Trypanosome Cyclic Nucleotide Phosphodiesterase 2B
Binds cAMP through Its GAF-A Domain*

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Trypanosoma brucei, the causative agent of sleeping sickness in humans and livestock, expresses at least three cAMP-specific class I phosphodiesterases (PDEs), all of which are essential for survival of the parasite. These PDEs have either one or two N-terminal GAF domains, which in other proteins function as signaling domains. However, neither the functional roles nor ligands for these domains in trypanosome PDEs are known. The present study shows that TbPDE2B, which contains two tandem GAF domains, binds cAMP with high affinity through its GAF-A domain. A purified re-combinant N terminus + GAF-A domain binds cAMP with an affinity (Kᵦ) of ~16 nM. It also binds cGMP but with a 15-fold lower affinity of ~275 nM. The TbPDE2B holoenzyme has a somewhat lower affinity (~55 nM) for cAMP but a greatly lower affinity (~10 μM) for cGMP. This suggests that both the selectivity and affinity for a ligand can be determined not only by the nature of the binding domain but also by the adjacent domains. Additionally, binding of cAMP to the holoenzyme showed positive cooperativity, with a Hill coefficient value of 1.75. However, binding of cGMP to the holoenzyme did not show any cooperativity, suggesting differences in the conformational changes caused by binding of these two cyclic nucleotides with the protein. Point mutation of a key predicted binding site residue (T317A) resulted in a complete loss of high affinity cAMP binding. This mutation increased the apparent Kᵦ of the mutant enzyme for substrate without altering the Vₘₐₓ. A truncated catalytic domain construct of TbPDE2B also exhibited an increased Kᵦ, strongly suggesting that cAMP binding to the GAF-A domain can regulate TbPDE2B by allowing the full activity of the enzyme to be expressed. These properties of the GAF-A domain of TbPDE2B thus suggest that it could be a new target for anti-trypanosomal drugs.

Cyclic nucleotide phosphodiesterases (PDEs)¹ regulate cAMP and cGMP signaling pathways by controlling the intracellular levels of cyclic nucleotides. In higher eukaryotes, PDEs are known to regulate a variety of processes including visual transduction, olfaction, control of metabolic activities, insulin secretion, fertility, and a host of other functions. The precise cellular profile of PDE expression is thought to influence the response of a tissue or organism to cyclic nucleotides.

In mammals, there are 11 distinct Class I PDE families (1, 2) that have conserved catalytic domains but different regulatory domains. These 11 mammalian PDE family members each have different kinetic and substrate characteristics, inhibitor profiles, amino acid sequences, and regulatory ligands (3). Other organisms such as Saccharomyces cerevisiae have a second class of phosphodiesterases, termed Class II PDEs, that have a different evolutionary origin and catalytic domains that are not homologous to mammalian PDEs (4). Five of the 11 mammalian Class I PDE family members contain regulatory segments with one or two tandem GAF domains (5). GAF domains are a very large family of small molecule-binding domains recently found to be present in nearly all organisms (5). Thus far, the only high affinity ligand identified for a PDE GAF domain has been cGMP. Binding of cGMP to the GAF domains of PDE5 (6) and PDE2 (7) is an important mechanism for regulating these enzymes. cGMP also tightly binds the photoreceptor PDE6 (8), where its precise role is being investigated (9). Recently, cAMP was found to bind and activate the GAF domain of the cytB1 adenylyl cyclase from the cyanobacterium Anabaena genus (10). Significant insights into the mechanism of cyclic nucleotide binding to these GAF domains was provided by the recent structure determination of the murine PDE2A regulatory GAF domains in complex with cGMP (11). A follow-up study also revealed a number of critical contacts enabling nucleotide binding and subtle discrimination between cAMP and cGMP (12). Two recent reviews discuss the evolutionary conservation, properties, and possible functions of these GAF domains in cyclic nucleotide phosphodiesterases and other proteins (13, 14).

In parasites of the Trypanosomatidae family, cAMP is an important mediator of cell transformation and proliferation. Intracellular levels of cAMP are known to vary during different life cycle stages of trypanosomes (15). In addition, a density-sensing mechanism that signals cell cycle arrest in trypanosomes and leads to differentiation appears to be via the cAMP pathway (16). The precise roles of PDEs in these parasites are still largely unexplored (17). However, it is known that the differentiation of Trypanosoma brucei from bloodstream forms to short, stumpy forms can be inhibited by non-selective PDE inhibitors (18), demonstrating the importance of PDEs in these organisms. Recently, three different Class I cAMP-specific PDEs, TbPDE2A, -B, and -C, were identified in T. brucei (19–21). All three were found to be essential for proliferation of the bloodstream form of T. brucei (21). These three also contained either one or two tandem GAF domains (19–21). TbPDE2B intriguingly did not appear to bind cGMP (19–21), despite containing a majority of the “critical residues” defined for binding cGMP in mammalian PDE2 (11). Neither a guanylyl cy-

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¹ The abbreviations used are: PDE, cyclic nucleotide-specific phosphodiesterase; TbPDE, PDE family member from T. brucei; HEK, human embryonic kidney; MOPS, 4-morpholinepropanesulfonic acid; cNMP, cyclic nucleoside monophosphate.

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clase nor a function for cGMP has yet been identified in trypanosomes. Considering the challenges being faced in present chemotherapy for human sleeping sickness (22) and the established success of PDE inhibitors as drugs in various human diseases, TbPDE2A, -B, and -C are attractive anti-trypanosomal drug targets. This provides much incentive for understanding their regulation and the function of their GAF domains. The current study reports high affinity cAMP binding to the TbPDE2B GAF-A domain, characterizes the binding, and provides data that suggest this cAMP binding is important for regulation of the trypanosome PDE activity.

**EXPERIMENTAL PROCEDURES**

**Materials**—[5,8-3H]cAMP and [8-3H]cGMP were purchased from PerkinElmer Life Sciences. cAMP and cGMP (sodium salts), phenylmethylsulfonyl fluoride, diithreitol, and isopropyl 1-thio-β-D-galactopyranoside were obtained from Sigma. Protein G-agarose beads were from Oncogene Research Products/Calbiochem.

**Cell Culture**—Human embryonic kidney (HEK293) or 293T cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin in a humidified 5% CO2 atmosphere. S/9 cells were grown at 27 °C in Grace’s insect cell medium (Invitrogen) supplemented with 10% fetal bovine serum.

**TbPDEB Constructs**—TbPDE2B holozyme in a pFastbac vector (21) was used as the template in all PCRs. TbPDE2B holozyme was PCR-amplified with Ffu polymerase (Stratagene) using primers 5'-CATATGACACACACGGTGCTGCT-3' and 5'-ACGAGTACGTGCTGTTGACGAAACT-3'. TbPDE2B N terminus + GAF-A domain was amplified using primers 5'-CATATGACACACACGGTGCTGCT-3' and 5'-ATCAGAATCGTACCTCTTTT-3', ending at amino acid Asp-395. The catalytic domain was amplified using primers 5'-CCAAGAATTTGGTTGCTGAGCAGG-3' and 5'-ACGAGTACGTGCTGTTGACGAAACT-3', starting at Pro-642 and to the end. All fragments were cloned into pcDNA3.1-V5his (Invitrogen). In addition, the N/H11032, starting at Pro-642 and to the end. All fragments were cloned into pcDNA3.1-V5his (Invitrogen) and digested with EcoRI and NotI were used to amplify the holoenzyme, CGCTCAACGAGTACTGCTGTTGTTGCCAG-3' of the holoenzyme, and lysed in lysis buffer (phosphate-buffered saline (Invitrogen) plus 10% glycerol, 2 mM phenylmethylsulfonyl fluoride, 5 mM dithiothreitol, and 0.5 mM EDTA (pH 7.5, 1 mM phenylmethylsulfonyl fluoride, 3 mM EDTA, 1 mM dithiothreitol). The cells were lysed using a Virsonic 100 sonicator for 4–5 s and centrifuged at 16,000 × g for 20 min at 4 °C. The supernatants were used for all further assays.

**Construction of a Baculovirus Expression Vector System for TbPDE2B Expression in S/9 Cells and Purification—**Recombinant TbPDE2B holoenzyme-expressing Baculoviruses were constructed using Baculogold DNA (Pharmingen) co-transfecting Baculogold DNA with the TbPDE2B-pAcGHLT-A vector into S/9 cells using the manufacturer’s transfection set and the manufacturer’s protocol (Pharmingen/BD Biosciences). After 5 days, the supernatants from the experimental co-transfection plates were collected, and co-transfection efficiencies were estimated using antibody dilution assays. The virus was amplified twice to generate high titer stock, and the viral titer was determined by plaque assay. S/9 cells were infected with the virus at a multiplicity of infection = 3. Maximum protein expression was seen after 3 days, when the cells were harvested. The cells were resuspended and lysed in lysis buffer (phosphate-buffered saline (Invitrogen) plus 10% glycerol, 2 mM phenylmethylsulfonyl fluoride, 2 mM dithiothreitol, and 0.5 mM EDTA (pH 7.5, 1 mM phenylmethylsulfonyl fluoride, 3 mM EDTA, 1 mM dithiothreitol). The supernatants were centrifuged at 16,000 × g for 30 min. The supernatant was subsequently purified on a glutathione uniflow resin (BD Biosciences) column (using the glutathione S-transferase epitope tag on TbPDE2B), and TbPDE2B was eluted using 10 mM reduced glutathione in the same buffer. The protein exhibited normal wild-type enzymatic activity and was stored at −20 °C for at least 3 weeks without any significant loss of activity.

**Immunoprecipitation—**Purified and control (untransfected) cell supernatants were first cleared by incubation with 15 µl of protein G-agarose beads at 4 °C for 45 min (with gentle mixing). These extracts were subsequently centrifuged at 16,000 × g for 5 min and the supernatants transferred to different tubes. Cleared supernatants were incubated with the anti-V5 antibody (Invitrogen) and 30 µl of protein G-agarose beads for 2–3 h in a volume of 1 ml with (gentle mixing). After a 30-min incubation, the immunoprecipitates were resuspended in homogenization or binding buffer or prepared in SDS sample buffer for SDS-PAGE resolution and Western blot analysis.

**Western Blot Analysis—**Samples were diluted 1:1 in a 2× SDS sample buffer (100 mM Tris, pH 6.8, 200 mM dithiothreitol, 4% SDS, 0.2% bromphenol blue, 20% glycerol), boiled at 100 °C for 5–10 min, and then applied to a 10 or 12% SDS-polyacrylamide gel. Resolved proteins were electroblotted onto nitrocellulose membranes and immunostained with anti-V5 antibodies. Immunoreactivity was detected by ECL using horseradish peroxidase-conjugated rabbit anti-mouse IgGs and a SuperSignal West Pico Chemiluminescent Substrate (Pierce).

**cNMP Competition Binding Assays and IC50 Curves—**To determine an IC50 value for cAMP or cGMP displacement of [3H]cAMP from the TbPDE2B N terminus + GAF-A domain in a total volume of 10 µl of binding buffer. Ten pmol of [3H]cAMP (1 nm) and ~6 pmol of purified bacterial N terminus + GAF-A protein (0.6 µg protein) were used/assay point, with increasing concentrations of cold cNMP (as indicated). The large 10 µl volume was used to keep the absolute concentrations of [3H]cNMP sufficiently low (because binding affinities for cAMP were ~16 nM), therefore retaining sufficient counts. Following an incubation of 20–30 min on ice, ammonium sulfate was added to a final concentration of either 1 or 3 M (as described under “Results”). For cNMP binding to the holoenzyme, the assay was done in a concentration of 1 µl of binding buffer (10 mM radioligand, 6 nM protein). These conditions for the assay were selected keeping in mind the assumptions and possible ambiguities of competitive binding curves, as described by Motulsky and Christopoulos (23) (i.e., ... if the concentration of labeled ligand greatly exceeds the equilibrium dissociation constant (Kd) obtained from the IC500 values (using the Cheng and Prusoff equation (24))) closely reflected the IC50 value itself. More detailed explanations are available in the online manuals on the Priam website resource library (www.graphpad.com). The solution was filtered on a nitrocellulose filter (Millipore), washed twice with the ammonium sulfate solution, dissolved in scintillation fluid (Filter-Count/PerkinElmer Life Sciences), and the counts bound meas...
Determination of Binding Equilibrium—To determine the time necessary for cNMP binding to reach equilibrium (for both the purified holoenzyme as well as the N terminus GAF-A construct), binding assays were done as described earlier with 10 nM [3H]cAMP and 6 nM purified protein. The samples were filtered onto a pre-moistened Millipore HAWP nitrocellulose membrane (pore size 0.45 mm, 24 mm diameter) and washed three times with 3 ml of 3 M ammonium sulfate. The filters were dissolved in 3 ml of Filter Count (Packard Instruments) and counted in a liquid scintillation counter. Data are expressed as counts/min bound/filter. wt, wild type holoenzyme. C, Western blot using an anti-V5 antibody detecting N terminus + GAF-A (lane 1), catalytic domain (lane 2), TbPDE2B holoenzyme in transfected cell lysates (lane 3). Data are representative of experiments repeated four times with independent transfections.

RESULTS
TbPDE2B Binds cAMP through Its GAF-A Domain—HEK293T cells were transiently transfected with a full-length native (wt) TbPDE2B expression plasmid or a TbPDE2B catalytic domain fragment plasmid. Both contained a C-terminal V5 epitope tag (Fig. 1A) allowing immunoprecipitation of the expressed protein using an anti-V5 antibody. Given the high sequence identity of TbPDE2B to mammalian PDE2A, especially within the GAF-A domain, it seemed likely that the TbPDE2B GAF-A domain should bind cyclic nucleotides. How-
ever, an earlier study (20) had not been able to detect cGMP binding. Yet this did not rule out binding of cAMP, which is an important signaling molecule in trypanosomids, nor was the binding to cGMP quantitatively determined in the previous study. To test this possibility, a Millipore filter-binding assay was carried out with [3H]cAMP and immunoprecipitated proteins from control (untransfected), catalytic domain, or TbPDE2B holoenzyme transfected cells. This was done in the presence of at least 5 mM EDTA (which inhibits enzymatic activity by chelating the Mg²⁺ ion required for hydrolysis) due to the absence of other effective inhibitors for this cAMP-specific PDE. [3H]cAMP bound to immunoprecipitated TbPDE2B but not to immunoprecipitates of control lysates or the catalytic domain alone, ruling out the possibility that the radioactivity detected was because of cAMP binding to the catalytic domain (Fig. 1B).

Given the primary amino acid sequence similarity of the TbPDE2B GAF-A to the PDE2A GAF-B domain (19) as well as the CyaB1 GAF domain of the Anabaena cyclase, the GAF-A domain seemed to be the region most likely to bind cAMP. To test this hypothesis, the N terminus + GAF-A fragment were visualized by Western analysis and the relative intensities measured by densitometry (Fig. 1C). It was not possible to determine the binding capacity, if any, of TbPDE2B B, because recombinant GAF-B in bacteria was insoluble. Similarly, constructs expressing either GAF-B or the GAF-B + catalytic domain in HEK293 cells were unstable and appeared to be degraded immediately upon lysis (data not shown).

cAMP Binds to Recombinant N terminus + GAF-A Domain with Nanomolar Affinity—To obtain enough purified protein to easily determine the binding affinity of cAMP to the GAF-A domain, the N terminus + GAF-A domain was cloned into pET28a (Fig. 1A) and expressed in RosettaDE3 Escherichia coli cells. Purification was then used in [3H]cAMP competition binding assays to obtain an IC₅₀ value, with increasing concentrations of cold cAMP as the competitive ligand. The IC₅₀ values for cAMP displacing [3H]cAMP were obtained by analyzing the competitive binding data by non-linear regression and fitting it to a one-site competition equation using Prism software. The actual affinities (Kᵣ values) were calculated as explained under “Experimental Procedures.”

2 The IC₅₀ of a ligand for a receptor depends on three factors: the affinity of the receptor for the competing drug (defined by the equilibrium dissociation constant Kᵣ), the concentration of the radioligand, and...
main was found to be of high affinity, with an IC_{50} value of 17.6 ± 1.8 nM (Fig. 2B). This high affinity cAMP binding is similar to that seen for cGMP binding to the mammalian PDEs 2, 5, and 6.

cGMP Binds to the TbPDE2B GAF-A Domain with Lower Affinity than cAMP—Given the high affinity binding for cAMP, as well as the close similarity of the TbPDE2B GAF-A domain to the mmPDE2A GAF-B domain, it seemed reasonable to expect at least some binding of cGMP to the GAF-A domain. Indeed, with the availability of larger amounts of purified bacterially expressed N terminus GAF-A protein, binding of [3H]cGMP could be detected (Fig. 2C). This purified protein was then used in [3H]cAMP competition binding assays to determine an IC_{50} for cGMP binding by competition analysis using increasing cold cGMP as the competitive ligand. cGMP was found to have at least 15-fold lower affinity for the GAF-A domain (compared with cAMP), with an IC_{50} value of 289 ± 5.4 nM (Fig. 2D). This discrimination in cyclic nucleotide affinity is just the opposite of the relative differences in the affinities for cGMP and cAMP of the mammalian PDE2A GAF-B domain (12).

Binding of cNMP to the Purified TbPDE2B Holoenzyme—Though the GAF-A domain of TbPDE2B bound cAMP with a high affinity, and cGMP with lower affinity, it was of interest to understand whether this phenomenon is representative of the TbPDE2B holoenzyme. To determine this, a baculovirus expression vector able to express the full-length TbPDE2B enzyme was constructed, baculovirus produced, TbPDE2B expressed in infected Sf9 insect cells, and purified (Fig. 3A). This purified TbPDE2B protein was subsequently used in competition binding studies (as described under “Experimental Procedures”) to determine cAMP and cGMP affinities. It was found that cAMP bound the TbPDE2B holoenzyme with an IC_{50} of
Table 1

| Domain                  | cNMP     | IC_{50}  | K_i  |
|-------------------------|----------|----------|------|
| N terminus + GAF-A      | cAMP     | 17.6 ± 1.8 nM | 16.6 ± 1.8 nM |
| N terminus + GAF-A      | cGMP     | 289.1 ± 5.4 nM | 273.5 ± 1.8 nM |
| TbPDE2B holoenzyme      | cAMP     | 62.5 ± 3.6 nM | 53.9 ± 3.6 nM  |
| TbPDE2B holoenzyme      | cGMP     | 12.24 ± 2.3 μM | 10.5 ± 2.3 μM  |

62.5 ± 3.6 nM, which was about 3-fold higher than the GAF-A domain alone (Fig. 3B). However, and perhaps more interestingly, cGMP had a much higher IC_{50} of 12.2 ± 2.3 μM for TbPDE2B. Thus the holoenzyme was able to discriminate more effectively for cAMP, as opposed to cGMP, than the GAF-A domain alone. The data were analyzed using Prism software and did not indicate a second high affinity site for cyclic nucleotide binding. The actual affinities of cNMP for the N terminus + GAF-A domain or the TbPDE2B holoenzyme were calculated from the IC_{50} using the Cheng and Prusoff equation (as explained earlier (24)). The equilibrium dissociation constants (K_i) and IC_{50} values for cNMP binding to the N terminus + GAF-A domain or to the TbPDE2B holoenzyme are shown in Table I.

The binding data were also reanalyzed in Prism by fitting it to a sigmoidal dose response curve with a variable slope (Fig. 3C), as explained under “Experimental Procedures.” Binding of cAMP to the holoenzyme showed positive cooperativity, with a Hill slope of 1.75 ± 0.1. However, binding of cGMP to the holoenzyme showed no cooperativity, with a Hill slope of 0.85 ± 0.1. Thus it seems that this enzyme is not only selective for cAMP, but the nature of the interactions of these two cyclic nucleotides with the protein appear to be different.

The N terminus + GAF-A Domain Alone Reaches cAMP Binding Equilibrium Faster than the TbPDE2B Holoenzyme—The time to reach binding equilibrium for [3H]cAMP was determined as described under “Experimental Procedures.” We found that, at low [3H]cAMP concentrations of 10 nM and low temperature (4 °C) and protein concentration of 6 nM, the purified N terminus + GAF-A domain reached cAMP binding equilibrium in <5 min (Fig. 4). However, the holoenzyme took ~45 min to reach binding equilibrium (Fig. 4). These conditions were subsequently used in the binding assays to the GAF domain or to the holoenzyme, respectively. As expected for bimolecular reactions, binding equilibrium was reached more rapidly at higher cAMP concentrations.

Effect of Ammonium Sulfate on cNMP-binding Stoichiometry—It was found that the concentration of ammonium sulfate used in the dilution/wash buffer was a factor for the amount of cNMP bound to the GAF domain. When high concentrations (3 M) of ammonium sulfate were used, an apparent binding stoichiometry of ~0.6 mol of cNMP bound/mol of (monomeric) protein was obtained; however, with 1 M ammonium sulfate, an apparent binding stoichiometry of ~0.2 mol of cNMP bound/mol of protein was seen (data not shown). Effects of ammonium sulfate on cGMP binding to other PDE GAF domains have been observed previously and its use in cNMP binding assays studied in detail (28, 29), although the magnitude of the effect seems to depend on the particular protein being investigated. It should be noted that the increased binding appears not to be due to greater loss of bound protein from the filter at the lower ammonium sulfate concentration, as multiple filters or multiple passes of the filtrate through several fresh filters did not increase binding. Therefore, the ammonium sulfate is altering some property of the GAF domain containing protein itself. The advantages and concerns of using ammonium sulfate in cGMP binding assays for other PDEs has been discussed extensively earlier (28–31). It also should be noted that the K_i values determined for cAMP binding were similar whether 1 or 3 M ammonium sulfate was used, indicating that only the maximal amount of cAMP retained on a filter, and not binding affinity, was affected.

A Point Mutation (T317A) in a Predicted Binding Site Results in Loss of cAMP Binding—Given the high similarity of this GAF-A domain with the cGMP-binding GAF-B domain of mammalian PDE2A, critical binding residues could potentially be predicted using the crystal structure of murine PDE2A GAF-B (11) as a model. Nine of the eleven “contact” residues of mmPDE2A GAF-B are well conserved in the TbPDE2B GAF-A domain as seen in a sequence alignment with mmPDE2A and the cyaB1 GAF-B domains (Fig. 5A), suggesting the likelihood of a cyclic nucleotide binding pocket in GAF-A (32). Using this information, as well as a homology model (Fig. 5B) of the TbPDE2B GAF-A domain based on the murine PDE2A GAF-B, the conserved Thr-317 in TbPDE2B (the equivalent of Thr-492 in mmPDE2A) was mutated into an alanine. In mmPDE2A, Thr-492 stabilizes cyclic nucleotide binding within the pocket through a critical contact with the ribose sugar of cGMP and was found to be essential for cGMP binding (12). In the TbPDE2B GAF-A model, the equivalent conserved Thr-317 appeared to function in a similar way. This region is also the most highly conserved region in the model. Therefore, the TbPDE2B T317A mutant was made and expressed in HEK293T cells. Binding assays were performed after immunoprecipitation of native and mutant proteins. We found that under our binding assay conditions, using either 1 or 3 M ammonium sulfate, the T317A mutation resulted in complete loss of detectable cAMP binding to the protein (Fig. 5C). The same result was also seen with a purified, bacterially expressed TbPDE2B N terminus + GAF-A T317A mutant (not shown). No binding could be detected for cGMP as well. This suggests that the cAMP-binding pocket of the TbPDE2B GAF-A domain is likely to have an overall structure similar to that of mmPDE2A GAF-B but with selectivity toward cAMP. It also suggests that Thr-317 forms a stabilizing contact with cAMP, thereby allowing cAMP to remain bound within the binding pocket. Additionally, because the point mutant T317A in GAF-A abolished all detectable cAMP binding in the holoenzyme, the presence of a second high affinity cAMP-binding site in TbPDE2B GAF-B seemed unlikely. However, the presence of a lower affinity site in GAF-B cannot be ruled out.

Binding Site Point Mutation Results in Decreased Enzymatic Activity at Low Substrate Concentrations—This observed loss in cAMP binding of the T317A mutant allowed us to test the hypothesis that the GAF-A domain might act as a regulator of...
FIG. 5. A single point mutation at a predicted binding site (T317A) results in loss of cAMP binding. A, sequence alignment of the TbPDE2B GAF-A domain, with the GAF-B domains of cGMP binding mmPDE2A and cAMP binding Anabaena cyaB1. The eleven critical residues shown to bind cGMP in mmPDE2A are indicated with arrows. Residues that differ between mmPDE2A and cAMP binding TbPDE2B GAF-A and CyaB1 GAF-B are shown in red arrows. The Thr→Ala mutation affecting binding and activity is marked with a green arrow. B, homology model of TbPDE2B GAF-A (blue) on mmPDE2B GAF-B (yellow) with cGMP bound, showing conserved regions between the two domains, differences in loops, and some residues that are present in PDE2A but absent in TbPDE2B (red). The mutated Thr-317 in TbPDE2B and the corresponding Thr-492 in mmPDE2A are also shown. C, [3H]cAMP binding of immunoprecipitated recombinant native (wt) TbPDE2B holoenzyme and the T317A mutant proteins. Immunoprecipitates of non-transfected cell supernatants were used as a control. The figure is representative of experiments repeated four times. D, Western blots showing band intensities of immunoprecipitated mutant T317A (lane 1), native wt TbPDE2B (lane 2) proteins and the control (immunoprecipitated non-transfected cell lysate, lane 3) used in the binding assay.
enzymatic activity through its ability to bind cAMP. When tested for activity, we found that the $K_m$ of the recombinant T317A mutant holoenzyme was ~4-fold higher than that of the native (wt) TbPDE2B holoenzyme (17.45 ± 1.7 μM versus 4.4 ± 0.8 μM, respectively) (Fig. 6A). This strongly suggests that the diminished cAMP binding to the GAF domain results in a decreased catalytic efficiency at the active site by low cAMP concentrations. Because the mutant protein is catalytically active with no significant change in the $V_{	ext{max}}$ (Fig. 6A), the data also indicate that the point mutation did not affect the global conformation or overall stability of the protein. The recombinant isolated catalytic domain had an even higher $K_m$ (≈44 μM) than the mutated holoenzyme, along with a possibly decreased maximal activity. Together these data suggest that cAMP binding to the GAF-A domain allows the full catalytic activity of the TbPDE2B holoenzyme to be expressed. Perhaps more importantly, binding of cAMP to the GAF-A domain allows more catalytic activity at lower cAMP concentrations in the cell. TbPDE2B is highly selective for cAMP as substrate, and cGMP did not affect cAMP-PDE activity when tested between 1 and 50 μM cGMP (data not shown), consistent with earlier reports (19–21).

Finally, no cooperative activity on its own hydrolysis was found for cAMP in the kinetic studies. A Hill coefficient of 1.0 was calculated from the kinetic curves for cAMP degradation. The reported Hill coefficient for a mammalian cGMP-stimulated PDE2 is 1.3 (7), which agrees with the maximal predicted theoretical $n$ value of 1.36 for dimeric enzymes showing this kind of regulation (33).

**DISCUSSION**

The data presented in this paper shows that cAMP binds to the TbPDE2B GAF-A domain with a high affinity. This is the first report of high affinity cAMP binding to any cyclic nucleotide phosphodiesterase GAF domain. The nucleotide binding is similar to what has been observed in the CyaB1 cyclase or mammalian PDEs 2 and 5 (with cGMP). Both the GAF-A domain alone and the holoenzyme bound cAMP with high affinity, with the GAF-A domain having a somewhat higher affinity than the holoenzyme. However, the holoenzyme shows much greater selectivity for cAMP binding over cGMP binding.

Importantly, there is an increase in the $K_m$ of the T317A mutant enzyme, which does not bind cAMP in its GAF domain. The $K_m$ increase is even higher for the recombinant catalytic domain (devoid of GAF domains), with an additional possible decrease in $V_{	ext{max}}$. These differences in kinetic properties indicate that there is likely to be communication between the GAF-A domain and the catalytic domain. Physiologically, this would suggest that cAMP binding to the intact GAF-A domain can increase the catalytic activity of TbPDE2B by increasing its affinity for substrate. For example, at 1 μM cAMP, the binding of cAMP to the GAF-A domain allows at least 10 times greater activity to be expressed compared with the isolated catalytic domain. This higher activity in turn could allow for greater feedback control over cAMP levels. A similar decrease in $K_m$ after cNMP binding and activation of the enzyme has been reported in activation studies on mammalian PDE5 (6). The exact molecular mechanisms by which this activation happens, however, remain unclear.

Although cGMP could bind to the GAF-A domain, it did so with much lower affinity than cAMP. This result was even more dramatic in the purified holoenzyme. The presence of the additional domains did not greatly alter the affinity of cAMP binding but significantly decreased the affinity for cGMP binding. Thus it appears that TbPDE2B has fine-tuned cyclic nucleotide selectivity preferentially for cAMP by evolving, not just the binding domain, but the regions around it as well. Additionally, positive cooperativity is seen for cAMP binding to the protein and not for cGMP binding. This suggests that the conformational changes caused by cyclic nucleotide binding to a GAF domain could possibly differ depending on the nucleotide. TbPDE2B, similar to many other PDEs, appears to be dimeric (from gel filtration and light scattering experiments, not shown), and it is conceivable that the cooperativity for cAMP binding exists between the GAF-A domains in the two subunits that compose the dimer. Therefore, it is plausible that the initial nucleotide interaction is at the catalytic site, with the GAF site being denied nucleotide access until the catalytic site is occupied. Such an interaction might then allow the GAF site to be exposed and allow the nucleotide to access the high affinity GAF site. This would be consistent with the binding and kinetic data obtained, because cGMP is not hydrolyzed by this PDE, nor does it affect cAMP hydrolysis by this highly cAMP-specific enzyme. This would also predict that cAMP binding to the holoenzyme would be slower than to the GAF-A alone, as is seen in Fig. 4. Finally, such a model could also fit with the differential effects of ammonium sulfate. At present, it is unclear whether the two nucleotides do cause different conformational changes within the GAF domains or if another mechanism is used to affect enzyme activity. It is also not clear whether any of the mammalian GAF domain-containing PDEs may also share a similar activation mechanism. Ultimately, the structures of the cyclic nucleotide bound and unbound forms of the enzyme should provide a better understanding of these regulatory mechanisms for TbPDE2B and for other GAF domain-containing PDEs.
The only other known cAMP-stimulated PDE (without GAF domains) is found in Dictyostelium (34), another lower eukaryote. In Dictyostelium, cAMP levels regulate chemotaxis and aggregation (35), and a cAMP-stimulated PDE was found to be important in controlling the level of aggregation (34). A similar role therefore may exist for TbPDE2B in trypanosomes. This selectivity for cAMP binding to the TbPDE2B GAF domain (compared with cGMP) and the fact that cGMP does not affect TbPDE2B activity is consistent with the absence of reports for a role of cGMP in T. brucei or any other member of the Trypanosomatidae family. A guanylyl cyclase is yet to be identified in these parasites, and there is no significant cGMP hydrolysis in trypanosome lysates. Given that cAMP levels regulate chemotaxis or any other member of the Trypanosomatidae family. A guanylyl cyclase is yet to be identified in these parasites, and there is no significant cGMP hydrolysis in trypanosome lysates. Given that cAMP levels regulate chemotaxis (34), another lower eu-

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