Conformation Changes, N-terminal Involvement, and cGMP Signal Relay in the Phosphodiesterase-5 GAF Domain*®

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The activity of phosphodiesterase-5 (PDE5) is specific for cGMP and is regulated by cGMP binding to GAF-A in its regulatory domain. To better understand the regulatory mechanism, x-ray crystallographic and biochemical studies were performed on constructs of human PDE5A1 containing the N-terminal phosphorylation segment, GAF-A, and GAF-B. Superposition of this unliganded GAF-A with the previously reported NMR structure of cGMP-bound PDE5 revealed dramatic conformational differences and suggested that helix H4 and strand B3 probably serve as two lids to gate the cGMP-binding pocket in GAF-A. The structure also identified an interfacial region among GAF-A, GAF-B, and the N-terminal loop, which may serve as a relay of the cGMP signal from GAF-A to GAF-B. N-terminal loop 98–147 was physically associated with GAF-B domains of the dimer. Biochemical analyses showed an inhibitory effect of this loop on cGMP binding and its involvement in the cGMP-induced conformation changes.

Cyclic nucleotide phosphodiesterases (PDEs)2 hydrolyze the second messengers cAMP and cGMP to 5'-AMP and 5'-GMP, respectively. The human genome contains 21 PDE genes that are categorized into 11 families and encode >100 isoforms of PDE proteins via alternative mRNA splicing (1–4). Family-selective PDE inhibitors have been widely studied as therapeutics for treatment of human diseases, including cardiotonics, vasodilators, smooth muscle relaxants, antidepressants, antithrombotic and antiasthmatic drugs, and agents for improvement of learning and memory and treatment of schizophrenia and psychosis (5–9). The most successful examples of this drug class are the PDE5 inhibitors of sildenafil (Viagra®), vardenafil (Levitra®), and tadalafil (Cialis®), which have been used for the treatment of male erectile dysfunction (5). Sildenafil has also been marketed as Revatio® for the treatment of pulmonary hypertension (10).

Mammalian PDEs contain a conserved catalytic domain at the C terminus and a regulatory domain at the N terminus. The regulatory domains of PDEs vary considerably and contain various structural/functional motifs. These include calmodulin-binding domains in PDE1; a PAS (Per-ARNT-Sim) domain in PDE8; two upstream conserved regions in PDE4; and two GAF domains in PDE2, PDE5, PDE6, PDE10, and PDE11. The term GAF was introduced in 1997 (11) as a common structural motif found in three proteins, cGMP-binding phosphodiesterases, cyanobacterial adenyl cyclases, and a formate-hydrogen lyase transcription activator from Escherichia coli, and now in >7000 proteins (12, 13). Certain GAF motifs have been shown to function as ligand-binding domains or to provide protein-protein interactions. GAF motifs are composed of 150–200 amino acids that are typically folded into a central six-stranded β-sheet flanked by two α-helices on both sides of the sheet. In the PDE superfamily, 5 of the 11 PDE families contain two GAF motifs that are arranged in tandem in their regulatory domains. For PDE holoenzymes, a certain type of cyclic nucleotide binds one of the two GAF motifs with high specificity: cAMP to GAF-B in PDE10; cGMP to GAF-B in PDE2; and cGMP to the GAF-A domains in PDE5, PDE6, and PDE11 (14–18). Binding of the cyclic nucleotides to the GAF domains in these PDEs regulates the catalytic activity and also impacts the phosphorylation status in PDE5 (15, 19–25). However, the molecular and structural features that provide for GAF functions are poorly understood.

Several x-ray crystal structures of PDE GAF domains have been determined: GAF-A + GAF-B of mouse PDE2A in complex with cGMP (14), GAF-A of chicken PDE6C in complex with cGMP (26), GAF-B of human PDE10A in complex with cAMP (27), and an unliganded PDE2A fragment containing both GAF and catalytic domains (28). The NMR structure of mouse PDE5A GAF-A in complex with cGMP has also been reported (18). These structures have provided insight into the functions of the GAF domains. For example, the PDE2 structures (14, 28) showed tight association of two molecules, implying that PDEs may work as a functional homodimer. However, many essential questions remain. How do the nucleotides bind to the closed GAF pockets of PDEs? How is the nucleotide signal propagated from the GAF domain to the catalytic site? Apparently, both unliganded and liganded...
structures of the GAF domains would be required to answer these questions. Here, we report structural and biochemical studies on human PDE5A1 fragments containing the N-terminal phosphorylation site, GAF-A, and GAF-B. These studies show a "lid" gating cGMP binding, a potential relay of the cGMP signal from GAF-A to GAF-B, and involvement of N-terminal segment 101–127 in the cGMP signaling.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—The cDNA of full-length human PDE5A1 (residues 1–875) was a gift from Drs. Jackie Corbin and Sharron Francis (Vanderbilt University). The coding regions for expression of GAF regulatory domains 29–518, 85–518, 89–518, 98–518, 116–518, 126–518, and 142–518 were subcloned into the modified expression vector pET32a. Oligonucleotide primers were synthesized for amplification of the PDE5A5 fragments by PCR. The amplified PDE5A cDNAs and the expression vector pET32a were separately digested by restriction enzymes NheI and XhoI, purified on agarose gel, and ligated by T4 DNA ligase. The resultant pET-PDE5A1 plasmids were confirmed by DNA sequencing and transferred into E. coli strain BL21-Codon-Plus for expression.

The E. coli cells carrying pET-PDE5A plasmids were grown in 2× yeast extract tryptone medium at 37 °C to A₆₀₀ = 0.7. Isopropyl β-D-thiogalactopyranoside (0.1 mM) was then added for further growth at 15 °C for 24 h. The recombinant PDE5A proteins were passed through a nickel-nitrotriacetic acid column (Qiagen), subjected to thrombin cleavage, and purified on Q-Sepharose and Sephacryl S-300 columns (GE Healthcare). The PDE5A fragments after thrombin cleavage contained 10 extra N-terminal residues (Gly-Ser-Ala-Met-Glu-His-Met-Ala-Ser-Met) and 2 C-terminal residues (Arg-Asn) from the expression vector. A typical purification batch yielded 20 mg of PDE5A fragments from 4 liters of cell culture. The purity of the proteins was estimated to be >95% by SDS-PAGE. To detect potential conformation or oligomerization changes, native polyacylamide gels (10%) were prepared by SDS-PAGE. To detect potential conformation or oligomerization changes, native polyacylamide gels (10%) were prepared by SDS-PAGE. SDS-PAGE.

RESULTS

Architecture of the Regulatory Domain of PDE5A—Two fragments of human PDE5A1 (amino acids 89–518 and 98–518; abbreviated as PDE5A98 and PDE5A98, respectively), both of which contain N-terminal loop 89/98–147, GAF-A (amino acids 148–323 with the C149S mutation), and GAF-B (amino acids 324–518), were crystallized in the same space group with similar unit cell parameters (Table 1). The two structures have very similar conformations, as shown by a root mean square deviation of 0.49 Å for the comparison of all Cα atoms in the structure.

Each of the crystallographic asymmetric units consists of a dimer of the respective fragments. Most residues in the structures have a good electron density and are traced without am-
bility, except for disordered residues 128–145, 395–406, and 436–445 in subunit A and residues 131–146, 209–217, 395–408, and 435–446 in subunit B in both structures. In addition, residues 89–100 in the PDE5A89 structure are disordered. Structural superposition between subunits A and B yielded root mean square deviations of 1.0 and 1.1 Å for the Cα atoms of the PDE5A89 and PDE5A98 structures, respectively. Detailed examination showed no substantial differences between the conformations of subunits A and B, except for the head and tail of segments, as well as residues 117–121. These residues did not contact the main body of the structures, and their positions were poorly resolved and may thus not be biologically significant.

The monomer of the PDE5A1 fragments contains eight α-helices and 13 β-strands that are assembled into GAF-A and GAF-B domains (Fig. 1A). In GAF-A, a central β-sheet that comprises six antiparallel strands (B1–B6) is associated with helices H2 and H5 to form a hydrophobic core. On the other side of the sheet, the β-strands constitute the bottom of the cGMP-binding pocket, whereas helix H4 and strand B3 appear to serve as two lids to cover the pocket (Fig. 1A). Similarly, the six-stranded antiparallel β-sheet (B7–B12) of GAF-B forms a hydrophobic core with helices H6 and H8 on one side. However, on the cGMP-binding side of GAF-B, residues 439–444 (indicated by D in Fig. 1A), which are equivalent to helix H4 of GAF-A, are disordered.

Two molecules of the PDE5A1 regulatory domain are tightly associated through hydrophobic and hydrophilic interactions with a parallel and symmetric dimer (Fig. 1A). The GAF-A dimer interface is composed of helices H1 and H5. The long helix H5 (residues 297–342) also contributes to formation of the dimer interface of GAF-B together with helix H8 but bends ∼15° around Cys310 (blue spheres in Fig. 1A). The tight association of both GAF-A and GAF-B domains in the crystal structure is consistent with results from biochemical studies in which the isolated GAF-A or GAF-B domain of PDE5 was a dimer and had a low dissociation constant: $K_D < 30 \text{ nM}$ for GAF-A and $K_D = 1–20 \text{ pm}$ for GAF-B (16). The smaller $K_D$ for GAF-B is supported by results of the present study demonstrating more hydrogen bonds in the GAF-B dimer interface.

The structure of the PDE5A1 GAF domain is similar to that of the dimer of the regulatory domain of unliganded PDE2A (28). However, these dimers superimposed poorly, as shown by a root mean square deviation of 4.4 Å for 459 Cα atoms when their secondary structures were compared using the CCP4 program (Fig. 1B). In addition, GAF-A and GAF-B of PDE5A communicate with one another through interactions between Ser194 of GAF-A and Asn485 of GAF-B (pink spheres in Fig. 1A), but no such noncovalent contacts between GAF-A and GAF-B exist in PDE2A.

The structural differences between the regulatory domains of PDE2A and PDE5A likely reflect differences in the cGMP signaling pathways in the two PDE families. The cGMP signal in PDE5 initiates from GAF-A, passes through GAF-B, and then stimulates the enzymatic activity. However, the cGMP signal starting from GAF-B of PDE2A may not require the involvement of GAF-A because GAF-B neighbors the catalytic domain. In addition, from a comparison of the isolated regulatory domain of cGMP-bound PDE2A versus the apo-form structure, cGMP binding elicits a switch of GAF-B locations (Fig. 1C). However, it is not clear if PDE5 undergoes a similar domain swap upon cGMP binding.

Conformation Changes upon cGMP Binding—Attempts were made to crystallize PDE5A fragments in complex with cGMP, but the crystals diffracted only to 5 Å resolution. Thus, to identify the cGMP-induced conformational changes, the x-ray structure of the unliganded PDE5A GAF-A domain was compared with the cGMP-bound GAF-A domain derived from NMR determination (18). This structural comparison
yielded an overall root mean square deviation of 2.7 Å for 130 Cα atoms after removal of 19 residues with differences of >6 Å. Five regions show large shifts (Fig. 2A): 6–8 Å for Cα atoms of the β-turn between strands B1 and B2 (human PDE5A1 residues 193–197), 6–13 Å for a loop after strand B2 (residues 207–214; indicated by L2 in Fig. 2A), 6–10 Å for a loop after strand B6 (residues 289–293; indicated by L6 in Fig. 2A), 4.3 Å for helix H4 (residues 254–259), and 3.2 Å for strand B3 (residues 216–222). Because loop L6 is the most variable loop in the NMR conformers (18), its positional difference from the x-ray structure may not be meaningful. Besides, helix H4 contacts symmetry-related helix H4 and strand B3 of another dimer in the crystal. We believe that this crystal lattice contact might not significantly change the structure as observed in many other crystal structures, but further structural study is needed for confirmation. The remaining positional differences were calculated from the well resolved NMR conformers and x-ray portions not involved in the crystal packing and may thus be statistically significant.

Functionally, loops L2 and L6 are distant from the binding pocket, and their biological influences are unclear. However, other changes appear to be biologically relevant. Helix H4 and strand B3 apparently serve as lids for the cGMP-binding pocket for opening and closing. The pocket is open in the unliganded state (Fig. 2B), as shown by ~10% of the solvent-accessible area of cGMP in the model of cGMP binding to unliganded PDE5A. In the cGMP-bound state, helix H4 and strand B3 moved toward one another by >3 Å relative to the unliganded state (Fig. 2C), whereas other elements of the binding pocket (helix H3 and strands B2–B6) did not significantly change their positions. These movements closed the pocket and thereby reduced the solvent-accessible area of cGMP to ~1.5%, as observed in the NMR structure of the cGMP-bound protein (18). Thus, helix H4 and strand B3 may play a key role in the transformation of the cGMP-binding pocket from an open to a closed state upon cGMP binding. However, a more detailed understanding of the conformational changes occurring
upon cGMP binding to PDE5 GAF domains must await x-ray crystal structure determination of the cGMP-bound form.

The β-Turn between Strands B1 and B2 of GAF-A May Serve as a Relay of the cGMP Signal—The cGMP-induced 6–8-Å movement of the β-turn between strands B1 and B2 of GAF-A also appears to be biologically relevant. This β-turn is the only region involved in the noncovalent interactions between GAF-A and GAF-B and may thus serve as a potential path of the cGMP signal. It makes both hydrophilic and hydrophobic interactions with the residues from N-terminal loop 101–127 and the loop near helix H8 of GAF-B (Figs. 1A and 3). Specifically, Oγ of Ser194 in the β-turn forms two hydrogen bonds with Oδ1 of Asp107 in the N-terminal loop and N82 of Asn485 in GAF-B, respectively, whereas the Ser194 nitrogen forms a hydrogen bond with Asp107 Oδ1 (Fig. 3, Center). Moreover, Glu192, Asp193, and Ser194 in the β-turn make van der Waals contacts with Asp107, Arg108, and Pro109 in the N-terminal loop, as well as with Asn485 and Asn487 in GAF-B.

The argument that the β-turn serves as a relay of the cGMP signal is indirectly supported by the results of an NMR study in which the dynamic GAF-A domain was stabilized by interactions with GAF-B in the unliganded state and these interactions were disrupted when cGMP was bound to GAF-A (18). However, other pathways for the cGMP signal may also be possible. For example, the long central helix H5 that covalently link GAF-A and GAF-B may serve as a pathway for the cGMP signal, although the comparison between the x-ray and NMR structures did not show a significant positional change in the top portion of helix H5.

Association of the N-terminal Loop with GAF-B—In the PDE5A98 structure, 8 residues of the expression vector (Ala-Met-Glu-His-Met-Ala-Ser-Met) along with 3 residues of PDE5A (Thr98, Arg99, and Lys100) form an amphipathic α-helix (H0, blue in Fig. 1A and pink in Fig. 3A). Because the corresponding residues of PDE5A1 (Asn91, Ser92, Val93, Pro94, Gly95, Thr96, and Pro97) are not predicted to form a helix, the positions of the PDE5A residues preceding Thr98 are uncer-
Structure of the PDE5 GAF Domain

An unusual observation is the physical association of N-terminal loop 101–127 with GAF-B. Residues 101–106 are located on the front surface of GAF-B, whereas residues 107–127 are at the back of GAF-B (Fig. 3, Left). Residues 101–114 exist in a coil conformation and interact mainly with residues in the GAF-B domain, including the hydrogen bonds between Arg108 N and Thr122 Oγ, Arg111 nitrogen and Gln326 Oε2, and Arg111 oxygen and Arg329 Nη2, as well as Asp107 Oδ1 and Ser194 nitrogen/Oγ in both subunits of the dimers (Fig. 3, Center). In addition, Phe124 forms a hydrogen bond with Asp335 from another subunit and hydrophobic interactions with Phe498 of the same subunit in the dimer (Fig. 3, Right).

However, the N-terminal loops in the dimer show some characteristics of asymmetry. Val115–Phe124 form two short antiparallel β-strands in subunit B of the dimer, but coils in subunit A. In addition, the hydrogen-bonding partners of Ser123–Asp127 are different in subunits A and B of the dimer. In subunit B, hydrogen bonds are formed between Ser123 Oγ and Asp335 Oδ2, Phe124 nitrogen and Asp335 Oδ1, Ser126 nitrogen and Gln331 Oε1, and Asp127 oxygen and Lys328 Nζ (Fig. 3, Right). In subunit A, hydrogen bonds are formed between Phe124 oxygen and Gln331 Nε2, Leu125 nitrogen and Asp335 Oδ2, Ser126 oxygen and Lys330 Nζ, and Asp127 Oδ1 and Lys330 Nζ. These asymmetric interactions appear to result from the resolution limit of the structures because the N-terminal loops have overall similar conformations and locations.

Inhibitory Effect of the N-terminal Loop on cGMP Signaling—To further study the conformational changes induced by cGMP binding, native PAGE was performed in the presence of various concentrations of cGMP. The gels showed that 20 μM cGMP converted a solid band of PDE5A89 to a smear, whereas 2 mM cAMP had no effect on the pattern (Fig. 4A). This conversion is likely caused by surface charge changes of the molecule as the consequence of the cGMP-induced conformation changes instead of the oligomerization state of the protein. FPLC showed that the PDE5A98 fragment was eluted as a sharp peak with an apparent molecular mass of 82.3 kDa in the absence of cGMP, which shifted to 125 kDa in the presence of 0.2 mM cGMP (supplemental Fig. 2). The 82.3-kDa peak basically matches the dimer molecular mass of 97.8 kDa. However, the 125-kDa peak is unlikely a trimer because the tight association of the PDE5 dimer in the crystal structure would predict a tetramer or other even oligomers. Very likely, the peak shift in the gel filtration reflects a change in the molecular shape of the PDE5 dimer.

To identify the segment of sequence that is most critical for the shifts on native PAGE, several fragments of the PDE5A GAF domain with various lengths were expressed, purified, and analyzed by native gel electrophoresis. In the presence of 2 mM cGMP, three PDE5A constructs (amino acids 116–528, 126–518, and 142–518) migrated slightly faster than in the absence of cGMP on the gel (Fig. 4B). This likely reflects the minor charge changes upon cGMP binding, but no major conformational changes. The high cGMP concentration used in the gel analysis might retain some cGMP in the binding pocket during electrophoresis, as reported by another group (34), thus impacting the band position. On the other hand, the bands for fragments 89–518 and 98–518 became smeared in the presence of 2 mM cGMP and migrated much slower, implying that N-terminal residues 98–115 affect the conformation of the GAF domains upon cGMP binding. This phenomenon is consistent with previous reports that the N-terminal fragments or full-length holoenzyme migrated slower in the presence of cGMP on native gels (16, 35).

To determine the impact of PDE5A N-terminal loop 98–147 on cGMP binding, we measured the cGMP dissociation constants of the various fragments. These experiments showed that the dissociation constant for cGMP was dependent on the length of the fragment (Table 2 and supplemental Fig. 3). Fragments 29–518, 85–518, and 98–518 had comparable dissociation constants that were at least of 17-fold lower affinity compared with those for fragments 116–518, 126–518, and 142–518. These constants are generally consistent with the previous report that small GAF-A fragments have high affinity with cGMP (16). However, our longer fragments showed weaker affinity compare with those reported previ-
 TABLE 2

Dissociation constants of the PDE5 GAF domains

| PDE5A1(C149S) fragment | $K_d$ (nM)* |
|------------------------|------------|
| 29–518                 | 515 ± 53   |
| 85–518                 | 1084 ± 100 |
| 98–518                 | 1420 ± 275 |
| 98–518b                | 1948 ± 184 |
| 116–518                | 24 ± 1     |
| 126–518                | 31 ± 2     |
| 142–518                | 24 ± 3     |

a The S.D. values were calculated from three repeated measurements.  
b This fragment contains 8 extra residues from the expression vector at the N terminus.

imply a conformational change upon cGMP binding. Apparently, other residues (yet to be identified), as well as this aspartic acid, are important for the specificity of cyclic nucleotide binding.

Despite two GAF domains in the PDE families that have quite similar overall folding, only one binds cyclic nucleotide. The structure of unliganded PDE5A reported herein suggests a clue to this puzzle. The cGMP-binding pocket in GAF-A of PDE5A is gated by helix H4 and strand B3, but residues 438–444, which correspond to helix H4 of GAF-A, are disordered in GAF-B. This disorder would be expected to make cGMP diffusion in/out of the pocket easier and significantly compromise the binding affinity of cGMP. Thus, our studies may imply that the three-dimensional conformation of the cGMP-binding pocket is another factor for binding of the nucleotides. However, this argument is weakened by the observation that the corresponding helix H4 in GAF-B of the unliganded PDE2A structure is disordered (28). Apparently, both the overall shape of the pocket and the chemical nature of individual residues in the pocket would determine nucleotide specificity and affinity, but further studies are required to draw a firm conclusion. In summary, our studies suggest a complicated and cooperative process of cGMP signaling, which is composed of conformation changes in the pocket upon cGMP binding, relay of the cGMP signal from GAF-A to GAF-B, and involvement of C-terminal segment 101–127 in the cGMP signaling.

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