The malaria parasite *Plasmodium falciparum* encodes a cGMP-dependent protein kinase G (*PfPKG*) that is critical for its life cycle. Specific cGMP analogs are able to act as partial agonists of *PfPKG*. Using the exquisite diagnostic power of NMR chemical shifts, Byun *et al.* demonstrate that the extent of agonism by these cGMP derivatives relates to the degree of stabilization of a unique inactive conformation that shares structural features with both the ligand-free, inactive and the cGMP-bound, active states. The observation of this third state helps to generalize a novel paradigm for the allosteric activation of kinase function and may open opportunities for the development of novel therapeutics.

The protozoan *Plasmodium falciparum*, the principal causative agent for malaria-related deaths, encodes more than 5 dozen proteins with sequence signatures characteristic of eukaryotic protein kinases (1). These include a cyclic guanosine 3′,5′-monophosphate (cGMP)-dependent protein kinase G (*PfPKG*) that has been shown to regulate multiple stages of the parasite’s life cycle (2). As a result, *PfPKG* has been explored as a target for the development of antimalarials; the promise shown by a series of imidazopyridine inhibitors validates that this is a functionally relevant strategy (3). *PfPKG* is a multidomain enzyme that, like its mammalian counterparts, is allosterically activated by cGMP. Targeting this allosteric site could enable the discovery of inhibitors with improved selectivity, although a rational exploitation of this strategy requires a more complete understanding of the mechanism of activation by cGMP and its derivatives. A new study from Byun *et al.* (4) delves into the details of this mechanistic question. Their studies reveal an unexpected inactive conformation that has broad implications for the allosteric activation of *PfPKG*.

The structural organization of *PfPKG* is more elaborate than in mammalian PKGs, in that it encodes four, rather than two, cyclic nucleotide–binding domains (CBD-A through D) attached to the N-terminus of a catalytic kinase domain (KD). CBD-D plays a central role in the cGMP-induced activation of the enzyme, making numerous contacts with the KD in stabilizing the auto-inhibited inactive state (5). A key conformational change in CBD-D, related to activation, is induced by cGMP binding and involves the displacement and rotation of a C-terminal helix (αC). This conformational change results in a key αC residue, Arg268, swapping partners, from forming a salt bridge with Asp597 (on the N-lobe of the KD) in the auto-inhibited state (5) to participating in an intra-domain hydrogen bond with Tyr480 in the cGMP-bound state (6). The cGMP-bound state of CBD-D is further stabilized by a unique “capping triad” that deviates from its human equivalent (6) and involves the αC residues, Gln532 and Asp533, that stack against the guanine moiety of cGMP, forming intradomain hydrogen bonds with Arg584 on the so-called phosphate-binding cassette. These various CBD-D residues may be considered diagnostic of the activation state of *PfPKG*. Byun *et al.* (4) exploit these features to great effect in a series of sophisticated NMR experiments on the isolated CBD-D, providing convincing evidence for the role of a hidden inactive state (or states), induced by cGMP analogs, that has a profound influence on enzymatic activity.

Byun *et al.* (4) analyzed the effects of the cGMP analogs, 8-NBD-cGMP (C8 of the imidazole ring modified by a bulky 2-[7-nitro-4-benzofurazanyl]aminooethylthio moiety) and 8-pCPT-cGMP (C8 modified by a less bulky 4-chlorophenylthio moiety) on the catalytic activity of a stripped down construct of *PfPKG* comprising CBD-D and the KD. They found that the degree of activation of this minimal enzyme, relative to that induced by cGMP, depended on the size of the C8 modification, with the bulkier 8-NBD modification leading to reduced activation. To probe the origins of this effect, the authors analyzed the chemical shift perturbations induced in CBD-D by these analogs relative to the absence (apo) or the presence of the natural activator, cGMP (holo). Making a reasonable assumption that the chemical shifts of the apo and holo states were indicative of the inactive (I) and active (A) conformations, respectively (Fig. 1), the authors performed a procedure termed chemical shift projection analysis (CHESPA) (7) using correlation spectra of CBD-D in the presence of the analogs. CHESPA provides a measure of the angle between two sets of two-dimensional vectors, one connecting the peak positions of corresponding residues in the spectra of the apo and holo states (reference) and a second that connects the peak in the holo spectrum to that in the presence of, in this case, a modified cGMP analog (perturbation). When an analog induces an active (or active-like) conformation, the angle between these two vectors is close to 180°. However, the authors found that several vectors corresponding to residues outside the binding site deviated from the expected antiparallel behavior, suggesting the presence of additional “hidden” states, represented by a single (third) conformation for simplicity. The authors then calculated the fractional shifts (i.e. the normalized projections of the perturbation vector onto the reference for each residue). These fractional shifts provide a measure of the relative population of active-like (close to 0 or positive) or inactive-like (negative) conformations represented by this third state. The fact that these projections were almost uniformly negative suggested that this third conformation (I3) was inactive-like. Further, the values of these projections in specific regions showed some very interesting trends.

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**Keep a lid on it: A troika in kinase allostery**

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of its allosteric activator, cGMP, comprises three discrete states. The resulting conformations of the cyclic nucleotide-binding domain (CBD-D) may be parsed into two inactive states (I₁, I₂) and an active state (A). I₁ may be represented by the unliganded (apo) form of the enzyme and is perhaps similar in overall geometry to that in auto-inhibited full-length PParkin, where the CBD-D is closely associated with the kinase domain (PDB entry 5DYK). A represents the cGMP-bound (holo) active form that is likely similar to the cGMP-bound form of the isolated CBD-D (PDB entry 4OFG). I₂ is a “hidden intermediate” state that contains features that are similar to A and to I₁. The major determinants of the differences between these states are linked to the C-terminal region of CBD-D and key residues therein (see text for details). The authors' NMR data indicate that A, I₁, and I₂ are populated to the extent of ~12, 48, and 40%, respectively, in the presence of the analog 8-NBD-cGMP, leading to a proportional activation of kinase activity compared with cGMP.

This was especially true for the so-called “pre-lid” and “lid” regions encompassing αC that populated inactive-like conformations to different extents in the presence of the two C8-modified cGMP analogs; the degree of kinase activation induced by these analogs could be reliably predicted from the NMR-determined populations of the A, I₁, and I₂ states. Finally, the authors used molecular dynamics simulations to probe the structural features characteristic of I₂, finding that the presence of a bulky substituent at the guanine C8 impairs formation of the key Tyr⁴⁸⁰→Arg⁵²⁸ hydrogen bond and results in nonideal interactions within the Arg⁴⁸⁴/Gin⁵³²/Asp⁵³³ capping triad. This results in the incomplete engagement of αC through intradomain contacts that characterize the A state. The authors concluded that the extent of agonism by a given cGMP analog depended on its ability to promote an I₂ state that is characterized by an engaged pre-lid but a disengaged lid; both regions are fully engaged in A and disengaged in I₁. Indeed, the influence of I₂ on the overall activity is somewhat reminiscent of the cAMP-induced partial agonism on PKG 1β (8). This partial agonism by related ligands through apparently similar mechanisms (i.e. through additional hidden conformations) could represent a general regulatory feature for this class of enzymes.

A question for the future is whether these observations can be exploited to design novel PParkin-centric antimalarial therapies. Targeting this bound-but-inactive state, I₂, discovered here by Byun et al. (4), could represent a viable strategy for allosteric inhibitor development. This possibility is buoyed by the observation that 8-NBD-cGMP engages PParkin with the same affinity as its natural activator, cGMP, suggesting that I₂ can indeed be stabilized without weakened binding. Nevertheless, additional fundamental work is needed toward achieving this goal, not least of which is to investigate whether the simple picture presented here, in terms of A, I₁, and I₂, is muddied in full-length PParkin by the presence of the other CBDs and additional regulatory elements (5).

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**Abbreviations**—The abbreviations used are cGMP, cyclic guanosine 3′,5′-monophosphate; PParkin, *P. falciparum* cGMP-dependent protein kinase G; KD, kinase domain; CBD, cyclic nucleotide-binding domain; 8-NBD-cGMP, 8-[7-nitro-4-benzofurazanyl]aminomethylthio) guanosine-3′,5′-cyclic monophosphate; 8-pCPT-cGMP, 8-(4-chlorophenylthio)adenosine-3′,5′-cyclic monophosphorothioate; CHESPA, chemical shift projection analysis; PDB, Protein Data Bank

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**Editors’ Pick Highlight:** Troika in kinase allostery

![Fig. 1. The simplest description of the conformational landscape of a stripped down PParkin construct in the presence of C8-modified analogs of its allosteric activator, cGMP, comprises three discrete states. The resulting conformations of the cyclic nucleotide-binding domain (CBD-D) may be parsed into two inactive states (I₁, I₂) and an active state (A). I₁ may be represented by the unliganded (apo) form of the enzyme and is perhaps similar in overall geometry to that in auto-inhibited full-length PParkin, where the CBD-D is closely associated with the kinase domain (PDB entry 5DYK). A represents the cGMP-bound (holo) active form that is likely similar to the cGMP-bound form of the isolated CBD-D (PDB entry 4OFG). I₂ is a “hidden intermediate” state that contains features that are similar to A and to I₁. The major determinants of the differences between these states are linked to the C-terminal region of CBD-D and key residues therein (see text for details). The authors' NMR data indicate that A, I₁, and I₂ are populated to the extent of ~12, 48, and 40%, respectively, in the presence of the analog 8-NBD-cGMP, leading to a proportional activation of kinase activity compared with cGMP.](image-url)