A GAF-domain-regulated adenylyl cyclase from *Anabaena* is a self-activating cAMP switch

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The gene *cyaB1* from the cyanobacterium *Anabaena* sp. PCC 7120 codes for a protein consisting of two N-terminal GAF domains (GAF-A and GAF-B), a PAS domain and a class III adenylyl cyclase catalytic domain. The catalytic domain is active as a homodimer, as demonstrated by reconstitution from complementary inactive point mutants. The specific activity of the holoenzyme increased exponentially with time because the product cAMP activated dose dependently and nucleotide specifically (half-maximally at 1 μ M), identifying cAMP as a novel GAF domain ligand. Using point mutants of either the GAF-A or GAF-B domain revealed that cAMP activated via the GAF-B domain. We replaced the cyanobacterial GAF domain ensemble in *cyaB1* with the tandem GAF-A/GAF-B assemblage from the rat cGMP-stimulated phosphodiesterase type 2, and converted *cyaB1* to a cGMP-stimulated adenylyl cyclase. This demonstrated the functional conservation of the GAF domain ensemble since the divergence of bacterial and eukaryotic lineages >2 billion years ago. In cyanobacteria, *cyaB1* may act as a cAMP switch to stabilize committed developmental decisions.

Keywords: adenylyl cyclases/*Anabaena*/evolution/GAF domain/PAS domain

Introduction

Essentially all cells generate cAMP in response to extracellular stimuli by intracellular adenylyl cyclases (ACs). As far as mammalian ACs (all class III ACs) are concerned, our state of knowledge is considerable (for reviews, see Sunahara *et al.*, 1996; Hanoune *et al.*, 1997; Smit and Iyengar, 1998). In bacteria, on the other hand, few biochemical details are known, although the cAMP-controlled catabolite repression in *Escherichia coli* (harbouring a class I AC) has been studied extensively for decades (Peterkofsky *et al.*, 1993; Amin and Peterkofsky, 1995). Many bacteria contain class III ACs, which possess catalytic domains with pronounced sequence similarities to their mammalian relatives (Barzu and Danchin, 1994; McCue *et al.*, 2000). The catalytic domains of bacterial class III ACs are mostly combined with non-catalytic domains, whose likely physiological role is to exert

regulatory, i.e. stimulatory or inhibitory, effects (Coudart-Cavalli *et al.*, 1997; Katayama and Ohmori, 1997; Kasahara and Ohmori, 1999; McCue *et al.*, 2000).

The cyanobacterium *Anabaena* sp. PCC 7120 has six genes coding for differently modularized AC isozymes (Katayama and Ohmori, 1997; Kasahara *et al.*, 2001). The gene for *cyaB1* codes for an AC, which at its N-terminal end has two GAF domains in tandem and a single PAS domain followed by an AC catalyst (Katayama and Ohmori, 1997; Ho *et al.*, 2000) (see scheme in Figure 1A). GAF domains [found in cGMP-phosphodiesterases, adenylyl cyclases and FhlA (formate hydrogen lyase transcriptional activator)] and PAS domains (found in period clock protein, aryl hydrocarbon receptor and single-minded protein) are ubiquitous small-molecule-binding domains present in signal transduction proteins in organisms from all phyla (Aravind and Ponting, 1997; Ho *et al.*, 2000; Anantharaman *et al.*, 2001). GAF domains, which have been identified in >1100 proteins, have been studied mostly in conjunction with the regulation of mammalian cyclic nucleotide phosphodiesterases (PDEs; McAllister-Lucas *et al.*, 1993, 1995; Soderling and Beavo, 2000). Five of the 11 mammalian PDE gene families contain GAF domains (PDE2, -5, -6, -10 and -11). PDE2, -5 and -6 possess two N-terminal GAF domains in tandem (termed GAF-A and GAF-B), which specifically bind cGMP, resulting in regulation of cAMP and cGMP hydrolysis (Soderling and Beavo, 2000). A number of mutational and recent structural and modelling studies attempted to pinpoint amino acids that are involved in nucleotide specification and cGMP binding (McAllister-Lucas *et al.*, 1995; Turko *et al.*, 1996; Granovsky *et al.*, 1998; Ho *et al.*, 2000; S.F.Martinez, A.Y.Wu, N.A.Glavas, X.B.Tang, S.Turley, W.G.J.Hol, J.A.Beavo, submitted for publication).

In the cyanobacterial AC *cyaB1*, the GAF domains formally fulfil the sequence requirements for cGMP binding. In bacteria, however, the presence of guanylyl cyclases has never been shown beyond a reasonable doubt and, therefore, remains contentious. In the present study, we sought to clarify the function of the GAF domains present in *cyaB1*. Expressing the presumed catalytic centre, we established that *cyaB1* AC activity was dependent on homodimerization. The GAF domains in the *cyaB1* holoenzyme operate as a high-affinity cAMP sensor and convey to the *cyaB1* AC the properties of a cAMP-specific, self-activating device, a cAMP switch. Finally, a replacement of the cyanobacterial GAF domain set with that from rat PDE2 generated a cGMP-activated AC and established a functional conservation of this small-molecule-binding domain over >2 billion years of evolution.

Fig. 1. (A) Scheme of the modular architecture of the cyanobacterial AC cyaB1. The domain abbreviations and approximate amino acid boundaries for each segment are indicated. (B) Sequence alignment of the catalytic domains of cyaB1 with C_{1a} from canine type V (VC1) and C₂ from rat type II (IIC2) ACs. The arrowheads mark the individually mutated amino acids (to alanine), which are involved in coordination of a divalent metal cation (D650), transition state stabilization (N728, R732) and substrate definition (K646 and T721); the latter replaces a canonical D in the catalytic fold of mammalian ACs. Residues conserved in all three sequences are shaded green. Residues conserved between cyaB1 and only one of the mammalian domains are red. The top line and the arrow mark a sequence gap and a single amino acid insertion, respectively, which are characteristic for this sub-family of class III ACs. The selection of the mammalian domains was based on the available X-ray structure of this heterodimer (Tesmer *et al.*, 1997). (C) Sequence alignment of cyaB1 GAF-A and GAF-B domains with their congeners from rat phosphodiesterase 2 and the yeast YKG9 GAF protein. Yellow columns denote residues in the NKX_nFX₃DE motif, which is conserved in the cyanobacterial and mammalian sequences. The site of individual point mutations (D190A in GAF-A, D360A in GAF-B) is indicated by an arrow. Residues conserved in at least four out of five sequences are red. The arrowheads pinpoint the amino acids assumed to define purine specificity (S.F.Martinez, A.Y.Wu, N.A.Glavas, X.B.Tang, S.Turley, W.G.J.Hol, J.A.Beavo, submitted for publication).

Fig. 2. Purification and activity of recombinant cyanobacterial AC proteins (SDS-PAGE analysis, Coomassie Blue staining). Two micrograms of each protein were applied, and molecular mass standards (in kDa) are indicated on the left. The specific AC activities indicated underneath were determined with 132 nM *cyaB1*_{595–859}, 265 nM K646A, 132 nM T721A, 3.3 μ M D650A, 0.66 μ M N728A and 248 nM R732A, and Mn-ATP as a substrate. The holoenzyme (right lane) was assayed at 5.2 nM protein, with Mg-ATP as a substrate in the presence of 100 μ M cAMP as an activator.

Results

Analysis and biochemical characterization of the *Anabaena cyaB1* AC catalytic domain

The *Anabaena cyaB1* gene codes for a protein with two tandem GAF, a single PAS domain and a predicted AC catalytic region (Figure 1A) (Katayama and Ohmori, 1997; Ho *et al.*, 2000). An alignment of the catalytic domain with the corresponding canine AC type VC1a and rat AC type IIC2 domains showed considerable similarities, justifying a class III classification (Figure 1B; Katayama and Ohmori, 1997). However, the replacement of a canonical aspartate (D1018 in AC-IIC2) by a threonine (T721 in *cyaB1*) may constitute a decisive difference because this residue was shown to be essential for substrate definition, and its mutation rendered the protein inactive (Tang *et al.*, 1995; Guo *et al.*, 2001). Yet, a threonine at this position is also present in other bacterial ACs, e.g. in *Stigmatella aurantiaca* and in four of 15 mycobacterial ACs (Coudart-Cavalli *et al.*, 1997; McCue *et al.*, 2000). A refined sequence analysis revealed that all open reading frames for predicted bacterial ACs, which have a threonine at this position, display a conserved pattern of changes: they contain a three amino acid gap in a highly conserved (F/Y)X₂(F/Y)D motif in C1a (marked by an overline in Figure 1B) and an insertion of a single variable amino acid in an otherwise invariant block in the mammalian C1a domains (arrow in Figure 1B). These critical length changes must be structurally compensated for in the folded protein to properly position the canonical amino acids in the dimeric catalytic cleft (Tesmer *et al.*, 1999), either to enable the known catalytic mechanism or to allow the cyclization to proceed via a different mechanism. Therefore, we expressed the catalytic domain of *cyaB1* (*cyaB1*_{595–781}) starting 11 amino acids prior to D₆₀₆, which, according to the structure of the catalytic fold

of mammalian ACs (Tesmer *et al.*, 1997, 1999; Zhang *et al.*, 1997), is essential for coordination of the metal cofactor. The recombinant *cyaB1*_{595–781} protein was invariably obtained in inclusion bodies, therefore we attempted to express *cyaB1*_{595–859}, i.e. including the C-terminus which was identified to have some similarity to a tetratricopeptide unit (TPR; amino acids 795–828) (analysed by the Simple Modular Architecture Research Tool, SMART; Schultz *et al.*, 1998). This construct was expressed as a partly soluble protein (~50%) and purified to homogeneity by Ni-NTA chromatography (Figure 2).

The kinetic properties of *cyaB1*_{595–859} were assayed with Mn²⁺ or Mg²⁺ as divalent metal ions. The *K_M* values for ATP, determined at 45°C and pH 8.5, were 11 ± 2 and 334 ± 18 μ M, and the *V_{max}* values were 309 ± 18 and 655 ± 38 nmol cAMP/mg/min for Mn²⁺ and Mg²⁺, respectively. The preference for Mg²⁺ with respect to *V_{max}* was unusual because, in most class III ACs, Mg²⁺-supported activity is rather low (Kasahara *et al.*, 1997; Hurley, 1999). The pH optimum was 8.5 (tested pH range 5.0–10), the temperature optimum an unusual 47°C and the activation energy derived from the linear arm of an Arrhenius plot was 96 kJ/mol (tested range 0–66°C). The Hill coefficient of 1.0 indicated no cooperativity of the two potential catalytic sites, which are probably formed by the symmetric homodimeric AC (Tang and Hurley, 1998; Guo *et al.*, 2001).

The time dependence of *cyaB1*_{595–859} was linear, yet the protein dependence was not. From 17 to 661 nM protein, the specific activity increased 8-fold, suggesting that a protein–protein interaction was involved in the formation of the catalytic site (Figure 3A). To prove the necessity for dimerization, we constructed two mutant proteins (*cyaB1*_{595–859}D650A and *cyaB1*_{595–859}N728A), which should be enzymatically inactive, yet complementary (Guo *et al.*, 2001). According to the known structure of the mammalian AC catalytic fold, D650 is involved in metal coordination and ATP positioning, and N728 in stabilizing a transition state (Tesmer *et al.*, 1997; Yan *et al.*, 1997; Zhang *et al.*, 1997). Both mutants were expressed and purified (Figure 2). Incubated individually, they were essentially inactive (<1% of wild type). Next we titrated 47 nM *cyaB1*_{595–859}N728A, functionally equivalent to a mammalian C2a AC domain, with *cyaB1*_{595–859}D650A, equivalent to a C1a domain (Figure 3B). AC activity was restored to a large extent, demonstrating that dimerization is required and sufficient for *cyaB1*_{595–859} activity, thus explaining the non-linear protein dependency (Figure 3A). In *cyaB1* AC, a threonine is at a position of a critical aspartate, which is involved in substrate definition by making a hydrogen bond to the N⁶ of the ATP (Tesmer *et al.*, 1999). We asked whether T721 could functionally replace this aspartate and generated a T721A mutant (Figure 2). *cyaB1*_{595–859}T721A had only 3% of wild-type AC activity, demonstrating that T721 participated in catalysis. Furthermore, T721 did not relax the substrate specificity for ATP because GTP was not accepted as a substrate.

To ascertain that the catalytic centre of *cyaB1* was otherwise in agreement with a canonical class III catalytic cleft, we generated the *cyaB1*_{595–859}K646A (substrate definition) and *cyaB1*_{595–859}R732A (transition state stabilization) mutant proteins (Figure 2). *cyaB1*_{595–859}K646A

Fig. 3. (A) Protein dependence of the specific activities of the cyanobacterial AC₅₉₅₋₈₅₉ catalyst (triangles; assayed at pH 8.5 and 45°C) and the holoenzyme (assayed at pH 7.5 and 37°C) with and without the addition of 100 μM cAMP (open and filled squares, respectively). cAMP addition did not affect the activity of AC₅₉₅₋₈₅₉. (B) Reconstitution of AC activity from the inactive AC₅₉₅₋₈₅₉D650A and AC₅₉₅₋₈₅₉N728A mutants. N728A (47 nM) was reconstituted with increasing amounts of AC₅₉₅₋₈₅₉D650A (open squares) and AC₅₉₅₋₈₅₉D650A alone (triangles).

had 5% and *cyaB1*₅₉₅₋₈₅₉R732A had ~25% residual AC activity relative to the wild-type enzyme. The latter result was in agreement with the observation that mutation of this critical arginine residue did not result in a full ablation of enzyme activity (Tang *et al.*, 1995; Yan *et al.*, 1997; Guo *et al.*, 2001). Because of the residual activity of these equivalents of a mammalian AC C2 domain, they could not be used for titration and complementation (see above).

cAMP activates *cyaB1* AC via its GAF domains

The catalytic domain of *cyaB1* AC carries two tandem GAF domains (GAF-A and GAF-B; Figure 1A) at its N-terminal end. An alignment with those from rat PDE2 shows prominent sequence conservation (Figure 1C). The cyanobacterial and rat GAF-A domains are 27% identical and 35% similar, and the GAF-B domains show 40% identity and 48% similarity, respectively. A noteworthy feature is the conservation of an NKX_nFX₃DE motif in the rat and cyanobacterial GAF-A and -B domains (Figure 1C;

Turko *et al.*, 1996). It was suggested that this motif is involved in cGMP binding because point mutations of the aspartate abolish cGMP binding to the respective GAF domains in PDE5 (McAllister-Lucas *et al.*, 1995). It is interesting that a recently crystallized putative yeast GAF domain, YKG9, lacks this conserved motif and does not bind cGMP (Ho *et al.*, 2000).

We expressed the *cyaB1* AC holoenzyme (*cyaB1*₁₋₈₅₉) in *E. coli*. Although >80% of the recombinant protein was in inclusion bodies, enough was soluble for purification and characterization (Figure 2). The pH optimum was 7.5 (tested range 5.0–9.5), the temperature optimum was 40°C (tested range 0–66°C) and the activation energy derived from an Arrhenius plot was 65 kJ/mol. As observed with *cyaB1*₅₉₅₋₈₅₉, Hill constants with a value of 1 indicated no cooperativity, and Mg²⁺ supported AC activity of the holoenzyme better than did Mn²⁺ (Table I). The values of the *V*_{max}/Michaelis constant were almost identical for Mg²⁺- and Mn²⁺-supported activity, indicating concerted changes in both constants (Table I). The protein dependence of *cyaB1* was non-linear and the specific activity increased with the protein concentration, reminiscent of the observations with *cyaB1*₅₉₅₋₈₅₉ (Figure 3A). However, the protein dependence of the holoenzyme may also be explained via a stimulation by cAMP formed during the assay. The cAMP concentrations at the end of the assays were 6.4 and 26.3 μM at 20 and 50 nM *cyaB1* (see below; Figure 5). When we examined the linearity of the enzymatic reaction over time, we observed an enhancement of cAMP biosynthesis irrespective of whether Mg²⁺ or Mn²⁺ was used as a divalent cation (Figure 4A). Calculating the specific AC activity at 1 min intervals, we noticed that, strikingly, it increased exponentially (Figure 4B). Because we were handling a recombinant, purified AC protein, we excluded the presence of activating protein factors and reasoned that the enzyme may be activated directly by the increasing cAMP product concentration, e.g. with Mg²⁺ as a cation the cAMP concentration in the assay increased from 0 to 1.2 μM over 10 min (Figure 4A). The addition of 100 μM cAMP to the assay linearized the time course and, surprisingly, the protein dependency over the concentration range, which was testable experimentally (Figure 3A). The determination of the Hill coefficients demonstrated no cooperativity (Hill constants were close to 1) and the *K*_M values with Mg²⁺ and Mn²⁺ in the presence of 100 μM cAMP remained essentially unchanged, yet *V*_{max} increased ~25-fold (Table I). Next, we examined the potency and nucleotide specificity of the activation (Figure 5). With Mg²⁺ as a cation, the dose–response curves for cAMP and cGMP yielded EC₅₀ values of 1 and 300 μM, respectively. Using Mn²⁺ as a divalent cation, the cAMP dose–response curve had a hump at ~100 μM cAMP and thus appeared biphasic, whereas with cGMP almost no stimulation of AC activity was observed (Figure 5). The stimulation by cAMP was highly specific, because adenosine, inosine, hypoxanthine, AMP, 2'-deoxy-3'-AMP, ADP, GMP, 2'-GMP, 2',3'-cGMP and GMPPNP (tested range 100 μM–1 mM) did not enhance *cyaB1* AC activity. A look at the dose–response curve for cAMP confirmed that the increase in the rate of cAMP biosynthesis over time was in full agreement with the concomitant increase in cAMP

Table I. Kinetic characterization of *cyaB*₁₋₈₅₉ AC

	$K_M(\text{ATP})$ (μM)	V_{max} (nmol/mg/min)	V_{max}/K_M	Hill coefficient
10 mM Mg^{2+}	38 ± 1	84 ± 8	2.2	1.0
2 mM Mn^{2+}	11 ± 0.3	24 ± 1	2.2	1.1
10 mM Mg^{2+} + 100 μM cAMP	24 ± 1	2244 ± 201	94	1.1
2 mM Mn^{2+} + 100 μM cAMP	4 ± 0.2	479 ± 5	120	1.0

With Mg^{2+} as a cation, the protein concentration used was 5.2 nM, and with Mn^{2+} it was 15 and 5.2 nM in the absence and presence of cAMP, respectively. Assays were carried out at 37°C and pH 7.5.

Fig. 4. (A) Time dependence of the *cyaB*₁ AC activity (*cyaB*₁₋₈₅₉ holoenzyme) assayed with Mg^{2+} (filled symbols) or Mn^{2+} (open symbols) as divalent metal cations. Note the time-dependent increase in cAMP formation in the absence of externally added cAMP (circles) and the linear time dependence in the presence of 100 μM cAMP (triangles). The protein concentrations in the assays with Mg^{2+} and Mn^{2+} were 7.6 and 102 nM, respectively, to account for different activities and to attain comparable conversion rates. (B) Changes in the specific activity of *cyaB*₁ over time. The specific activity at each time point in (A) was calculated for 1 min intervals. Note the exponential increase in specific AC activities without the addition of cAMP and the constant specific activity in the presence of 100 μM cAMP [symbols are as in (A)].

(0–1.2 μM). This corroborated the conclusion that the reaction product cAMP was an autoactivator.

We attributed the activating effect of cAMP to an interaction with the GAF domains and investigated

whether cAMP activated via the GAF-A or GAF-B domain. Mutational and modelling studies suggested that the aspartate residue in the NKX_nFX₃DE motif may be involved in cGMP binding (McAllister-Lucas *et al.*, 1995; Turko *et al.*, 1996; Ho *et al.*, 2000). This motif is conserved in both cyanobacterial GAF domains (Figure 1C). Considering the cAMP preference, this suggestion may not be sustainable as far as cyclic nucleotide specificity is concerned. One may, however, infer that the aspartate of this motif is either involved in cyclic nucleotide binding in an as yet unknown manner or that its mutation may result in a local conformational deterioration, which affects ligand binding of the affected GAF domain (McAllister-Lucas *et al.*, 1995; Turko *et al.*, 1996). Therefore, we took those data as an experimental opportunity and mutated the corresponding aspartate residues in the cyanobacterial GAF-A and GAF-B domains to alanine, generating *cyaB*_{1D190A} and *cyaB*_{1D360A} (marked by the arrow in Figure 1C). Both constructs were poorly expressed, with most of the protein ending up in inclusion bodies. The soluble portion of the recombinant proteins was purified by Ni-NTA chromatography. We could not stain a protein on an SDS-polyacrylamide gel, yet a western blot confirmed expression and purification (Figure 6). In *cyaB*_{1D190A} (GAF-A mutation), basal activity was almost undetectable, whereas 100 μM cAMP activation was comparable to the unmutated *cyaB*₁. The low basal activity in this instance may be the result of the low enzyme concentration available to us because of poor expression. In contrast, *cyaB*_{1D360A} (GAF-B mutation) had a basal activity like *cyaB*₁ wild type and cAMP addition was without effect. This indicated that the stimulatory effect of cAMP is exerted via the GAF-B domain.

This was further substantiated with cAMP-binding studies using 0.5–10 μM cAMP in a binding assay employed earlier (McAllister-Lucas *et al.*, 1995; Ho *et al.*, 2000). *cyaB*₁ bound cAMP in a concentration-dependent manner, whereas *cyaB*_{1D360A} maximally bound 3% of cAMP when comparing all corresponding data points with the unmutated protein, i.e. *cyaB*_{1D360A} had lost essentially all cAMP-binding capacity. These studies also unequivocally demonstrated that, in *cyaB*₁, GAF-A did not bind cAMP because GAF-A was unaffected in the *cyaB*_{1D360A} mutant. It cannot yet be decided whether the loss of cAMP binding was due to an inaccessibility or destruction of the cAMP-binding pocket or to changes in the k_{on} or k_{off} rates. Irrespective of this, both loss of cAMP stimulation and cAMP binding by the *cyaB*_{1D360A} mutation exclusively implicated the GAF-B site as responsible for the cAMP effect.

Fig. 5. Dose-response curve of cAMP- and cGMP-stimulated *cyb1*. Closed symbols indicate measurements with Mg^{2+} and open symbols measurements with Mn^{2+} as a divalent metal cation. cAMP, squares; cGMP, circles.

Conversion of *cyb1* to a cGMP-activated AC by GAF domain replacement

Although GAF domains occur in proteins from all phyla, a direct involvement in enzyme regulation has only been demonstrated for FhlA and several mammalian PDEs (Hopper and Böck, 1995; Turko *et al.*, 1999; Ho *et al.*, 2000). In the latter, cGMP specifically binds and regulates cAMP and cGMP PDEs. The tandem arrangement of the GAF domains in mammalian PDE2, -5, -6 and -10 is identical to that in *cyb1*. Cyanobacteria and mammals are about as distant in evolutionary terms as possible, therefore it was particularly interesting whether the GAF domains are sufficiently conserved to be interchangeable. We replaced the GAF domain ensemble in *cyb1* with the tandem GAF arrangement present in the rat PDE2. We used the GAF domain region from rat PDE2 because it is the only isoform, which has been demonstrated unequivocally to be directly activated by cGMP binding to the GAF domains, and because the structure of the tandem GAF domains of the mouse PDE2 has been elucidated most recently (S.F.Martinez, A.Y.Wu, N.A.Glavas, X.B.Tang, S.Turley, W.G.J.Hol, J.A.Beavo, submitted for publication; mouse and rat PDE2 GAF domains are virtually identical). The chimeric rat-cyanobacterial protein *cyb1*_{rat-GAF} was expressed in *E.coli* and affinity purified on Ni-NTA (Figure 7). Basal AC activity of the recombinant rat-*Anabaena* protein chimera was 2.4 nmol cAMP/mg/min. Addition of cGMP dose dependently activated the *cyb1* AC (Figure 7). Thus, we generated a cGMP-activated *cyb1* AC and demonstrated that the GAF domains were truly exchangeable. The EC_{50} for cGMP of 3 μ M corresponded precisely to that reported for cGMP stimulation of PDE2 activity (Sonnenburg *et al.*, 1991). cAMP, on the other hand, did not stimulate the *cyb1* any longer. In view of the huge evolutionary distance between cyanobacteria and mammals, the observed decrease in specific activity of *cyb1*_{rat-GAF} compared with that of *cyb1* may be rationalized in terms of the fact that the substitution boundaries were not optimally placed and/or that the intramolecular signal transduction to the catalytic domain was relaxed.

Fig. 6. cAMP activation of *cyb1* is mediated through the GAF-B domain, as demonstrated by GAF-inactivating mutations of Asp190 and Asp360 (D190A and D360A) in GAF-A and GAF-B domains, respectively. The assays were carried out with 83 and 25 nM protein for GAF-AD190A and GAF-BD360A, respectively, and 75 μ M Mg^{2+} -ATP at pH 7.5, 37°C $\pm$ 100 μ M cAMP as an activator. The insert shows a western blot of the purified proteins.

Discussion

A new arrangement of the catalytic fold in the *cyb1* AC

The catalytic centre of class III ACs is usually formed by two similarly folded protein domains, which donate amino acids to a 2-fold symmetric catalytic cleft (Tang and Gilman, 1995; Yan *et al.*, 1996, 1997). In agreement with mutational studies, the 2.8 Å AC structure identified the amino acids involved in catalysis (Tesmer *et al.*, 1997, 1999; Zhang *et al.*, 1997). In the *cyb1* AC, a threonine (T721) replaces a highly conserved aspartate, which in mammalian ACs serves as a hydrogen acceptor towards the N⁶ atom of the adenine moiety of ATP and is indispensable for activity, as shown by alanine replacement (Tang *et al.*, 1995; Tesmer *et al.*, 1997; Sunahara *et al.*, 1998). Our data suggest that threonine functionally replaces aspartate, because in a *cyb1*T721A mutant, AC activity was reduced by >95%. Most likely, T721 functions as a hydrogen acceptor as a result of subtle changes in the fine structure of the purin-binding pocket caused by peculiar *cyb1* sequence features, such as a single amino acid insertion (position 715) in an otherwise length-invariant segment. This interpretation is supported by the fact that *cyb1*T721A was a V_{max} mutant (K_M was unchanged; data not shown). This indicates that the amino acid at position T721 is less important for substrate docking and probably more involved in the correct orientation of the substrate towards the transition state. Substrate specificity in *cyb1* would then mainly rely on lysine 646 (supported by the finding that K646A or K646E mutants were K_M and V_{max} mutants; data not shown). It is noteworthy that a guanylyl cyclase from *Paramecium*, with a mammalian AC topology, has a serine at the position corresponding to T721. In this instance, the serine most likely serves as a hydrogen donor toward the O⁶ of the guanine moiety, whereas GTP substrate specification is achieved by a glutamate located at the position of K646 in *cyb1* (Linder *et al.*, 2000).

Fig. 7. Dose–response curve of cGMP- and cAMP-stimulated cyanobacterial AC activity, which contains the tandem GAF assembly from rat PDE2. The insert shows a western blot of the purified protein (100 ng applied/lane). cAMP, squares; cGMP, circles.

cAMP activates *cyaB1* via its GAF domains

GAF domains were identified in a formate-activated transcription factor in *E.coli* and in mammalian PDEs (Hopper and Böck, 1995; Aravind and Ponting, 1997). In the latter, they are involved in regulation of PDE activity and serve as cGMP receptor domains (Sonnenburg *et al.*, 1991; McAllister-Lucas *et al.*, 1993; Soderling and Beavo, 2000). Using bioinformatics, GAF domains have now been detected in >1100 proteins from all kingdoms of life and are expected to serve as receptors for a variety of as yet unidentified small molecules (Aravind and Ponting, 1997; Anantharaman *et al.*, 2001). Our data establish cAMP as a new ligand and define GAF domains as a novel cAMP receptor in the terminology used to describe cGMP receptors (Ho *et al.*, 2000). The differences in the $V_{\max}$ values of *cyaB1* in the absence and presence of the activator cAMP, and the uniform increase in the quotient of $V_{\max}/K_M$ (Table I), would argue that cAMP binding to the GAF-B domain has a direct bearing on the catalytic site. The nature of this coupling is a pressing question that awaits investigation.

Based on mutational (GAF domains of PDE5) and structural efforts (YKG9), the cGMP binding to PDE GAF domains was modelled to involve the NKX_nFX₃DE motif (McAllister-Lucas *et al.*, 1995; Turko *et al.*, 1996; Granovsky *et al.*, 1998; Ho *et al.*, 2000). A most recent X-ray structure of the PDE2 GAF domains shows that none of the amino acids of this motif makes contact with cGMP (S.F.Martinez, A.Y.Wu, N.A.Glavas, X.B.Tang, S.Turley, W.G.J.Hol, J.A.Beavo, submitted for publication). In the novel structure, cGMP specificity is based on hydrogen bonds established by an aspartate not previously implicated in ligand binding (to the protonated N¹ via the carboxyl and to O⁶ via the backbone amide) and a threonine (D434 and T483 in the rat GAF-B domain, marked by arrowheads in Figure 1C). In the cyanobacterial GAF-B domain, the threonine is conserved (T323), whereas an alanine (A275) takes the position of the

aspartate (Figure 1C). An attempt to change the cyclic nucleotide specificity of the *cyaB1* GAF-B domain from cAMP to cGMP in a *cyaB1A275D* mutant did not materialize (data not shown). This was not completely unexpected because the conserved threonine, which forms a hydrogen bond with the N²-amino group of cGMP in PDE2-GAF-B, cannot similarly connect to cAMP as it does not have an N²-amino group. Therefore, different binding modes for cyclic nucleotides are likely to exist in the rat and cyanobacterial GAF-B domains.

This is the second instance in this study where we cannot entirely reconcile excellent high resolution X-ray structures with our experimental data. We believe that the X-ray structures applicable to this work, the mammalian class III AC catalyst (Tesmer *et al.*, 1997, 1999; Zhang *et al.*, 1997) and the GAF domains of PDE2 (S.F.Martinez, A.Y.Wu, N.A.Glavas, X.B.Tang, S.Turley, W.G.J.Hol, J.A.Beavo, submitted for publication), describe the overall domain conformation of the *cyaB1* catalyst and GAF domain ensemble adequately, albeit in a frozen state. Our observations underline the fact that rigid structures often cannot be extended unmodified to the flexible fine structure of a small-molecule-binding site, such as a substrate- or ligand-binding site, which actually may be akin to a fingerprint of a particular protein.

cyaB1 distinctly prefers Mg²⁺ over Mn²⁺ as a metal cofactor for catalysis, in contrast to most other ACs, yet the almost identical ratios of $V_{\max}/K_M$ for Mg²⁺- and Mn²⁺-supported activities indicate an equal catalytic efficiency. Interestingly, with Mn²⁺ as a divalent cation, the cAMP activation dose–response curve is biphasic. This suggests that with Mn²⁺, cAMP may bind at two different sites, a low- and a high-affinity site, whereas with Mg²⁺, only a single high-affinity site seems to be occupied. Conceivably, a divalent cation may enhance or even be required for cAMP binding to the GAF-B domain in addition to its function in the cyclization reaction. Mg²⁺ and Mn²⁺ may serve differently in these roles.

Anabaena sp. PCC 7120 has at least six genes coding for functional ACs (Katayama and Ohmori, 1997; Kasahara *et al.*, 2001). For the maintenance of intracellular cAMP levels, the most relevant AC isoform seems to be *cyaC*, which consists of a response regulator, a histidine kinase, a second response regulator and a single catalytic domain (Katayama and Ohmori, 1997). What then are the possible physiological functions of the self-activating *cyaB1* AC? Looking at the multiple differentiation potentials of *Anabaena*, there seems to be room for all AC isoforms, as they considerably expand the regulatory possibilities. As a filamentous bacterium, *Anabaena* assimilates carbon dioxide. Under light, yet without fixed nitrogen, it terminally differentiates about every tenth cell into a heterocyst, which provides an environment for oxygen-sensitive nitrogen assimilation (Adams and Duggan, 1999; Wolk, 2000). In addition, proheterocysts are formed, which, contrary to heterocysts, can dedifferentiate when necessary. Furthermore, akinetes (spore-like cells) are developed that survive dry periods, and hormogonia exist, which are short mobile filaments used for dispersal. These multiple possibilities for differentiation in response to environmental conditions certainly involve intra- and intercellular signalling processes at many levels. In such a scenario, a role for an autoactivating AC can easily

be envisaged because such a behaviour would tend to stabilize committed developmental decisions. In this sense, *cyaB1* may constitute a cAMP switch that senses and interprets signals of changing environmental conditions, and accordingly initiates and maintains lasting developmental programs. Obviously, it will be interesting to see how such a switch is turned off.

Generation of a cGMP-activated *cyaB1* AC

GAF domains are not known to possess an intrinsic enzyme activity. They fit the classification as a regulatory small-molecule-binding domain (Aravind and Ponting, 1997; Anantharaman *et al.*, 2001). The distribution of GAF domains among all phyla suggests that this module has been optimized early in evolution and been reused in differing regulatory contexts. So much can be deduced from bioinformatic studies. We now provide a striking experimental example of this conclusion. Replacing the cyanobacterial GAF tandem with that from rat PDE2, we generated a cGMP-activated AC. The cGMP concentration needed for half-maximal activation was 3 μ M, i.e. identical to that for PDE2 activation, whereas cAMP had no effect (Sonnenburg *et al.*, 1991). This raises the question of how the signal generated by binding of the ligand to the GAF domain is transmitted to the catalytic domain. This is particularly intriguing because the rat PDE2 GAF domains operate in a protein background of *cyaB1*, in which an intervening PAS domain and an extended linker region separate the catalyst from the GAF assemblage (Figure 1A). We propose that the mechanism of GAF-domain activation, as accomplished by the movement of structurally rigid protein domains by a fixed angle and distance, is largely context independent (S.F.Martinez, A.Y.Wu, N.A.Glavas, X.B.Tang, S.Turley, W.G.J.Hol, J.A.Beavo, submitted for publication). The principle of such a simple and successful regulatory switch seems to have been maintained for >2 billion years and spread to all phyla, to be used in conjunction with many different effector domains.

Materials and methods

Recombinant DNAs

The *cyaB1* gene (DDBJ/EMBL/GenBank accession No. D89623; gift of Professor M.Ohmori, University of Tokyo) was assembled into the *Bam*HI–*Sma*I sites of pQE30 via subcloning steps involving pBluescriptII SK(–). With *cyaB1* as a template, *cyaB1*_{595–781} and *cyaB1*_{595–859} were amplified by PCR using specific primers. All recombinant proteins were fitted with an N-terminal MRGSH₆GS affinity tag. Single amino acid mutations (K646A, K646E, D650A, N728A, R732A and T721A in the catalytic domain, and D190A, D360A and A275D in the GAF domain) were introduced by site-directed mutagenesis using respective PCR primers and the DNA coding for *cyaB1*_{1–859} as a template. Restriction sites located nearby were used where appropriate or were inserted by silent mutations.

The GAF domain from rat PDE2 (E207–N546) was amplified from a rat intestine cDNA library using specific primers. *Sfi*I and *Xho*I sites were added at the 5′- and 3′-ends, respectively. The PCR product was inserted into *Sfi*I and *Xho*I sites of *cyaB1*, which were introduced by silent mutations, and replaced the cyanobacterial sequence from Q50 to L385 in *cyaB1* (see Figure 1C). The fidelity of all constructs was verified by double-stranded sequencing. Primer sequences are available on request.

Expression and purification of bacterially expressed proteins

The constructs in the pQE30 expression plasmid were transformed into *E.coli* BL21(DE3)[pREP4]. Cultures were grown in Lennox L broth at 25°C containing 100 mg/l ampicillin and 25 mg/l kanamycin. For

*cyaB1*_{595–859} and its mutants, cultures were induced with 300 μ M isopropyl- β -D-thiogalactopyranoside and incubated at 27°C for 3 h. For *cyaB1*_{1–859}, its mutants and the *cyaB1*–ratPDE2 GAF chimera (*cyaB1*_{rat-GAF}), the cultures were induced with 7.5 or 15 μ M isopropyl- β -D-thiogalactopyranoside at an *A*₆₀₀ of 0.6 and grown overnight at 20°C. Bacteria were harvested and washed once with 50 mM Tris–HCl, 1 mM EDTA pH 8.5 and stored at –80°C. For purification, frozen cells were suspended in 20 ml of cell lysis buffer (50 mM Tris–HCl pH 8.5, 50 mM NaCl, 10 mM 1-thioglycerol and 2 mM MgCl₂) and sonicated (3 $\times$ 10 s). Cell debris was removed by centrifugation (48 000 g for 30 min). Nickel-NTA slurry (200 μ l; Qiagen, Hilden, Germany) was added to the supernatants. After gentle rocking for 60 min at 0°C, the resin was poured into a column and washed (2 ml/wash) (wash buffer A: 50 mM Tris–HCl pH 8.5, 10 mM 1-thioglycerol, 2 mM MgCl₂, 400 mM NaCl, 5 mM imidazole; wash buffer B: wash buffer A with 15 mM imidazole; wash buffer C: wash buffer A with 10 mM NaCl and 15 mM imidazole). Proteins were eluted with 0.5–1 ml of buffer C containing 150 mM imidazole. For *cyaB1*_{1–859}, its mutants and the *cyaB1*_{rat-GAF} chimera, all buffers contained 20% glycerol. Because of the poor stability of *cyaB1*_{595–859} and *cyaB1*_{1–859} in the presence of 150 mM imidazole, the eluates were immediately dialysed overnight against 50 mM Tris–HCl pH 8.5, 10 mM NaCl, 2 mM MgCl₂ (for *cyaB1*_{595–859} with 2 mM 1-thioglycerol) and 20% glycerol. The purified proteins could be stored in dialysis buffer at –20°C.

AC assay

The AC activity of *cyaB1*_{595–859} and its mutants was measured for 4 min at 45°C in a final volume of 100 μ l (Salomon *et al.*, 1974). The reactions usually contained 22% glycerol, 50 mM MOPS–Na pH 8.5, either 2 mM MnCl₂ or 10 mM MgCl₂, and 75 μ M [α -³²P]ATP (25 kBq) and 2 mM [2,8-³H]cAMP (150 Bq) to determine yield during product isolation. Creatine phosphate (3 mM) and 1 U of creatine kinase were used as an ATP-regenerating system when assaying crude extracts. To determine kinetic constants, the concentration of ATP was varied from 2 to 1600 μ M, with a fixed concentration of 2 mM Mn²⁺ or 10 mM Mg²⁺. The protein concentration was adjusted to keep substrate conversion to <10%. The reaction was started by addition of substrate.

For *cyaB1*_{1–859}, its mutants and the *cyaB1*_{rat-GAF} chimera, the samples were incubated for 4 min at 37°C in a buffer containing 22% glycerol, 50 mM Tris–HCl pH 7.5, either 2 mM MnCl₂ or 10 mM MgCl₂, and 75 μ M [α -³²P]ATP (25 kBq). [2,8-³H]cAMP was added after stopping the reaction together with the stop buffer (Salomon *et al.*, 1974). To determine kinetic constants, the concentration of ATP was varied from 2 to 240 μ M with a fixed concentration of 2 mM Mn²⁺ or 10 mM Mg²⁺. Usually, <10% of the ATP was consumed at the lowest substrate concentrations. The data are means from several experiments. Unless indicated, the standard deviations (SD) were usually <5%.

cAMP binding assays

Binding of cAMP was assayed using a modification of previously described protocols (McAllister-Lucas *et al.*, 1995; Ho *et al.* 2000). Briefly, 2 μ g of affinity-purified *cyaB1* or *cyaB1D360A* were incubated for 1 h at 0°C in the presence of 10 mM sodium phosphate buffer pH 6.8, 1 mM EDTA and various concentrations (0.5–10 μ M) of cAMP (with trace [³H]cAMP). Samples were applied to pre-moistened HAWP filters (0.45 μ m; Sartorius, Göttingen, Germany) and rinsed with 10 ml of 10 mM phosphate buffer pH 6.8 containing 1 mM EDTA. Dry filters were counted for ³H using scintillation counting. Background levels of nucleotide binding to filters, as determined with 2 μ g of BSA/incubation instead of enzyme, were subtracted.

Western blot analysis

Protein was mixed with sample buffer and subjected to SDS–PAGE (12.5%). Proteins were blotted onto PVDF membranes and sequentially probed with a commercial anti-RGS-His₄ antibody (Qiagen) and with a 1:5000 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, Freiburg, Germany).

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