with either 8-NBD-cGMP or 8-pCPT-cGMP extend throughout the PfD structure, whereas the least inhibitory analog, PET-cGMP, perturbs only a relatively limited region near the cGMP-binding pocket (Fig. 3, A and C–E).

Overall, the data of Fig. 3 suggest that chemical shifts are ideally suited to investigate the dynamical allosteric transitions underlying PfPKG inhibition. This notion is fully consistent...
with the fast exchange between the inactive and the active states of CBDs (27–33), whereby the observed NMR peak positions are population-weighted averages of the pure inactive and active ppm values. Hence, chemical shifts report on the position of the dynamic inactive-active CBD conformational equilibrium. Chemical shift projection analysis (CHESPA) is an effective means to evaluate the perturbation of such dynamic equilibria caused by ligand modifications (28, 29, 31, 32). In the CHESPA analysis, the NMR peak positions of the cGMP analog–bound sample are evaluated relative to a reference vector connecting the peaks of the apo and cGMP-bound samples. The secondary structure of PfD is depicted at the top of the plot. The cGMP-binding regions BBR and PBC as well as the adjacent β2–3 loop are highlighted in gray background. G, local apo (PDB code 4OFF) versus cGMP-bound (PDB code 4OFG) root mean square deviation for PfD. The secondary structures obtained from the apo and cGMP-bound crystals are reported as red and black dashed lines, respectively. H, Cβ chemical shift values of cGMP-bound PfD obtained from NMR triple-resonance experiments versus the theoretical Cβ chemical shift values predicted from the structure (PDB code 4OFG) using the ShiftX software (54). I, similar to H but for the Ca chemical shifts.

**CHESPA of the cGMP analog–bound PfDs reveals the presence of a third “mixed” conformer with an engaged pre-lid but a disengaged lid**

The CHESPA of 8-NBD-cGMP shows that the majority of residues exhibit negative X values (Fig. 4B), suggestive of most residues shifting toward the inactive state upon binding of 8-NBD-cGMP. However, the magnitudes of the X values are highly residue-dependent, pointing to deviations from the pure inactive-active two-state equilibrium, which predicts similar X values for residues outside the binding site. Another characteristic of a two-state model is the co-linearity of the perturbation and reference vectors (i.e. |cos(θ)| ~ 1) for residues outside the binding site. However, the CHESPA of 8-NBD-cGMP reveals that several residues not in the binding site exhibit |cos(θ)| values significantly less than 1 (Fig. 4C), suggesting deviations from the two-state model and pointing to the presence of additional state(s) sampled by the PfD: 8-NBD-cGMP complex. Similar trends are observed for the 8-pCPT-cGMP–bound sample (Fig. 4E and F).
The presence of multiple conformational equilibria is further supported by the width of the X value distributions (Fig. 4G). The X distributions measured for 8-NBD-cGMP– and 8-pCPT-cGMP–bound PfD are significantly wider compared with PET-cGMP (Fig. 4G), which is nearly a full agonist and samples mainly the active state (Fig. S2). These observations suggest that the PfD:8-NBD-cGMP and PfD:8-pCPT-cGMP complexes sample a multistate ensemble, including not only the inactive and active conformers represented by the apo and cGMP-bound crystal structures, but also an additional state or states.

To simplify the analysis, we focused on the C-terminal helices that are directly linked to the catalytic domain and are one of the allosteric elements most critical in the inhibition and activation of the kinase (14, 21, 24). The C-terminal helices span two regions with clearly distinct average X values (⟨X⟩) in the PfD:8-NBD-cGMP complex: residues 515–530, denoted as the pre-lid with ⟨X⟩ = −0.48, and residues 534–535, denoted as the lid with ⟨X⟩ = −0.88 (Fig. 4, B, H, and I). This means that for the PfD:8-NBD-cGMP complex, the pre-lid only shifts about halfway toward the inactive state, whereas the lid region shifts almost completely to the inactive state. A similar trend is observed for the PfD:8-pCPT-cGMP complex with average X values of −0.30 and −0.64 in the pre-lid and the lid regions, respectively.

One of the simplest models to explain both the enzymatic and the NMR data in Figs. 1L and 4, respectively, is a three-state equilibrium mechanism involving an inactive, an active, and an intermediate state (Fig. 5A). The inactive state is similar to the apo-PfD structure, where both the pre-lid and the lid are disengaged. The active state is similar to the cGMP-bound structure, where both the pre-lid and the lid are engaged. The intermediate state exhibits a mixed character, as the pre-lid is engaged similarly to the active state, but the lid is disengaged, similarly to the inactive state (Fig. 5A). Next, we checked whether it is possible to utilize this three-state model to back-calculate the relative kinase activities.

**The CHESPA-based three-state model of PfD inhibition explains the experimental kinase activities**

Using the average X values for the pre-lid and the lid regions (Fig. 4, B and E) and Fig. S2A), the populations of the inactive, intermediate, and active states were estimated. Because the pre-lid is disengaged only in the inactive state, the average X of the pre-lid reflects the population of the inactive state. Similarly, the lid is engaged only in the active state. Hence, the population of the active state is estimated as 1 − ⟨X⟩_{lid}. The population of the intermediate is the percentage not accounted for by the other two states. Using this approach, the populations of the inactive, intermediate, and active states for the PfD:8-NBD-cGMP complex are 48 ± 12, 40 ± 12, and 12 ± 1%, respectively (Fig. 5A and Table S1). Similarly, the three state populations can be calculated for the 8-pCPT-cGMP– and PET-cGMP–bound PfD complexes based on the respective CHESPA data (Fig. 4E, Fig. S2, and Table S1). Using these populations and assuming that the inactive and intermediate states are fully inhibitory,
given the lid disengagement (14), while the active state is non-inhibitory, the relative kinase activity at ligand saturation induced by these cGMP analogs was predicted. The predicted activation values correlate well with the experimental kinase activity values (Fig. 5B).

The ability of our three-state model to recapitulate the enzymatic data has three key implications. First, the measurements on the PfD construct are relevant for the longer PfPKG construct used in the kinase assays to probe the PfPKG function. Second, the NMR chemical shift projection analysis and the resulting fractional shift values are suitable for predicting the populations of different functional states. Third, the mixed intermediate state, where only the lid region is disengaged, is inhibitory to an extent similar to the inactive state, further corroborating the pivotal role of the lid region in activation. Hence, we further probed the inhibitory mixed intermediate stabilized by the 8-NBD-cGMP analog.

**8-NBD-cGMP binds to PfD in a syn conformation and perturbs interactions critical for activation**

As a first step toward investigating the mixed intermediate, we examined the base orientation of 8-NBD-cGMP as bound to PfD. Typically, the base of cyclic nucleotides is oriented either in *syn* or *anti* relative to the ribose ring (34–38). To determine whether the guanine base of 8-NBD-cGMP is *syn* or *anti*, we could not rely on NOE-based assessments, as this cGMP analog lacks nonexchangeable protons in the base. Hence, we focused on the chemical shifts of the Cγ2 methyl of Ile465, which in the PfD:cGMP complex is in direct contact with the guanine base, and of the Cγ methyl of Thr493, whose hydroxyl interacts with the amino group of cGMP (Fig. 1C). The chemical shifts of both Ile465 Cγ2 and Thr493 Cγ show only marginal changes when cGMP is replaced by 8-NBD-cGMP (Fig. 6A and B), suggesting that the 8-NBD-cGMP guanine base binds in a *syn* conformation, similar to cGMP. This conclusion was confirmed also by...
the 3D map of 8-NBD-cGMP versus cGMP chemical shift changes occurring at or near Tyr^{480} (Fig. 3A). The Tyr^{480} NH HSQC cross-peak of the cGMP:PfD complex moves completely toward the apo position when cGMP is replaced by 8-NBD-cGMP (Fig. 6F), confirming that the 8-NBD moiety perturbs the Tyr^{480}–Arg^{528} interaction. To further corroborate this result, we generated the R528K mutant and compared the R528K versus WT chemical shifts for both cGMP– and 8-NBD-cGMP–bound samples (Fig. 6I). For the cGMP:PfD complex, the largest R528K versus WT ΔCCS values cluster primarily close to Tyr^{480} (Fig. 6I), as expected based on the Arg^{528}–Tyr^{480} interaction observed in the cGMP:PfD structure (14). However, when cGMP is replaced by 8-NBD-cGMP, the R528K versus WT CCS changes in the vicinity of Tyr^{480} are markedly suppressed (Fig. 6I), confirming that in the 8-NBD-cGMP:PfD complex, the Arg^{528}–Tyr^{480} interaction is significantly weakened compared with the cGMP:PfD structure.

Similar analyses were carried out to probe the effect of 8-NBD-cGMP on the capping triad interactions between Arg^{484}, Gln^{532}, and Asp^{533}, which are also critical for activation (14). We found that the Arg^{484}–Asp^{533} interaction is perturbed in the simulated intermediate structure of the 8-NBD-cGMP: PfD complex (Fig. S3A). To confirm such perturbation, we measured the CCS changes between WT and the R484A mutant for
both the cGMP– and 8-NBD-cGMP–bound samples (Fig. S3B). Fig. S3B reveals that the R484A versus WT CCS changes in the C-terminal helices, especially in the vicinity of Gln532–Asp533, are suppressed when cGMP is replaced with 8-NBD-cGMP, confirming that 8-NBD-cGMP perturbs the capping interaction between Arg484 and the C-terminal helices. These results are in full agreement with the MD simulations, suggesting that the 8-NBD substituent destabilizes also the capping triad.

Hence, we conclude that upon introduction of the NBD group at the 8-guanine position, the syn base orientation is preserved but the Tyr480–Arg528 contact and the capping triad interactions are significantly perturbed, thus compromising the full engagement of the C-helix and explaining why 8-NBD-cGMP functions as an effective allosteric antagonist of the PfPKG kinase.

Sampling a mixed intermediate state is beneficial for maximizing kinase inhibition while minimizing affinity losses

The three-state model with the mixed intermediate state featuring an engaged pre-lid and a disengaged lid (Fig. 5A) pro-
vides an excellent strategy for PfPKG kinase inhibition without significantly compromising affinity. Reconciling high inhibitory efficacy and potency would be more challenging for a simple two-state model. In this case, the disengagement of the lid necessary for inhibition also implies the concurrent disengagement of other PFD elements necessary for high-affinity binding, such as the PBC in its entirety. On the contrary, in the mixed intermediate state (Fig. 5A), which exhibits an inhibitory effect similar to that of the inactive state, it maintains the pre-lid and the adjacent PBC engaged. For example, whereas the capping carboxylate of Asp533 is displaced away from Arg484 in the PBC, the side chain of this arginine in the mixed intermediate remains in an orientation similar to the active conformation (Fig. S3A), thus minimizing perturbations of the interactions with the cyclic nucleotide. Hence, based on the three-state model of Fig. 5A, we hypothesize that the 8-NBD-cGMP preserves an affinity comparable with the endogenous allosteric effector, cGMP.

Our hypothesis is confirmed through surface plasmon resonance (SPR) measurements (Fig. 7) showing that the affinities of 8-NBD-cGMP and cGMP are comparable (i.e. \( K_d = 59 \pm 4 \text{ nM} \text{ versus } 51 \pm 7 \text{ nM}; \text{Table S3} \)), despite their markedly different efficacies in terms of kinase activation (Fig. 1L and Fig. S1A). This conservation of affinity is especially important, because 8-NBD-cGMP acts as a competitive antagonist (Fig. S1B). Hence, 8-NBD-cGMP serves as an excellent example for how sampling a mixed intermediate state enables significant inhibition without major affinity losses by selectively disengaging only structural units strictly necessary for kinase activation. Similar inhibition strategies are anticipated to inform the design of future PfPKG allosteric inhibitors, which may serve as anti-malaria drug leads.

**Experimental procedures**

**Expression and purification**

The PfPKG CBD-D construct (residues 401–542) (i.e. PfD) within a His-tagged expression vector pQTEV (14) was expressed in the *Escherichia coli* strain BL21 (DE3). The transformed *E. coli* was grown in a minimal medium enriched with \(^{15}\)NH\(_4\)Cl and \(^{13}\)C glucose and supplemented with \(\alpha\)-biotin, thiamine HCl, MgSO\(_4\), and CaCl\(_2\). The cells were grown at 37 °C to an optical density of 0.7 (at \(\lambda = 600 \text{ nm} \)), induced with 0.5 mM isopropyl 1-thio-\(\beta\)-galactopyranoside, and then incubated for an additional 18 h at 18 °C. Cells were harvested, resuspended in the lysis buffer (50 mM Tris, pH 7.4, 500 mM NaCl, 1 mM BME), and lysed using a cell disruptor. The cell debris was removed through centrifugation, and then the supernatant was loaded onto an Ni\(^{2+}\)-Sepharose resin. The cell lysate was allowed to flow through, and the column was rinsed with 50 mM Tris, pH 7.4, 500 mM NaCl, 1 mM BME, and 20 mM imidazole. After thorough rinsing, the protein was eluted with 50 mM Tris, pH 7.4, 500 mM NaCl, 1 mM BME, and 300 mM imidazole. The eluted protein was then dialyzed in 50 mM Tris, pH 7.4, 100 mM NaCl, 1 mM BME with tobacco etch virus protease for 24 h at 4 °C. The cleaved products were loaded on the Ni\(^{2+}\)-Sepharose resin. The protein without the His-tag was obtained through collecting the flow-through and was further purified with a HiLoad 16/60 Superdex 75 gel filtration column (GE Healthcare) equilibrated with NMR buffer (50 mM Tris, pH 7, 100 mM NaCl, 10 mM MgCl\(_2\), 1 mM DTT). The His-tagged PfPKG 401–853 construct including the catalytic domain was expressed in *E. coli* TP2000 and purified using Ni-NTA and size-exclusion chromatography, as described previously (21).

**NMR spectroscopy**

NMR experiments were performed on a Bruker Avance 700-MHz spectrometer equipped with a 5-mm TCI cryoprobe or a Bruker Avance 850-MHz spectrometer equipped with a triple-resonance TXI probe. All experiments were acquired at 306 K in NMR buffer (50 mM Tris, pH 7, 100 mM NaCl, 10 mM MgCl\(_2\), 1 mM DTT) with 5% D\(_2\)O. All NMR spectra were processed with NMRPipe (42) and analyzed using Sparky (43). Three-dimensional triple-resonance NMR experiments (i.e. HNCA CB(\((\text{CO})\)_NH, HN(CO)CA, HNCO, HCC(\((\text{CO})\)_NH) and \((\text{H})\text{CC(\((\text{CO})\)_NH) were acquired and used to assign the two-dimensional \(^{1}H\text{-}^{15}N\text{ HSQC and }^{1}H\text{-}^{13}C\text{ HSQC NMR spectra of cGMP-bound PfD. Because the apo sample was prone to precipitation, 2D }^{15}N\text{-exchange, 2D difference }^{15}N\text{-exchange, and 2D }^{13}C\text{-exchange experiments with mixing time of 250 ms were used to transfer the chemical shift assignments from the cGMP-bound PfD to the apo-PfD spectra. The cGMP analog-bound spectra were assigned through spectral comparison with**
cGMP-bound spectra, if no ambiguities were present, and through 2D N\textsubscript{2} exchange and 2D difference N\textsubscript{2} exchange with the apo state. Chemical shift assignments of R528K-cGMP and R528K-8-NBD-cGMP were obtained through spectral comparison with WT spectra if no ambiguities were present.

**Chemical shift analyses**

All spectra utilized for chemical shift analyses were acquired with 100 \( \mu \)M PfD and saturating amounts of cGMP or cGMP analogs (2 mM) or without any cNMP addition for apo spectra. For accurate chemical shift comparisons, an internal reference (i.e. 15\textsuperscript{N}-labeled N-acetylglycine) was used to align the spectra in Sparky, and the resulting chemical shift values were used to compute the compounded chemical shift differences and perform CHESPA following protocols described previously (29, 30, 34, 44, 45). The fractional shift \((X)\) values and \(\cos(\theta)\) values were computed for residues with cGMP analog–bound versus cGMP-bound CCS difference \(>0.05\) ppm. The average \((X)\) values for the pre-lid and the lid were calculated using residues that were assigned in all five samples (i.e. apo, cGMP-bound, 8-NBD-cGMP-bound, 8-pCPT-cGMP-bound, and PET-cGMP-bound PfD), had \(X\) values between 0 and \(-1\), and had \(\Delta\)CCS (cGMP analog–bound versus cGMP-bound) values greater than \(0.05\) ppm, ensuring reliable \(X\) and \(\cos(\theta)\) values. The distribution of \(X\) values for cGMP analogs was computed using the residues that exhibited \(\cos(\theta) > 0.75\) to minimize biases from nearest-neighbor effects.

**Site-directed mutagenesis**

The R528K mutation was created via site-directed mutagenesis using a KOD Hot Start Master mix (Novagen) and primer pairs (synthesized by IDT): forward, CTTGGCCACTTGGAAGAGAGATATGTCAGGATAC; reverse, GTATTGCGATGCTGAGCAG. The R528K mutation was created through site-directed mutagenesis using a KOD Hot Start Master mix (Novagen) and Site-directed mutagenesis versus cGMP analogs was computed for residues with cGMP analog–bound versus cGMP-bound CCS difference \(>0.05\) ppm. The average \((X)\) values for the pre-lid and the lid were calculated using residues that were assigned in all five samples (i.e. apo, cGMP-bound, 8-NBD-cGMP-bound, 8-pCPT-cGMP-bound, and PET-cGMP-bound PfD), had \(X\) values between 0 and \(-1\), and had \(\Delta\)CCS (cGMP analog–bound versus cGMP-bound) values greater than \(0.05\) ppm, ensuring reliable \(X\) and \(\cos(\theta)\) values. The distribution of \(X\) values for cGMP analogs was computed using the residues that exhibited \(\cos(\theta) > 0.75\) to minimize biases from nearest-neighbor effects.

**Kinase assays**

Specific kinase activities were determined using an enzyme-coupled spectrophotometric assay (46). In a quartz cuvette, purified PfPKG 401–853 was mixed with the reaction buffer (100 mM MOPS, pH 7.0, 10 mM MgCl\textsubscript{2}, 5 mM \( \beta \)-mercaptoethanol, 1 mM ATP, 1 mM phosphoenolpyruvate, 230 \( \mu \)M NADH, 15 units/ml lactate dehydrogenase, 8.4 units/ml pyruvate kinase). The reactions were started by adding 1 mM PKStide (GRTGRNSSL; GeneCust, Luxembourg) and monitored in the absence and presence of a 100 \( \mu \)M concentration of each cyclic nucleotide (cGMP, 8-pCPT-cGMP, and PET-cGMP) for at least 1 min. Specific kinase activity was calculated according to the Lambert–Beer law.

Kinase activity induced by 8-NBD-cGMP was measured by microfluidic mobility shift assay using a Caliper Desktop Profiler (PerkinElmer Life Sciences). Here, 100 \( \mu \)M cGMP or 8-NBD-cGMP was mixed with assay buffer (20 mM MOPS (pH 7), 300 mM NaCl, 10 mM MgCl\textsubscript{2}, 1 mM ATP, 1 mM DTT, 990 \( \mu \)M PKStide, 10 \( \mu \)M FITC–PKStide (FITC–GRTGRNSSL; GeneCust, Luxembourg, 0.05% L-31) and a fixed protein concentration and continued as described previously (21). The assay was also conducted with multiple concentrations of 8-NBD-cGMP or cGMP, ranging from 0.1 nM to 300 \( \mu \)M. For the competition assay, the protein was preincubated with 350 nM cGMP, varying concentrations of 8-NBD-cGMP were added to the reaction, and substrate conversion was monitored over 2 h. The combined kinase inhibition data were further analyzed and normalized using GraphPad Prism 8.3.0 (GraphPad Software).

**SPR**

Direct binding data were obtained via SPR using a Biacore T200 (GE Healthcare Life Sciences). Briefly, 200–300 RU of Ni\textsuperscript{2+} (5 mM NiCl\textsubscript{2} in running buffer) were immobilized on a poly-NTA–derivatized sensor chip (NIHC 1000M; XanTec Bioanalytics GmbH). Subsequently, His-tagged PfPKG 401–853 in running buffer (20 mM HEPES, 150 mM NaCl, 50 \( \mu \)M EDTA, 0.01% P20, pH 7.3) was captured at a flow rate of 10 \( \mu \)l/min to an immobilization level of 500–2500 RU. Next, association was measured by injection of a dilution series of the respective cyclic nucleotide for 45–75 s. Then running buffer was injected for 60–180 s to initiate dissociation. Regeneration of the sensor chip surface was achieved by consecutively injecting 0.5 M EDTA (pH 8) for 600 s, followed by injections of 3 M guanidinium HCl, 0.5% SDS, and 3 M guanidinium HCl for 240 s each. Unless stated otherwise, all kinetic measurements were performed at 25 °C in running buffer and a flow rate of 50 \( \mu \)l/min. Data were evaluated with the Biacore T200 Evaluation Software 3.0 using a global fit and assuming a 1:1 Langmuir binding model. Further data processing was performed using GraphPad Prism 8.3.0 (GraphPad Software).

**Molecular dynamic simulations: initial model preparation**

The structure of 8-NBD-cGMP in a syn conformation was generated using RDKit (47). A model of the mixed intermediate state sampled by the PfD:8-NBD-cGMP complex was then built using residues 401–533 from the active apo-PfD crystal structure (PDB code 4OFG) (14) and lid region residues 534–542 from the inactive apo-PfD crystal structure (PDB code 5DYK) (24). The 8-NBD-cGMP ligand was docked into the resulting mixed structure of PfD using AutoDock4 with default parameters generated by AutoDockTools (48). Residues 463–494, where the PBC and BBR regions are located, were assigned as the binding pocket for AutoDock. The structure in which the ribose-phosphate and guanine moieties of the docked ligand were best aligned with the position of cGMP in the cGMP-bound active structure (PDB code 4OFG) (14) was selected as the initial coordinates for MD simulations.

**Molecular dynamics simulation protocol**

All simulations were performed using the AMBER 16 software PMEMD.CUDA (49) on the Shared Hierarchical Aca-
demic Research Computing Network (SHARCNET). The Amber ff99SBnmr force field was employed for the protein, whereas RESP charge and the general Amber force field were applied to generate the parameters for 8-NBD-cGMP using the Gaussian09 program (50) and the antechamber module implemented in AMBER 16, as described previously (51). The TIP3P water model was applied in the form of a rectangular MD solvent box surrounding the PfD8-NBD-cGMP structure, with a minimum distance of 12 Å between the PfD8-NBD-cGMP structure and the box edge. To mimic the experimental conditions, the charged residues and N/C termini were set to the protonation states expected at pH 7, following a protocol similar to that implemented previously (52), and Na⁺ and Cl⁻ ions were added to mimic a 100 mM salt concentration.

The simulations started with an energy minimization performed with harmonic restraints on the protein backbone (with a force constant of 5 kcal/mol·Å²). The system was then gradually heated from 0 to 100 K at constant volume and then heated from 100 to 306 K in the NPT ensemble, and all of the heating process employed harmonic restraints on protein backbone (with a force constant of 3 kcal/mol·Å²) for a total of 1 ns followed by an equilibration period of 1.2 ns with main-chain termini restrained with a force constant of 1 kcal/mol·Å². Then a 1-µs production MD trajectory was generated in the NPT ensemble at 306 K and 1 atm with a weak-coupling algorithm, saving structures every 10 ps for subsequent analysis. A cluster analysis for 20,000 frames recorded from the MD trajectory (i.e., the structures recorded at 50-ps intervals) was performed, with β-core alignment, using the CPPTRAJ protocol (53).

Data availability

The NMR chemical shift assignment for cGMP-bound PfD has been deposited in the Biological Magnetic Resonance Data Bank (BMRB) with the code 50203. All remaining data are contained within the article.

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EDITORS’ PICK: Mechanism of PKPKG allosteric inhibition