The cyanobacterial tandem GAF domains from the cyaB2 adenylyl cyclase signal via both cAMP-binding sites

Sandra Bruder*, Jürgen U. Linder*, Sergio E. Martinez‡, Ning Zheng‡, Joseph A. Beavo‡, and Joachim E. Schultz*

*Pharmazeutisches Institut, Universität Tübingen, Morgenstelle 8, D-72076 Tübingen, Germany; and ‡Department of Pharmacology, University of Washington, Seattle, WA 98195

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The tandem GAF domains from the cyanobacterium *Anabaena* PCC7120 cyaB2 adenylyl cyclase form an antiparallel dimer with cAMP bound to all four binding sites. cAMP binding causes highly cooperative allosteric enzyme activation (>500-fold; EC50 = 1 μM; Hill coefficient >2.0). The cyaB2 GAF domains, like those of the cyclic nucleotide phosphodiesterases (PDEs), contain conserved NKFDE motifs that when mutated in the PDEs abrogate cyclic nucleotide binding. We mutated the aspartic acids within this motif in cyaB2 to determine which domains were required for signaling. Constructs containing an Asp/Ala mutation in either GAF domain still showed positive cooperative cAMP stimulation but with reduced Hill coefficients. The cyaB2 GAF domain NKFDE motifs contain inserts of 14 (GAF-A) and 19 (GAF-B) amino acids not present in PDE2 or cyaB1. Constructs having these inserts deleted could still be activated by cAMP (23- to 100-fold) but lost all positive cooperative activation, suggesting that the inserts play an important role in domain interaction and/or stabilization of the cAMP-binding pockets. In the shortened constructs, even those with a single Asp/Ala mutation in the NKFD motifs could still be activated by cAMP. However, in a double Asp/Ala mutant of the shortened construct, stimulation by cAMP was almost completely lost, and the EC50 shifted far to the right. Overall, the data suggest that in GAF domains without these inserts, only the canonical lysine:aspartate salt bridge keeps the α4-helix and the α4-β5 linker that close over the cyclic nucleotide properly oriented, thereby stabilizing the binding pocket. The cyaB2 GAF ensemble appears to be an evolutionary intermediate where both GAF domains still participate in allosteric activation by cAMP.

PDE | phosphodiesterase

GAF domains are widespread small-molecule-binding domains that are present in >1,600 proteins from all kingdoms of life (1–4). The acronym is derived from those proteins in which these domains were initially identified, i.e., in cGMP-regulated mammalian phosphodiesterases (PDEs), cyanobacterial adenylyl cyclases (ACs), and a formate-hydrogen lyase transcriptional activator. In the mammalian PDEs 2, 5, 6, 10, and 11 and in the cyanobacterial ACs cyaB1 and cyaB2, two GAF domains are located N-terminally in tandem (4). Cyclic nucleotides serve as allosteric regulators that activate these C-terminally located effectors domains (2, 4–9).

In 2002, the structure of a PDE2 tandem GAF domain was solved at 2.9 Å (10). It showed a parallel dimer in which the N-terminal GAF-A domains were physically dimerized, whereas the GAF-B domains each contained a molecule of bound cGMP and were 65 Å apart. Mutagenesis experiments confirmed that GAF-B is the cyclic-nucleotide-binding domain in PDE2 (11). Most surprisingly, the tandem GAF domains of this mammalian PDE2 could functionally replace those of the cyanobacterial AC, cyaB1 (9). That is, a CAMP-regulated cyanobacterial AC was converted into a cGMP-regulated enzyme. The elucidation of the structure of the tandem GAF domains from the closely related cyanobacterial AC, cyaB2, at a 1.9 Å resolution provided another surprise (12). These GAF domains formed a more closely packed antiparallel dimer that has a molecule of CAMP bound to all four individual GAF domains. In both structures, however, the regions around each of the cyclic-nucleotide-binding sites was rather well conserved and in all instances the bound cyclic nucleotides were almost totally buried inside the proteins. Here, we present a structure-guided biochemical analysis of cAMP signaling by the cyaB2 tandem GAF domain based on a functional activation assay. For technical reasons a chimera of cyaB2 GAF domains fused with the cyaB1 catalytic domain was used as an analytical tool (9) because the cyaB2 homolog which also is cAMP stimulated, was difficult to express, unstable and could not be purified. Because in the crystal structure all four potential ligand-binding domains contained cAMP, we wanted to determine whether all of these binding sites actually contribute to intramolecular signaling.

As part of this investigation we characterized the role of a lysine:aspartate salt-bridge found in the conserved NKFDE motif [a conserved signature motif of the amino acids Asn(N), Lys(K), Phe(F), Asp(D), and Glu(E) found in all GAF-domain-containing enzymes that have cyclic-nucleotide-binding capacity] that has been identified in the GAF domains of five mammalian PDEs and the two cyanobacterial ACs, cyaB1 and cyaB2 (4, 13). Both available GAF domain structures, the parallel one from PDE2 and the antiparallel one from cyaB2, established that the signature NKFDE motif that contains a conserved lysine:aspartate salt bridge is adjacent to the binding sites and not directly involved in ligand binding. In the PDEs and cyaB1, mutation of the conserved aspartates within this motif to alanine reduced or eliminated ligand binding or activation and thus has been used to identify which of the potential cyclic-nucleotide-binding sites actually mediates intramolecular signaling (2, 6, 9, 14, 15). The cyaB2 tandem GAF domains contain as unique structural features either 14- or 19-aa inserts between the conserved Lys and Phe of the NKFDE motif in GAF-A and -B, respectively (Fig. 1) (12). By deletion mutagenesis, we now establish that these inserts are essential for positive cooperative cAMP signaling. In addition, they complement the function of the stabilization function of the lysine:aspartate salt bridge. Finally, the biochemical experiments provide evidence that in cyaB2, both the GAF-A and the GAF-B ligand-binding regions participate in AC activation as suggested by the crystal structure and the large Hill coefficient.

Materials and Methods

CyaB2-GAF-cyaB1-Chimera. The cyaB2 gene from *Anabaena* PCC7120 was a gift of M. Ohmori (University of Tokyo). The cyaB2 tandem GAF domain (amino acids 1–441) was amplified by PCR with specific primers. It was cloned into pBluescriptII

Abbreviations: AC, adenylyl cyclase; PDE, phosphodiesterase; sGAF, shortened GAF.

To whom correspondence should be addressed. E-mail: sandra.bruder@uni-tuebingen.de.

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CyaB2-GAF, shGAF-Asp224Ala, -Asp416Ala, and -Asp224Ala/Asp416Ala-Adenylyl Cyclases. A shortened version of cyaB2 GAF [cyaB2-shGAF (A220–215/Lys386Gly/A339–407)-cyaB1-chimera; see alignment in Fig. 1 for further details] was generated by site-directed mutagenesis with deletion primers and the cyaB2-GAF-cyaB1-chimera in pBluescriptII SK(−) as a template. The constructs were cloned into the BamHI and Smal sites of pQE30 attaching an N-terminal MRGSH6GS affinity tag. The fidelity of all constructs was verified by double-stranded sequencing. Primer sequences are available on request.

Expression and Purification of Bacterially Expressed Proteins. All pQE30 constructs were transformed into Escherichia coli BL21(DE3)[pRep4] and pET16b clones into E. coli BL21(DE3)[pLysS]. Cultures were grown in Lennox L broth at 30°C containing 100 mg/liter ampicillin and 25 mg/liter kanamycin. Expression was induced at an A600 of 0.6 with 25–40 μM isopropyl-β-thiogalactopyranoside for 4 to 5 h at 16–19°C. Bacteria were harvested and washed once with 50 mM Tris-HCl (pH 8.5) at 4°C and stored at −80°C. For purification, frozen cells were suspended in 25 ml of cell lysis buffer (50 mM Tris-HCl, pH 8.5/50 mM NaCl/7.5 mM imidazole/protease inhibitors (20%/glycerol) at 4°C and passed through a French press at 1 psi (1 psi = 6.89 kPa). Cell debris was removed by centrifugation (48,000 × g for 45 min). Fifty to 200 μl of Ni²⁺-NTA slurry (Qiagen) was added to the supernatants. After binding for several hours at 0°C, the resin was poured into a column and washed (2 ml per wash). Wash buffer A was 50 mM Tris-HCl (pH 8.5)/2 mM MgCl₂/400 mM NaCl/5 mM imidazole/20% glycerol; wash buffer B contained 15 mM imidazole. Wash buffer C was as A with 10 mM NaCl and 25 mM imidazole. Proteins were eluted with 0.2–0.4 ml of buffer C containing 300 mM imidazole. Full AC activity is detectable in the eluate; however, imidazole impaired stability, and samples were dialyzed for 2 h against 50 mM Tris-HCl (pH 8.5)/10 mM NaCl/2 mM MgCl₂/35% glycerol (9). Protein was stored at −20°C.

Adenylyl Cyclase Assay. AC activity routinely was measured in 100 μl at 37°C for 4 or 10 min (16). The reactions contained 22% glycerol, 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, and 75 μM cAMP (25 kBq) [32P]ATP (25 kBq) [2,8-3H]cAMP (150 Bq) was added after stopping the reaction to determine yield during product isolation. The protein concentration was adjusted to keep substrate conversion below 10%. The reaction was started by addition of the substrate.

Western Blot Analysis. Protein was mixed with sample buffer and subjected to SDS-PAGE (10%). Proteins were blotted on PVDF membranes and sequentially probed with a commercial anti-RGS-H₂-antibody (Qiagen) and with a 1:5,000 dilution of a peroxidase-conjugated goat anti-mouse IgG secondary antibody (Dianova, Hamburg, Germany). Peroxidase detection was carried out with the ECL Plus kit (Amersham Pharmacia).

Results and Discussion

In a construct consisting of the cyaB2 tandem GAF domain and the cyaB1 AC catalytic domain, cAMP stimulated enzyme activity >500-fold in a dose-dependent manner and with very high specificity. The EC₅₀ concentration for half-maximal activity was 1.3 μM cAMP (Fig. 2A and Table 1), i.e., almost identical to that for cyaB1 (9). cGMP had almost no effect up to 100 μM. Remarkably, the cAMP dose–response curve showed that full activation was accomplished over only a 10-fold concentration range from 0.3 to 3 μM. Accordingly, a Hill coefficient of 2.2 indicated strong positive cooperativity (Table 1). This differed from cyaB1 GAF-domain-mediated activation that had a Hill coefficient of 0.7, i.e., it had no positive cooperativity. AC activation via cyaB2 GAF domains was not affected by...
preincubation with cAMP on ice for up to 45 min before substrate addition. Similarly, the activation was reversible instantaneously, i.e., the on- and off-rates and presumably the allosteric changes in response to binding were very rapid.

The cyaB2 tandem GAF domain is similar to its cyaB1 congener and also to the tandem GAF domains from mammalian phosphodiesterases PDE2 (24% identity and 43% similarity) and PDE5 (22% identity and 43% similarity), which are stimulated by cGMP via their respective GAF-B and GAF-A domains (8, 10, 11, 17). All of these tandem GAF domains share a conserved NKFDE motif that is present in both GAF-A and -B (Fig. 1A). Because these motifs are not close to the binding pockets they clearly are not directly involved in cyclic nucleotide binding or discrimination (10, 12). However, they must indirectly affect signaling from the binding site because several studies have successfully used mutations of the invariant aspartate in the NKFDE motif (2, 6, 9, 14) to determine which of the GAF domains, GAF-A or -B, mediate binding of cyclic nucleotide or activation of the associated target enzyme. Such studies have shown that GAF-B in PDE2, GAF-A in PDE5, and GAF-B in cyaB1 are the cyclic-nucleotide-binding domains (9–11, 17). Surprisingly, in the crystal structure of cyaB2, both domains, GAF-A and GAF-B, were found to contain bound cAMP. Therefore, the question arose whether both cAMP-binding sites also contribute to activation.

A comparison of the structures of the cyaB2 and PDE2 GAF domains (10, 12) showed that the salt bridges between the invariant Lys and Asp of the NKFDE motif in GAF-A and -B were retained in all four cyaB2-GAF domains of the dimer (Fig. 1B and C). The distances were 2.6 Å from Lys-200 to Asp-224 in GAF-A and 3.3 Å from Lys-387 to Asp-416 in GAF-B. A superimposition of the NKFDE motif between the structures of PDE2 GAF-B and both GAF domains of cyaB2 matched excellently with rms deviation values of 0.91 Å (GAF-A of cyaB2 to GAF-B of cyaB2), 0.98 Å (GAF-A of cyaB2 to GAF-A of PDE2), and 2.06 Å (GAF-A of cyaB2 to GAF-B of PDE2). The 14- and 19-aa inserts, compared with PDE2, between Lys and Asp in cyaB2 form distinct additional elements that do not, by themselves, affect the positions of the NKFDE amino acids (Fig. 1) (12). However, from the cyaB2 tandem structure, a distinct function for these inserts could not be deduced (12).

In contrast to cyaB1, the cyaB2/cyaB1 chimeric AC showed highly positive cooperative activation by cAMP (Hill coefficient of 2.2; Table 1). These data suggested that both GAF domains might be required for full activation. To examine biochemically whether both cyaB2 GAF domains mediate cAMP activation, we mutated Asp-224 in GAF-A and Asp-416 in GAF-B to Ala according to established strategies (6). As mentioned above, in all other GAF domains this mutation greatly weakened or completely abolished binding or activation. Therefore, the question arose whether both cAMP-binding sites also contribute to activation.

![Fig. 2](https://example.com/fig2.png)

**Table 1. EC50 values and Hill coefficients for all constructs examined in this study**

<table>
<thead>
<tr>
<th>Construct</th>
<th>EC50, μM</th>
<th>cAMP, Fold stimulation</th>
<th>Hill coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>1.3</td>
<td>500–1,000</td>
<td>2.2</td>
</tr>
<tr>
<td>D224A</td>
<td>0.5</td>
<td>430</td>
<td>1.4</td>
</tr>
<tr>
<td>D416A</td>
<td>0.3</td>
<td>850</td>
<td>1.6</td>
</tr>
<tr>
<td>D224A/D416A</td>
<td>0.3</td>
<td>625</td>
<td>1.7</td>
</tr>
<tr>
<td>shGAF</td>
<td>1.1</td>
<td>100</td>
<td>0.7</td>
</tr>
<tr>
<td>shGAF D224A</td>
<td>1.6</td>
<td>90</td>
<td>0.5</td>
</tr>
<tr>
<td>shGAF D416A</td>
<td>65</td>
<td>76</td>
<td>0.6</td>
</tr>
<tr>
<td>shGAF D224A/D416A</td>
<td>450</td>
<td>6</td>
<td>0.7</td>
</tr>
</tbody>
</table>

Data are means of two to four separate experiments for each condition. Hill coefficients are based on linear regression analysis of data plotted in EXCEL (Microsoft). The correlation coefficient for all constructs was 0.9 or higher.
GAF-A Asp224Ala and GAF-B Asp416Ala were similar to the unmutated cyaB2 GAF domains (Fig. 2B). More surprisingly, in the double mutant, Asp224Ala/Asp416Ala, the potency of cAMP to activate the cyclase was fully sustained (>600-fold activation) and the EC50 of 0.3 μM cAMP was again identical to the unmutated domains (Fig. 2C). The Hill coefficient of 1.7 was reduced from that of the unmutated construct. The specificity for cAMP compared to cGMP was retained for all these mutants (Fig. 2).

The surprising lack of an effect of the Asp/Ala mutations at these positions that was in stark contrast to observations with respective mutations in PDE2, PDE5, and cyaB1 (2, 6, 9, 11, 14) demonstrated that the invariant aspartates are not required for a functional coupling between the tandem GAF domain and the catalytic region of the cAMP cyclase. Furthermore, it is likely that the Hill coefficients 1 are due to differences in the crystal structure contained bound cAMP. The data also support the idea that the conserved aspartate residues of the NKFDE motif are not required for signal coupling between the regulatory and catalytic domains in this cyclase. Furthermore, it is likely that the Hill coefficients <1 are due to differences in cAMP affinities of the GAF-A and GAF-B domains (Fig. 3B).

![Figure 3](image_url)

**Fig. 3.** Stimulation of cAMP formation via a cyaB2 tandem GAF domain with shortened NKFDE region (shGAF). (A) Stimulation by cAMP (squares) and cGMP (circles) via cyaB2 shGAF. (B) Stimulation in shGAF-Asp224Ala (circles) and shGAF-Asp416Ala (squares). The inset shows the cAMP stimulation of the shGAF-Asp224Ala/Asp416Ala quadruple mutant (same scale as main figure). The corresponding Western blots are to the right.

It has been postulated that the conserved lysine:aspartate salt bridge may stabilize the cyclic-nucleotide-binding pocket as mutations of these residues greatly reduced or abolished cGMP binding in PDEs 2, 5, and 6 (2, 6, 11, 14). Similarly, mutations of the conserved Asn of the NKFDE motif also reduced or abolished cGMP binding (11, 15). Examination of the structure of either of the cyaB2 GAF domains showed that Asn-386 in GAF-B or Asn-199 in GAF-A (i.e., the conserved Asn in each NKFDE motif) makes multiple hydrogen bonds to backbone amides and a carbonyl in the α4-β5 linker (Fig. 1 B and C), thereby presumably stabilizing the pocket. The neighboring lysine:aspartate salt bridge therefore provides a strong interaction with the α5-helix, thereby stabilizing the orientation of these two structural elements. However, the data from the mutations in the cyaB2 GAF domains described above were not consistent with the data from the same mutations described for the PDEs because both Asp/Ala mutants in the cyaB2-GAF-cyaB1 AC construct had essentially undiminished signaling capacity. The activation factor for cAMP was >400-fold for all full-length cyaB2 GAF constructs (Table 1). Therefore, we suspected that the inserts of 14 and 19 aa between the conserved Lys and Phe of the NKFDE motif in the cyaB2 GAF-A and GAF-B domains, respectively, might be responsible for these unexpected results. These additional amino acids are located close to the α4 helical rim of the cAMP-binding pocket, particularly in GAF-B. As shown in Fig. 1B, there are also multiple contacts between several of these amino acids and the α4-β5 linker that connects the α4-helix with the β-sheet or a more rigid structural unit composed of the β-sheet and helices α2 and α5. Therefore, the possibility existed that these extra loops stabilized the position and orientation of the α4-helix and the important Thr-176 and Thr-363 side chains in the cAMP-binding pockets such as to make the otherwise necessary lysine:aspartate salt bridges dispensable.

Therefore, we deleted the intervening sequences from Lys-202 to Ile-215 in GAF-A and from Lys-389 to Cys-407 (Lys-388 was replaced by a Gly; see Fig. 1A) in GAF-B to bring the cyaB2 tandem GAF domain to length-conformity with the PDE2 congener. This construct was termed cyaB2-shGAF and used to test the question of whether the shortened AC constructs could be activated by cAMP. Because formation of the lysine:aspartate salt bridge still should be possible, it was predicted that the dose–response curves for cAMP and cGMP ought to be identical to the unabridged version. In fact, the EC50 value for activation was similar (1 μM cAMP); however, maximal stimulation was only 100-fold, and cGMP was essentially inactive as an activator (Fig. 3A and Table 1). Notably, the Hill coefficient dropped to 0.7 (Table 1). This result was more akin to previous data seen with the cyaB1 tandem GAF domains that also showed a Hill coefficient of 0.7 and the PDE2-GAF chimera with the cyaB1 AC with a Hill coefficient of 0.9. Therefore, the additional 14 and 19 aa appeared not to be required for activation, yet their presence was required for the positive cooperativity of cAMP as an activator (Table 1). Next, we generated single Asp/Ala mutations at either Asp-224 in shGAF-A or Asp-416 in shGAF-B (Fig. 3B). Somewhat surprisingly, each individual mutant AC construct was subject to cAMP stimulation albeit to different extents (Fig. 3B). In the cyaB2-shGAF-Asp224Ala mutant, the stimulation was ~20-fold, and the EC50 for cAMP was 1.6 μM, i.e., only somewhat less efficacious than for the unmutated protein (Table 1). In the cyaB2-shGAFB-D416A mutant, the stimulation was ~70-fold, but the EC50 was 65 μM cAMP, i.e., 30-fold higher than in the cyaB2-shGAF-A-Asp224Ala construct. The Hill coefficients were 0.5 and 0.6, respectively, i.e., the loss of cooperativity was also apparent in these mutations and corresponded to that observed in the shGAF-cyclase construct. Nevertheless, in both mutant proteins a functional coupling between the tandem GAF domain and the catalytic region was retained. Taken together, the data strongly imply that both cAMP-binding GAF domains participate in cooperative activation as predicted by the Hill coefficient of the unmutated construct and the fact that all four GAF domains in the crystal structure contained bound cAMP. The data also support the idea that the conserved aspartate residues of the NKFDE motif are not required for signal coupling between the regulatory and catalytic domains in this cyclase. Furthermore, it is likely that the Hill coefficients <1 are due to differences in cAMP affinities of the GAF-A and GAF-B domains (Fig. 3B).

Such differences would result in an apparent Hill coefficient of <1 unless a strong positive cooperativity still existed. Therefore,
the data suggest that little or no positive cooperativity exists between GAF-A and GAF-B in these mutants.

Next, we mutated the aspartate residues in both GAF domains by introducing one or more mutations into shGAF-A D224A (wild-type numbering), thereby generating a cyaB2 shGAF-Asp224Ala/Asp416Ala-cyaB1 cyclase mutant. The basal activity of this shortened double mutant was 0.4 ± 0.1 nmol of cAMP·min⁻¹·mg⁻¹ (SD; n = 6), which is at the lower end of the basal activities of the other mutant proteins. Cyclase activity was linear with respect to protein concentration and time. Furthermore, enzyme activity of this mutant was inhibited >75% by 1 mM βγ-yi-mido-GTP (GMPPNP), as were the activities of the wild-type construct, the individual shGAF single Asp/Ala mutants, and the cyanobacterial cyaB1 AC (data not shown). These findings suggest that the basic enzymatic properties of the catalytic domain were functionally identical to those of the other mutant cyclases. Finally, a dose–response curve with cAMP showed that this quadruple mutant could be stimulated ~6-fold but only by very high concentrations of cAMP (Fig. 3B Inset). The EC₅₀ was 450 μM cAMP and the Hill coefficient for activation was 0.7, in line with a general loss of positive cooperativity in all of the shGAF constructs (Table 1). The removal of the inserts invariably resulted in the absence of positive cooperativity (Table 1), as also observed for the cyaB1 AC and the chimeras containing PDE2 and PDE5 GAF domains and the cyaB1 AC (ref. 9 and unpublished data). Furthermore, the stimulation factor in all shGAF constructs dropped dramatically (Table 1). It appeared that each cyaB2 GAF domain was still capable of individually activating AC activity, albeit with different affinities and potencies. Only when both aspartates were mutated to Ala in cyaB2 shGAF was most activation lost; moreover, the small remaining activation did not show positive cooperativity.

Considering the similarity at the amino acid level within individual GAF domains of mammalian PDEs and cyanobacterial ACs, it is highly likely that the tandem domains evolved by gene duplication. Probably, a single, functional GAF domain was duplicated, resulting in a tandem GAF ensemble with two equally functional GAF domains. This, in turn, likely allowed positive cooperativity to be expressed, thereby making such a signaling unit immensely responsive to subtle changes in ligand concentration (e.g., see Fig. 2 A). Over time, such tandem GAF domains presumably further evolved by mutation, with the result that in some cases only one of the two domains signals, as is the case in the tandem GAF domains of mammalian PDE2 and probably PDE5 (10, 11, 17). This leaves the possibility for the second GAF domain to acquire a new function, e.g., in dimerization or binding to another molecule. Therefore, along the way of such an evolutionary process, we may expect several functional intermediates. It would appear that the tandem cyaB2 GAF domains are such an early evolutionary intermediate where both GAF domains still participate in allosteric activation by cAMP.

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