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Structures of the cGMP-dependent protein kinase in malaria parasites reveal a unique structural relay mechanism for activation

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The cyclic guanosine-3′,5′-monophosphate (cGMP)-dependent protein kinase (PKG) was identified >25 y ago; however, efforts to obtain a structure of the entire PKG enzyme or catalytic domain from any species have failed. In malaria parasites, cooperative activation of PKG triggers crucial developmental transitions throughout the complex life cycle. We have determined the cGMP-free crystallographic structures of PKG from Plasmodium falciparum and Plasmodium vivax, revealing how key structural components, including an N-terminal autoinhibitory segment (AIS), four predicted cyclic nucleotide-binding domains (CNBs), and a kinase domain (KD), are arranged when the enzyme is inactive. The four CNBs and the KD are in a pentagonal configuration, with the AIS docked in the substate site of the KD in a swapped-domain dimeric arrangement. We show that although the protein is predominantly a monomer (the dimer is unlikely to be representative of the physiologic form), the binding of the AIS is necessary to keep Plasmodium PKG inactive. A major feature is a helix serving the dual role of the N-terminal helix of the KD as well as the capping helix of the neighboring CNB. A network of connecting helices between neighboring CNBs contributes to maintaining the kinase in its inactive conformation. We propose a scheme in which cooperative binding of cGMP, beginning at the CNB closest to the KD, transmits conformational changes around the pentagonal molecule in a structural relay mechanism, enabling PKG to orchestrate rapid, highly regulated developmental switches in response to dynamic modulation of cGMP levels in the parasite.

cyclic GMP | signal transduction | malaria | Plasmodium | structure

Malaria remains a serious global health problem, with close to 500,000 deaths and hundreds of millions of new infections annually. Reports of prolonged parasite clearance times and treatment failures using artemisinin combination therapies (ACTs) are increasingly frequent in parts of Southeast Asia (1). New targets to supply the next generation of antimalarial drugs are being studied with urgency to tackle this growing trend, particularly in anticipation of the spread of ACT resistance to Africa. Among promising drug targets are protein kinases encoded by the genomes of Plasmodium parasites responsible for the disease (2). Previous work has demonstrated that one particular kinase, known as cyclic guanosine-3′,5′-monophosphate (cGMP)-dependent protein kinase, or protein kinase G (PKG), has essential roles in multiple stages of the parasite life cycle. Selective pharmacological inhibition of Plasmodium falciparum PKG (PfPKG) blocks the egress of merozoites (3) and gametocytes (4) from erythrocytes, as well as inhibits the motility of oocokites in the mosquito (4, 5) and invasion of hepatocytes by sporozoites (6). PKG orchestrates the progression of these key differentiation events in Plasmodium via a complex system of second messenger signaling, involving phosphoinositide metabolism and calcium mobilization (7, 8). A phosphoproteomic study has linked PfPKG activity to 107 phospho-sites on 69 different proteins in the P. falciparum proteome, including some implicated in invasion and egress (9). Regulation of cGMP has also been associated with calcium flux and egress in the related apicomplexan pathogens Toxoplasma and Eimeria (10). These lines of evidence support PKG as a promising target for antiparasitic drug discovery, as well as a gateway to a deeper understanding of parasite signaling.

The cellular functions of parasite PKG are regulated by allosteric and cooperative binding of cGMP (11), similarly to how mammalian PKG is activated. Allosteric and cooperativity are also the hallmarks of another eponymous member of the AGC protein kinase family, protein kinase C (PKC) (12). PKC isozymes are involved in a wide range of cellular processes, which have been exploited to develop diverse therapeutic strategies, including PKC inhibitors as cancer therapies (13, 14). PKG is also a member of the AGC protein kinase family, which includes PKC isozymes, and its structural and functional properties have been compared to those of PKC (11). Comparative analysis of PKG and PKC structures reveals a unique structural relay mechanism for activation, in which conformational changes are transmitted cooperatively through four cyclic nucleotide-binding domains, or CNBs (3). These structural features are also the hallmarks of another eponymous member of the AGC protein kinase family, protein kinase C (PKC) (12). PKC isozymes are involved in a wide range of cellular processes, which have been exploited to develop diverse therapeutic strategies, including PKC inhibitors as cancer therapies (13, 14). PKG is also a member of the AGC protein kinase family, which includes PKC isozymes, and its structural and functional properties have been compared to those of PKC (11). Comparative analysis of PKG and PKC structures reveals a unique structural relay mechanism for activation, in which conformational changes are transmitted cooperatively through four cyclic nucleotide-binding domains, or CNBs (3). These structural features are also the hallmarks of another eponymous member of the AGC protein kinase family, protein kinase C (PKC) (12). PKC isozymes are involved in a wide range of cellular processes, which have been exploited to develop diverse therapeutic strategies, including PKC inhibitors as cancer therapies (13, 14). PKG is also a member of the AGC protein kinase family, which includes PKC isozymes, and its structural and functional properties have been compared to those of PKC (11). Comparative analysis of PKG and PKC structures reveals a unique structural relay mechanism for activation, in which conformational changes are transmitted cooperatively through four cyclic nucleotide-binding domains, or CNBs (3). These structural features are also the hallmarks of another eponymous member of the AGC protein kinase family, protein kinase C (PKC) (12). PKC isozymes are involved in a wide range of cellular processes, which have been exploited to develop diverse therapeutic strategies, including PKC inhibitors as cancer therapies (13, 14).

Significance

Despite being one of the first protein kinases discovered, cyclic guanosine-3′,5′-monophosphate (cGMP)-dependent protein kinase (PKG) has not been successfully crystallized previously, leaving many unanswered questions about its mechanism of activation. We report herein the structure of cGMP-free PKG from Plasmodium parasites, the causative agents of malaria, one of the world’s deadliest infectious diseases. The structures, combined with data from biochemical and biophysical experiments, provide insight into a mechanism of activation that involves previously unpredicted interdomain communication via a structural relay system. In addition to the full structure of PKG, our work contributes important functional information for a key antimalarial drug target.


The authors declare no conflict of interest.

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Data deposition: The crystallography coordinates have been deposited in the National Center for Biotechnology Information’s Protein database, https://www.ncbi.nlm.nih.gov/protein [PDB ID codes SDY1 (PfPKG), SDYK ( PfPKG), and SDZC (PfPKG-AMPPNP)].

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PNAS Latest Articles | 1 of 10
subfamily of protein kinases, adenosine 3',5'-cyclic mono-
phosphate (cAMP)-dependent protein kinase (PKA). The reg-
ulatory and catalytic subunits of PKA are distinct, separately
encoded proteins that form tetrameric complexes composed of
two regulatory subunits and two catalytic subunits (R2C2) (12, 13).
In contrast, the regulatory and catalytic domains of PKG
cocexist within a single polypeptide encoded by a single gene. In
mammals, this assembly contains two cyclic nucleotide-binding
(CNB) sites, compared with the four CNB domains in apicom-
plexan PKG isoforms (14). Even though PKA and PKG were
both among the earliest kinases identified, understanding of how
the structural divergence of PKG from PKA affects cGMP-
mediated allostery and cooperativity (15) in different organ-
isms is at best fragmentary.

Structural biology is an essential tool in the study of protein
kinases. PKA, the first to be crystalized (16), is the archetypal
protein kinase in structural and mechanistic studies. There are
now a number of PKA structures revealing the standalone catalytic
and regulatory subunits, as well as the engaged R1C1 dimers and
R2C2 tetramers (12, 17, 18). In contrast, for both mammalian and
parasite PKG, only the structures of isolated regulatory domains
have been reported (19–26). A report disclosing the structure of
one of the cGMP-binding domains of PfPKG (CNB-D) identified
a triad of residues in its C-terminal helix that are essential for
regulation of enzyme activity (23). How this domain interacts
with the remainder of the PKG structure and, more generally, how
Plasmodium PKG mediates cGMP signaling are unknown.

Here, we describe the complete atomic structure of PKG from
two human malaria parasite species, P. falciparum and Plasmodi-
mum vivax. To date this has not been achieved for PKG from any
other organism. The resulting structures provide insights into
how the CNBs interact with the kinase domain (KD) to keep it in
a largely autoinhibited state and how the parasite maintains its
cGMP signaling system in the off state. Combining our structural
findings with biophysical and biochemical data allows us to
propose a structural relay model of cooperative activation of
parasite PKG that may also have important implications for
regulation of mammalian PKG.

Results

*Plasmodium PKG* Is a Member of a Distinct Class of PKG Enzymes.

Mammalian PKGs are classified into types Iα, Iβ, and II (also
known as PKG-Iα, -Iβ, and -II), all featuring a single polypeptide
comprising a regulatory domain of two CNBs fused to the N-
terminal flank of a single catalytic KD. Known to form inactive
dimeric holoenzymes when free of cGMP, they have an effective
regulatory stoichiometry (i.e., a CNB-to-KD ratio) of 4:2, similar
to that in a PKA R2C2 tetramer. In contrast, PKG enzymes of
Plasmodium and other apicomplexan parasites have an extended
N-terminal domain that features four CNBs (Fig. L4) upstream
of the KD and are monomeric (27), resulting in an effective
regulatory stoichiometry of 4:1. There is thus significant struc-
tural divergence between *Plasmodium* and mammalian PKGs,
prompting us to formally define kinases with more than two in-
tegrated cGMP-binding sites as type III or PKG-III.

A search of the National Center for Biotechnology Information
us to identify multiple subclasses of type III PKGs. In all apicom-
plexan PKGs examined, one of the four CNBs is similar in sequence
to canonical cGMP-binding domains but lacks one or more critical
residues (23). We refer to such a domain as a pseudo-CNB (pCNB)
and the corresponding PKG as type IIIα. There are organisms,
including green algae such as *Ostreococcus*, in which PKG contains
four CNM-binding CNBs—that we call type II PKG-IIIβ. Finally,
type IIIy PKGs, such as those found in *Paramaecium* and *Tetrahymena*
(both of which have multiple paralogues of PKG), contain three
predicted CNBs. Type III PKGs are observed only in protist
genomes. In contrast, animals possess only types I and II PKGs.
The architectures of these subtypes, along with those for types I and II as
well as PKA, are shown for comparison in Fig. L4.

*Plasmodium PKG* Adopts a Pentagon-Shaped Architecture.

We expressed and purified full-length recombinant *Pf* PKG and
*Pv* PKG (*Pf*PKG). They crystallized as dimers in orthorhobi-
cic and monoclinic space groups respectively, and each yielded
2.4-Å apo structures [Fig. 2A and SI Appendix, Fig. S1 and Table
S1; Protein Data Bank (PDB) ID codes: 5DYK and 5DYL].
The two orthologs are 92% identical in sequence; furthermore,
alignment of their structures resulted in rmsds of 0.8 Å—
representing negligible differences (SI Appendix, Fig. S1). P-PKG
also cocrystallized with adenylyl-imidodiphosphate (AMPNNP),
a nonhydrolyzable analog of adenosine triphosphate (ATP),
again as a dimer (PDB ID code 5DZC, 2.3 Å; SI Appendix, Table
S1). In the ensuing description, unless stated otherwise, impor-
tant details are common to all of the structures. To ensure clarity,
important differences in residues are mentioned in the
text and highlighted in an alignment of the sequences of the two
orthologs (SI Appendix, Fig. S2).

In the *Plasmodium* PKG crystal structures, each protomer in
the dimers can be described as four CNBs, which, together with the N
lobe of the KD, form a pentagonal arrangement (Fig. 1 B and C),
with the KD C lobe locked in the center. CNB-A and -B make
direct contact with and constrain (and are constrained by) the C
lobe of the KD from functionally essential movement (Fig. 1 B
and C), resulting in a substructure of these three domains that
mirrors a common feature of the mammalian kinase dimer (Fig. 1 D).
Interestingly, all three *Plasmodium* structures display a swapped
domain arrangement, in which the N terminus of each protomer in
the dimer interacts with the active site of the other (Fig. 2A).

The N-Terminal Segment of *Plasmodium PKG* Has an Autoinhibitory
Role. To study the significance of occupation of the *Plasmodium*
PKG active site by the N-terminal residues, we generated a trunc-
cated form of *Pf*PKG which starts at position S30, and so lacks the
swapped N-terminal motif (Fig. 2A). Kinase activity assays (Fig.
2B) showed that the truncated recombinant enzyme (*Pf*PKG
ΔAIS) had equivalent activity to the full-length protein in the pres-
ence of cGMP; however, only the truncated form demonstrated
activity in the absence of cGMP (Fig. 2B). This suggested an
inhibitory role for the N-terminal segment of *Plasmodium* PKG
that is likely similar to that performed by an equivalent motif in
PKA (18) and human PKG (28). Accordingly, we validated that the
trans-binding of the N-terminal motif does not contribute to in-
hibition of the kinase (Fig. 2D), suggesting an intrametric mode of
action as demonstrated by preincubation of the catalytically active
natively truncated recombinant form of *Pf*PKG (PKGΔAIS) with an
inactive full-length *Pf*PKG–E589A enzyme (ATP-binding-site
mutant). Furthermore, the activity curve of *Pf*PKG–ΔAIS is sig-
moidal, with a Hill coefficient of 1.9 (Fig. 2C), indicative of the
same homotropic positive cooperativity reported for full-length
PKG in *Plasmodium* and other type IIIα PKGs.

The dimeric crystal structure was unexpected and required in-
vestigation of its relevance, which was performed by using multi-
angle laser light scattering (full-length *Pf*PKG and *Pv*PKG; SI
Appendix, Fig. S3), analytical ultracentrifugation (full-length *Pf*PKG
and *Pv*PKG; full-length *Pf*PKG with AMPPNP; and *Pv*PKG–ΔAIS;
SI Appendix, Fig. S4), and immunoblot analysis of epitope-tagged
native *Pf*PKG from parasite lysates (SI Appendix, Fig. S5). In all
cases, the dimeric fraction ranged from very little (e.g., the strongest
dissociation constant was found to be ~32 μM in area-under-the-
curve experiments, indicative of a very small dimer fraction) to
undetectable. Collectively, the activity assays and biophysical char-
acterizations indicate that (i) cGMP is required for full activation
of *Pf*PKG; (ii) the interaction between the N terminus of the protein
and the active site is required for complete autoinhibition (but not
for cooperativity), prompting us to name this region the auto-
inhibitory segment (AIS) for parasite PKG; and (iii) *Plasmodium*
PKG is predominantly monomeric [consistent with what has been
reported for the closely related *Eimeria* PKG (29)]—i.e., the
swapped-domain dimer is unlikely to be representative of the
physiological form of the inactive or active protein. To date, our
attempts to crystallize the AIS-truncated form of *Plasmodium* PKG,

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**2 of 10**

El Bakkouri et al.
the monomeric state of the full-length (or truncated) protein, or its cGMP-bound configuration have been unsuccessful.

**Deviations of the KD of Plasmodium PKG from the PKA Catalytic Subunit Have Functional Implications.** Similar to PKA, the KD of P/PKG adopts a classical bilobal structure flanked by an N-terminal helix (αK) and a C terminus (Fig. 3A). Despite being locked in an overall autoinhibited state, P/PKG–KD surprisingly exhibits many structural features of an active kinase, including: (i) interaction between the catalytic lysine K570 and E589 (on helix αC'; Fig. 3A); (ii) positioning of the helix αC toward the ATP-binding site (Fig. 3A); (iii) an open (active) conformation of the activation loop (Fig. 3A); and (iv) intact hydrophobic spines (Fig. 3B). We further note that, compared with fully closed KDs (e.g., PDB ID

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**Fig. 1.** *Plasmodium* PKG displays a pentagonal architecture. (A) PKA is made up of two proteins: The regulatory subunit (R) contains CNB-A and -B and the catalytic subunit (C) includes an N-terminal helix (pink), the classical bilobal KD (yellow and green), and a C terminus (brown). Mammalian PKG-I and -II feature a PKA-like catalytic KD concatenated with a tandem of CNBs. In type III PKG, there are three or more CNB or CNB-like domains. *Pf* PKG, an exemplar of type III, contains four integrated CNBs, one of which does not bind cGMP. In contrast, type IIIβ PKG has four functional cGMP-binding sites, while type IIIγ members have exactly three CNBs. An inhibitory substrate sequence flanks the N terminus of the first CNB in each case. In *Pf* and mammalian PKG, there is an additional region of dimerization and docking motifs. The absence of this in type III PKGs suggests that they are likely monomeric. (B) Surface rendering of *Pf*PKG colored as in A. PKG adopts a pentagonal architecture composed of the four CNB domains and the N lobe KD in the outer rim, and the C lobe KD locked in the middle. The CNB-A and -B domains make contact with the C lobe of the KD, similar to what is seen in all PKA heterodimers. CNB-D also makes contact with the C terminus (brown), whereas pCNB-C does not make direct contact with any part of the KD. CNB-D shows a unique arrangement not seen in PKA, making contacts with both the N lobe and the C terminus. (C) Structure of *Pf*PKG drawn in cartoon representation. The long helix connecting CNB-A and -B is similar to that seen in PKA. We hypothesize that, inside the parasite, the AIS would occupy the KD’s active site to form a fully autoinhibited monomer. (D) Overlay of *Pf*PKG and PKA heterodimer (PDB ID code 2QC5; in gray) depicts structural homologies. CNB-A, -B, and the KD form a substructure that closely resembles the heterodimer of PKA.
Fig. 2. Biochemical and biophysical assays. (A) The representation of the asymmetric unit from the PPKG crystal illustrates a swapped domain dimer arrangement in which the N terminus of each protomer interacts with the active site of the other. The pseudosubstrate (red and gray surfaces) from the dimeric partner is shown binding (and inhibiting) the substrate site between the N and C lobes. These interactions contribute to autoinhibition of the protein. (B) The AIS inhibits PKG activity in vitro. In the absence of cGMP, the full-length protein exhibits negligible activity, whereas the truncated mutant protein (AIS removed) was partially active. Both samples were equally active in the presence of cGMP. wt, wild type. (C) Both the full-length and AIS-truncated constructs demonstrate homotropic positive cooperativity (Hill coefficient is 1.7 for the full-length sample and 1.9 for the truncated sample) when activated by addition of cGMP. The AIS is not required for full kinase activity under cGMP activation. (D) The AIS functions intrastERICally to suppress kinase activity. The kinase activity of the catalytically active PPKG–ΔAIS truncation mutant (1 μM) is monitored in the absence of cGMP with incremental concentrations of full-length PPKG–E589A dead enzymes (1–8 μM). The AIS of the dead enzyme does not occupy/inhibit the active site of PPKG–ΔAIS. (E) Using the dimeric structure to hypothesize a model for the autoinhibitory domain of monomeric PPKG. The KD of one PPKG-a is shown in yellow and green surface, along with its adjacent CNB-A domain aligned with the CNB-A of all four types of PKA (PDB ID codes 3FHI, 2QVC, 4DIN, and 4X6Q; all drawn in gray). The AIS of PPKG-b (dark blue) overlays with the inhibitory segment of all four PKA structures (RIα in purple, RIIα in cyan, RIβ in pink, and RIIβ in yellow). In a monomer, we hypothesize that PPKG-a would extend its own AIS (colored in red) in the same position (instead of in the KD of PPKG-b). In all cases, a short helical overpass hovers above the long helix connecting the first two CNBs follows this inhibitory motif. The overpass of PPKG-b is shown here to indicate how PPKG-a might be different structurally as a monomer. It is also interesting that all of the structures are closely aligned from helix X:N onward, including Plasmodium PKG. This, along with all experimental data, supports the relevance of the rest of our crystal structure.
code 1ATP), the KD in all our structures can be described as partially open, with the AMPPNP-bound structure less open than the nucleotide-free versions.

All three of our Plasmodium PKG structures feature an activation loop that is both in its open conformation and unphosphorylated—an uncommon combination previously observed in rho kinases and rho-associated kinases (30) such as ROCK and MRCK. This is in contrast to PKA (and other members of the AGC subfamily and the majority of available S/T kinases crystallized with the activation loop in the open position), where T917 is phosphorylated (16). In recombinant PfPKG, we found that T695 (homologous to T917 in PKA) would become phosphorylated in the presence of cGMP, Mg²⁺, and ATP. The same modification has also been identified in a phosphoproteomics study (31). On the other hand, we found that the recombinant protein maintained the same activity profile (i.e., inactive without cGMP and fully active with cGMP) when T695 was mutated to either Ala or Gln (SI Appendix, Table S2). Rationalizing the significance of this putative phosphothreonine requires additional experiments.

The C terminus is a hallmark of AGC kinases (32) and an essential cis-regulatory component. It is partially disordered in our PfPKG structure, similar to apo PKA structures (e.g., PDB ID code 1ICTP). It is made up of four segments (Fig. 3C)—helix α, the C-lobe tether (CLT), the active-site tether (AST), and the N-lobe tether (NLT). In the CLT, the first proline in the PxxP motif in PKA (and PKC) known to play a role in interlobe movement—is conserved in PfPKG (Fig. 3C and D). In the AST, PfPKG uses a motif starting with tyrosine (Fig. 3C and D) to interact with the ATP-binding pocket, similar to the role of the FDDY motif in PKA, as well as in mammalian PKG (32). In the NLT, the hydrophobic motif (HF) features WxF in place of FxF (Fig. 3C), a minor deviation conserved in all types of PKG. In our structure, W849 is disordered, but F853 engages a hydrophobic pocket at the top of the N lobe (Fig. 3D), similar to what has been reported in

![Fig. 3. KD of PfPKG.](Image)
PKA (32). In addition, the C tail makes interactions with the N-terminal helix αA and water-mediated contacts with CNB-D (Fig. 3E), the former of which is described below.

The N-terminal helix, αA and PKG, is the only major structural element to deviate in position noticeably from its PKA counterpart (Fig. 3F). As shown in Fig. 3G, αA in PfPKG makes multiple contacts with the N and the C lobes, some of which involve R809 as well as the C terminus of PKG–KD, and none of which have been observed in PKA. The contact between the two termini features a salt bridge between R528 and D597 as well as a π- bond between H524 and R809. Substitution with alanine of H524, R528, or R809 (PKG–H524A, R528A, and R809A; SI Appendix, Table S2) resulted in polyepitides that were either unstable (i.e., prone to precipitate) or inactive, establishing the significance of these interactions.

The PfPKG and PfCNB structure is related to other adenylate kinase (AK) domains, with the C-terminal region forming a helix-loop-helix (HLH) motif, which is conserved in all AKs (33, 34). The N3A bundle (helices αN and αA, with a 3.6 loop in between) in the N terminus, an eight-stranded β-barrel, followed by a C-terminal hinge made up of helices αB and αC (Fig. 4A). In the middle of the β-barrel of CNB-A, -B, and -D is a 24-residue-long phosphate-binding cassette (PBC) featuring a conserved glutamate and arginine residue, as well as a short flexible helix, sometimes referred to as the B’ helix (35). Their interactions with cGMP were determined by isothermal titration calorimetry (ITC), revealing 14, 17, and 0.17 μM binding affinities for the CNB-A, -B, and -D when expressed as a standalone recombinant protein samples, respectively (Fig. 4B).

The third CNB, pCNB-C, stands out because a hydrophobic network consisting of Y363, H373, F374, and F359 occludes the cGMP-binding pocket. Accordingly, we were not able to detect any binding activity toward cGMP due to a degenerate binding site (Fig. 4B). Furthermore, D361 and P370 take the places of glutamine and arginine, respectively, which are universally conserved in determined cAMP- and cGMP-binding CNBs (35). Our crystal structures and binding affinities strongly support previous suggestions that only three of the four CNB domains in Plasmodium PKG bind cyclic nucleotides (36).

CNB-D stands out from the other regulatory domains in a different way: its C-terminal helices (αBD and αCD) are closer to their cGMP-capping positions than in the other CNB domains, as confirmed by comparison with existing structures of cGMP- and cAMP-binding CNBs (35). This domain also has the highest affinity for cGMP by two orders of magnitude, compared with CNB-A and -B (Fig. 4B), such that this regulatory unit is likely the first domain in PfPKG to become occupied as the cellular concentration of cGMP rises. This is consistent with a model proposed by Kim et al. (23).

Interdomain Contacts Provide Insight into Regulation of Plasmodium PKG by cGMP. To study the interaction between different domains in the Plasmodium PKG pentagon, we divided the structure into two overlapping halves. The left half consists of CNB-A, -B, and the KD. As shown in Fig. 1D, this trio of domains is arranged in a way that is highly similar to the PKAR–PKAC heterodimer. Close analysis reveals that some of PKA’s interdomain contacts, previously cataloged into four sites (12, 18), are also conserved in Plasmodium PKG, as described immediately below.

In site 1, the basic AIS in PfPKG is docked against the activation loop of the KD in a manner reported for PKA, with key lysines (K15 and K16) replacing homologous arginines (R94 and R95 in PKAR–R1a) in interacting with the P1+1 loop on the KD (SI Appendix, Fig. S7A). Sites 2 and 3 (SI Appendix, Fig. S7B) are the main interdomain contacts. In the active form, P1+1 helix (Fig. 4A), along with other contacts between CNB-D and the C terminus, the C-terminal helix (αC) of the first CNB and the starting helix of the second CNB (αD). In this arrangement, the helix αN makes contact with the preceding CNB, including interactions with the PBC. These connecting helices not only constrain neighboring domains, but are also likely the mediator of interdomain communication, propagating movement in one domain to the other. To further investigate the cGMP allosteric regulation transmitted along connecting helices, we attempted solving the crystal structures of other isolated CNB domains bound to cGMP, including CNB-A and -B. The PKG–A domain bound to cGMP yielded high-quality crystals diffraction to 1.65 Å resolution (PDB ID code 5E16) and illustrates conformational changes imposed by the repositioning of the helix αBD, to lock the cGMP in place within the binding pocket (Fig. 4E). This conformational change is accompanied by a larger displacement of the connecting helix αC (42° rotation) that is anticipated to redraw the neighboring interaction between CNB-A and KD in the full-length protein (Fig. 4F). Especially, we denote the probable rearrangement of key interactions upon cGMP activation, particularly the K157–Y694 π-bond interaction (site 2) between the connecting helix αC and the activation loop in the immediate vicinity of the kinase active site (Fig. 4 E and F and SI Appendix, Table S2). This clearly suggests a mechanism by which conformational modifications induced by binding of cGMP to one CNB could be relayed to adjacent domains.

Discussion

In mechanistic studies of protein kinases, obtaining their inactive and active structures are important landmarks representing two key states in a regulatory or signaling system. Using a combination of structural biology with biochemical and biophysical assays, our study has established that cGMP-free Plasmodium PKG is a monomeric protein held in an autoinhibited state by four main features: (i) intrasteric regulation effected by the cis-binding of the AIS in the substrate site; (ii) immobilization of the two lobes by the CNBs using a number of interdomain contacts; (iii) interaction...
The CNBs of PfPKG. (A) Surface rendering of the four PfPKG CNB domains, shown with their cGMP-binding pocket in gray surfaces. A cGMP molecule is manually docked in their putative binding sites for illustration. The third CNB, pCNB-C, is similar overall to the other CNBs, except for the cGMP-binding pocket, which is occluded due to a network of hydrophobic residues. (B) ITC binding curves for the CNB-A, -AB, -C, and -D constructs to cGMP. Upper and Lower display the ITC titration curves and the binding isotherms, respectively. As the CNB-B domain did not yield soluble recombinant protein, its binding activity was tested within a CNB-AB tandem construct. The binding affinity for the CNB-A, -B, and -D domains is 1.4, 1.7, and 0.17 μM, respectively; the CNB-C domain does not bind to cGMP. (C) Alignment of the CNB-D in our apo PfPKG structure (in color) with the cGMP-bound costructure (PDB ID code 4OFG; in gray) shows that the helix αC_D undergoes noticeable displacement when cGMP is engaged. The triad (R484, Q532, and D533) key to activation of the enzyme is also displaced to (R484′, Q532′, and D533′). (D) Orthogonal representation of C. (E) Alignment of the CNB-A in our apo PfPKG structure (in color) with the cGMP-bound costructure (PDB ID code 5E16; in gray) shows the rearrangement of helices αB_A and αC_A in response to cGMP binding. Upon cGMP activation, the helix αC_A undergoes a large rotation of 42°, but does not contribute to cGMP capping as observed in PKA and PyCNB-D; instead, the cGMP ligand is stabilized by interactions with the backbone of helix αB_A. (F) Alternative representation of E that includes the CNB-A neighboring domains of PfPKG (apo form). The cartoon illustration depicts rearrangements of the connecting helix αC_A relative to the CNB-B and kinase activation loop, with the disruption of the important K154–Y694 π-bond interaction.
between the N-terminal helix and the C terminus of the KD in a mutually locking arrangement; and (ii) arrangement of connecting helices between CNB-B and pCNB-C, pCNB-C and CNB-D, and, most significantly, the conjoined helix between CNB-D and the KD. To the best of our knowledge, the last two features are not only distinct from PKA, but entirely unique among kinases to date.

**IntrastERIC Regulation Is Mediated by an N-Terminal AIS.** Autoinhibition in mammalian PKA and PKG involves interactions between the regulatory and catalytic domains (18, 38). The proposed conformational changes mediated by cGMP binding are thought to release PKG from its autoinhibition and allow it to become active. Although the autoinhibitory domains of PKA and PKG do not have a universally conserved sequence, they all share a consensus small residue (most commonly glycine, replaceable by alanine, serine, or valine; underlined in SI Appendix, Fig. S7A) in the so-called P$p$ position, where a phosphorylation target—namely, serine or threonine—might be found in a pseudosubstrate peptide (e.g., the pseudosubstrate bound in the AIS site in one of the first PKA structures). Alignments of sequences and structures indicate that this residue (Ala18 in $P_f$/PKG) is conserved in the AIS of apicomplexan PKGs. This adds weight to the notion that the Plasmodium AIS displays an autoinhibitory function similar to the inhibitory segment (IS) in PKA (18). The mammalian PKG-III inactivation in vivo may involve the observable increase in basal activity (i.e., in the absence of cGMP) of the truncated version of $P_f$/PKG missing this motif.

Although the domain-swapped dimer in our crystal structures suggests the intriguing possibility of an intermolecular regulatory system (in which each protomer extends N-terminal residues to block the substrate-binding site of its dimeric partner), the totality of our biochemical and biophysical data indicates cGMP-free (i.e., inactive) *Plasmodium* PKG to be predominantly a monomer. Accordingly, we were not able to detect transinhibition between a full-length and a truncated form of $P_f$/PKG, as illustrated in Fig. 2D. This suggests that the dimeric interaction observed in our crystal structures is likely a crystallographic artifact. Attempts to crystallize *Plasmodium* PKG in a monomeric form have been unsuccessful, allowing us only to speculate on how the AIS might mediate autoinhibition in monomeric PKG-III. We observe that in all available PKA holoenzyme structures, as well as in our *Plasmodium* PKG structures, a short helix follows the inhibitory region and passes over the long helix, connecting the first two CNBs. In comparing the two PKG molecules in the dimer with the PKA structures (Fig. 2E), we observe that there is a rearrangement of connecting helices which enables the binding of cGMP at CNB-D to facilitate binding at CNB-B and -A and ultimately results in all inhibitory constraints from the KD. Specifically, we propose the following mechanism of cooperative activation (a sequence that likely results in changes in the observable increase in basal activity). In *Plasmodium* parasites, stringent timing is required for cGMP-mediated events, including egress of merozoites (41) and gametocytes (4) from erythrocytes. A key trigger in the regulation of this timing is cooperative binding of cGMP and activation of PKG—a property confirmed by the sigmoidal shape of the activity curves of our samples and a Hill coefficient of 1.9 observed in our experiments. Whereas cooperativity in PKA is conferred by the additional constraints imposed in the tetrameric holoenzyme (13), our data indicate that, in *Plasmodium* PKG, and likely PKG-IIs in general, it is mediated by a network of connecting helices which enables the binding of cGMP at CNB-D to facilitate binding at CNB-B and -A and ultimately results in all inhibitory constraints from the KD. Specifically, we propose the following mechanism of cooperative activation (a sequence that may also represent four potential stable or metastable states of $P_f$/PKG). (i) When cGMP and ATP concentrations are low, all three functional CNBs and the ATP-binding site are ligand-free. The substrate-binding site of the KD is occupied by the AIS. This is the totally autoinhibited state of $P_f$/PKG. (ii) As the level of cGMP rises, the domain with the highest affinity, CNB-D, becomes occupied first, resulting in a series of conformational changes that involves displacement of $\alpha_{A_{C}}$. This releases the N-lobe of the KD from some of its constraints. $\alpha_{P_{G}}$ is only basally active in this state because the AIS continues to inhibit the KD, and the C lobe remains constrained by the first two CNBs. $P_f$/PKG (with ATP) with cGMP-bound CNB-D may be a structurally metastable state, one that is primed to engage cGMP at CNB-B. (iii) The movement initiated at CNB-D is propagated to CNB-C via the connecting helix between the domains. In return, this propels movement of the helix shared by CNB-A and -B in a manner seen in PKA and enabling binding of cGMP to CNB-A. (iv) Binding of cGMP in CNB-A disrupts interactions between this domain and the KD, including the hydrophobic stack. This can release the AIS from its auto-inhibitory position and the C lobe from all constraints, and free the KD, which is already in its active conformation.

**A Structural Relay Model for Activation of *Plasmodium* PKG.** In *Plasmodium* parasites, stringent timing is required for cGMP-mediated events, including egress of merozoites (41) and gametocytes (4) from erythrocytes. A key trigger in the regulation of this timing is cooperative binding of cGMP and activation of PKG—a property confirmed by the sigmoidal shape of the activity curves of our samples and a Hill coefficient of 1.9 observed in our experiments. Whereas cooperativity in PKA is conferred by the additional constraints imposed in the tetrameric holoenzyme (13), our data indicate that, in *Plasmodium* PKG, and likely PKG-IIs in general, it is mediated by a network of connecting helices which enables the binding of cGMP at CNB-D to facilitate binding at CNB-B and -A and ultimately results in all inhibitory constraints from the KD. Specifically, we propose the following mechanism of cooperative activation (a sequence that may also represent four potential stable or metastable states of $P_f$/PKG). (i) When cGMP and ATP concentrations are low, all three functional CNBs and the ATP-binding site are ligand-free. The substrate-binding site of the KD is occupied by the AIS. This is the totally autoinhibited state of $P_f$/PKG. (ii) As the level of cGMP rises, the domain with the highest affinity, CNB-D, becomes occupied first, resulting in a series of conformational changes that involves displacement of $\alpha_{A_{C}}$. This releases the N-lobe of the KD from some of its constraints. $\alpha_{P_{G}}$ is only basally active in this state because the AIS continues to inhibit the KD, and the C lobe remains constrained by the first two CNBs. $P_f$/PKG (with ATP) with cGMP-bound CNB-D may be a structurally metastable state, one that is primed to engage cGMP at CNB-B. (iii) The movement initiated at CNB-D is propagated to CNB-C via the connecting helix between the domains. In return, this propels movement of the helix shared by CNB-A and -B in a manner seen in PKA and enabling binding of cGMP to CNB-A. (iv) Binding of cGMP in CNB-A disrupts interactions between this domain and the KD, including the hydrophobic stack. This can release the AIS from its auto-inhibitory position and the C lobe from all constraints, and free the KD, which is already in its active conformation.
Collectively, our structural-relay model of cooperative activation proposes that cGMP-mediated activation of *Plasmodium* PKG involves a series of conformational changes, initiated by binding to CNB-D, which are propagated around the Uniprot database 7

Materials and Methods

Protein Expression and Purification. Synthetic DNA for PPKG and PPKG from *G. max* (sequences are in SI Appendix) was amplified by PCR and subcloned into the pET-15-MHL vector—a baculovirus expression vector with an N-terminal Histag followed by a TEV cleavage site [https://www.thegene.com/reagents/vector]. Individual PCBN domains (CNB-A, -B, -C, and -D) were subcloned into the pET-15-MHL vector—a bacterial expression vector with an N-terminal Hexaf in a fully active KD in a yet-to-be-determined structural arrange-ment proposes that cGMP-mediated activation of *PKG* as a search model; the structure was refined by using REFMAC to a final R factor of 22.1%.

The PBCNDB–cGMP structure was solved by using Phaser for molecular replacement using the CNB-D domain coordinates from PPKG as a search model; the structure was refined by using REFMAC to a final R factor of 19.6%. The geometry of the final models was checked by using MolProteo (Schrödinger, LLC). The coordinates have been deposited in the NCBI protein structure database [https://www.ncbi.nlm.nih.gov/protein; PDB ID codes SDYL (PPKG), SDYK (PPKG), and SDZC (PPKG–AMPNNP)].

Enzymatic Assays. Kinase activity was characterized by using an NADH/ATPase coupled assay (52). The reactions were performed at 25 °C in a 384-well plate by using the Synergy 4 plate reader (Biotek). The reaction mix typically contained the enzyme at a concentration of 25 nM, 500 μM Peptide 7 (RRRAPSFYAK), 150 μM NADH, 300 μM phosphoenolpyruvate, 1 mM ATP, a lactate dehydrogenase/pyruvate kinase mix from Sigma (3 units/mL), 20 mM Hepes (pH 7.5), 30 mM NaCl, 10 mM MgCl₂, 1 mM CaCl₂, 1 mM dithiothreitol, and 0.01% Tween 20. The reaction was started by adding cGMP (0–200 μM) and monitored for 1 h by using the rate of NADH absorbance decrease at 340 nm, which is proportional to the rate of ATP hydrolysis.

Results

ITC. The binding constant and thermodynamic parameters of cGMP binding to the CNB domains of PPKG were assessed by using a nano-isothermal titration calorimeter (TA Instruments). Experiments with PCBN-A, -B, -C, and -D were performed at 25 °C. The sample cell was filled with 169 μL of purified protein samples prepared at a concentration of 60 μM in buffer A (25 mM Hepes, pH 7.5, 300 mM NaCl, and 0.5 mM Tris(2-carboxyethyl)phosphine (TCEP)) and stirred constantly at 350 rpm. The syringe was filled with 50 μL of cGMP at a concentration of 0.7 mM in buffer A and titrated into the sample cell by using 3-μL injections at 180-s intervals. The net binding data were fitted by using the NanoAnalyzer Software (TA Instruments) to calculate the binding parameters.

Analytical Ultracentrifugation. Sedimentation equilibrium analytical ultracentrifugation experiments were performed by using an Optima XL-A ana-lytical ultracentrifuge (Beckman). Protein samples at concentrations of 0.4, 0.8, and 1.2 mg/mL were prepared in a buffer containing 25 mM Hepes (pH 7.5), 150 mM NaCl, and 1 mM TCEP. The samples were spun for 36 h at 6,000, 8,000, and 10,000 rpm by using an An-60 Ti analytical rotor until equilibrium was reached at 4 °C. Absorbance was monitored at 280 nm. The partial specific volume, solvent density, and solvent viscosity were estimated by using the Sednterp program (University of New Hampshire; server located at http://asmb.org/sednterp/). Data analysis was done with the Origin MicroCal XL-A/C/Li Data Analysis Software Package (Version 4.0).

Multimode Light Scattering. The molecular size of purified PPKG was measured at 25 °C by using a Viscotek Tetra detection system equipped with detectors for static light scattering, UV, and refractive index (Malvern Instruments) connected downstream of a size-exclusion chromatography system with a Superdex 200 HR 10/30 column mounted. The column was equilibrated in Equilibrium Buffer (25 mM Hepes, 150 mM NaCl, and 0.5 mM TCEP). Protein samples and the bovine serum albumin standard were dialyzed overnight against Equilibration Buffer. Protein was diluted to a concentration of 6 mg/mL. A volume of 100 μL of each was sequentially injected by using an auto-sampler into the chromatography system at a flow rate of 0.2 mL/min. Molar weight determination was performed by using the OmniSes software (Malvern Instruments).
Native Gel Electrophoresis and Immunoblotting. Proteins sample were mixed with 2x native sample buffer (62.5 mM Tris-HCl, pH 6.8, 25% glycerol, and 1% SDS) or sodium dodecyl sulfate/PAGE sample buffer + DTT and resolved on an 8% Tris-HCl (pH 8.8) polyacrylamide gel run in Tris glycine buffer (pH 8.3) for 3 h at 150 V in an ice-water bath or on an 8% Tris.HCl (pH 8.8) polyacrylamide gel run in Tris-glycine SDS buffer (pH 8.3), respectively. Proteins were transferred to nitrocellulose and the PPKG-HA fusion protein visualized with anti-hemagglutinin antibody (clone 3F10, Roche; diluted to 1:3,000) followed by horseradish peroxidase-conjugated anti-rabbit (catalog no. SC-2006, Santa Cruz, at 1:6,000). The blot was reacted with enhanced chemiluminescence plus substrate (Pierce) and exposed to X-ray film.

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