

A subset of GAF domains are evolutionarily conserved sodium sensors.

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1 Abstract.

2 Most organisms maintain a transmembrane sodium gradient for cell
3 function. Despite the importance of Na⁺ in physiology, no directly Na⁺ responsive
4 signalling molecules are known. The CyaB1 and CyaB2 adenylyl cyclases of the
5 cyanobacterium *Anabaena* PCC 7120 are inhibited by Na⁺. A D360A mutation in the
6 GAF-B domain of CyaB1 ablated cAMP mediated autoregulation and Na⁺ inhibition. Na⁺
7 bound the isolated GAF domains of CyaB2. cAMP blocked Na⁺ binding to GAF domains
8 but Na⁺ had no affect on cAMP binding. Na⁺ altered GAF domain structure indicating a
9 mechanism of inhibition independent of cAMP binding. Δ *cyaB1* and Δ *cyaB2* mutant
10 strains did not grow below 0.6 mM Na⁺ and Δ *cyaB1* cells possessed defects in Na⁺/H⁺
11 antiporter function. Replacement of the CyaB1 GAF domains with those of rat
12 phosphodiesterase type 2 revealed that Na⁺ inhibition has been conserved since the
13 eukaryotic/bacterial divergence. CyaB1 and CyaB2 are the first identified directly Na⁺
14 responsive signalling molecules that function in sodium homeostasis and we propose a
15 subset of GAF domains underpins an evolutionarily conserved Na⁺ signalling mechanism.

16 Introduction.

17 The ability to maintain appropriate intracellular inorganic ion
18 concentrations when challenged by extracellular fluctuations is among the most ancient
19 and fundamental cellular processes. Sodium is an essential cation whose intracellular
20 levels can be maintained by primary and secondary transport, for example, by Na⁺-
21 ATPases and Na⁺/K⁺-ATPases in animal cells and H⁺-ATPases, ion channels, and co-
22 transporters in plant cells (Pedersen, 2005; Zhu, 2003). Typically, intracellular
23 concentrations of Na⁺ are maintained much lower than in the extracellular milieu. This
24 transmembrane Na⁺ gradient can be used as the basis for electrical signalling or can be
25 coupled to electrochemically unfavourable solute flow.

26 Na⁺ homeostatic response mechanisms enable cells to adapt to increases or
27 decreases in environmental Na⁺ and their study is of great interest in both agriculture and
28 medicine. Salinity stress is one of many abiotic decertifying stressors that reduces
29 agricultural output by 50% worldwide and more than 50% of global arable lands may be
30 under salinity stress by 2050 (Boyer, 1982; Wang et al., 2003) Plants respond to salinity
31 stress through a number of distinct mechanisms including the homeostatic transport of
32 ions, control of water flux, and osmolyte biosynthesis (Hasegawa et al., 2000). Among the
33 human population, many normotensive and hypertensive patients are “salt sensitive” and
34 these patients show large fluctuations in blood pressure in response to salt repletion or
35 depletion (Franco and Oparil, 2006). Despite these fundamental roles for Na⁺ in biology,
36 no molecular mechanism by which fluctuations in Na⁺ concentration are directly detected
37 and signalled has been identified. Identification of such a direct detection mechanism
38 would be vital to understanding the biology of many medically and environmentally
39 important Na⁺-dependent processes.

40 The cyanobacteria are an excellent model for studying Na⁺ stress response
41 mechanisms. The identification of six specific histidine kinases and five response

42 regulators required for the full stimulation of salt-induced genes of *Synechocystis* PCC
43 6803 demonstrates that the bacterial two component system has a key role in Na⁺
44 detection in this organism (Murata and Suzuki, 2006). The demonstration that NaCl
45 dependent enhancement of heterocyst development in the filamentous N₂ fixing
46 cyanobacterium *Anabaena* PCC 7120 was dependent upon cAMP production by the
47 multi-domain CyaC adenylyl cyclase (AC) *in vivo* also raises the possibility that an
48 individual protein domain may detect a Na⁺ signal (Imashimizu *et al.*, 2005). A major
49 component of CyaC is a dual GAF (found in cGMP phosphodiesterases, adenylyl
50 cyclases, and FhlA [formate hydrogen lyase transcriptional activator]) domain motif. The
51 ubiquitous GAF domain is an important site of signal perception in many eukaryotes and
52 prokaryotes (Aravind and Ponting, 1997; Hurley, 2003). GAF domains from diverse
53 species have equally diverse ligands including bilin chromophores in plants and
54 cyanobacteria, haem in the DoS sensor of *Mycobacterium tuberculosis*, NO in the NorR
55 sensor of *Escherichia coli*, 2-oxoglutarate in NifA of *Azotobacter vinelandii*, and the
56 cyclic nucleotides cAMP and cGMP in cyanobacteria, unicellular parasitic eukaryotes,
57 and mammals (D'Autreaux *et al.*, 2005; Fischer *et al.*, 2005; Gross-Langenhoff *et al.*,
58 2006; Kanacher *et al.*, 2002; Laxman *et al.*, 2005; Little and Dixon, 2003; Rybalkin *et al.*,
59 2003; Sardiwal *et al.*, 2005).

60 The mammalian cyclic nucleotide phosphodiesterases (PDE) are integral to the
61 regulation of cellular levels of cAMP and cGMP by controlling the rate of degradation
62 (Martinez *et al.*, 2002a; Zoraghi *et al.*, 2004). At least eleven distinct families of PDE exist
63 whose activity can be regulated by their N-termini (Francis *et al.*, 2001; Houslay and
64 Adams, 2003). Of these regulatory domains PDEs types 2, 5, 6, 10, and 11 possess GAF
65 domains regulated by cyclic nucleotides (Erneux *et al.*, 1981; Gross-Langenhoff *et al.*,
66 2006; Mullershausen *et al.*, 2003; Rybalkin *et al.*, 2003; Zoraghi *et al.*, 2005). The CyaB1
67 and CyaB2 ACs of *Anabaena* PCC 7120 also bind cAMP through one (CyaB1) or two

68 (CyaB2) N-terminal GAF domains to mediate positive feedback regulation of a carbon
69 dioxide responsive AC domain (Bruder et al., 2005; Hammer et al., 2006; Kanacher et al.,
70 2002; Martinez et al., 2005). Although cyclic nucleotide binding to GAF domains in
71 multiple species is well characterized, molecules that control cyclic nucleotide regulation
72 of GAF domain function are not known.

73 Here we demonstrate that Na^+ binds to and inhibits cAMP dependent GAF domain
74 mediated positive feedback in the CyaB1 and CyaB2 ACs through blocking a
75 conformational change associated with cAMP binding to the GAF domain. Genetic
76 ablation of both the *cyaB1* and *cyaB2* genes gives strains defective in sodium homeostasis
77 mediated by a defect in Na^+/H^+ antiporter function. Na^+ regulation of GAF domain
78 function is conserved in the GAF domain of a mammalian PDE. CyaB1 and CyaB2 are
79 therefore the first ever identified signalling molecules that respond directly to Na^+ and
80 function in sodium homeostasis. Na^+ regulation of GAF domain function has also been
81 evolutionarily conserved over a period of several billion years and may represent a
82 widespread mechanism for Na^+ detection.

83 **Results.**84 **Na⁺ inhibits the CyaB1 adenylyl cyclase of *Anabaena*.**

85 The *cyaB1* (alr2266; <http://www.kazusa.or.jp/cyano/Anabaena/>) gene of
86 *Anabaena* PCC 7120 codes for a protein consisting of two tandem GAF (GAF-A and
87 GAF-B) domains, a PAS (found in periodic clock protein, aryl hydrocarbon receptor, and
88 single minded protein) domain, and a C-terminal AC catalytic domain. Purified full length
89 recombinant CyaB1 holoenzyme (CyaB1₁₋₈₅₉) is activated by inorganic carbon at pH 7.5
90 added as 10 mM KHCO₃ but not NaHCO₃ (Cann et al., 2003). This suggested that Na⁺
91 blocked an aspect of intramolecular signalling and we therefore assessed the response of
92 CyaB1₁₋₈₅₉ and a protein corresponding to only the CyaB1 AC catalytic domain
93 (CyaB1₅₉₅₋₈₅₉) to a range of monovalent cations. Li⁺ inhibited CyaB1₅₉₅₋₈₅₉ specific
94 activity but there was no significant influence of Na⁺ compared to basal activity (Figure
95 1A). In contrast CyaB1₁₋₈₅₉ specific activity was reduced two-fold in the presence of 20
96 mM Na⁺ when compared to basal activity and other cations (Figure 1B). CyaB1₁₋₈₅₉
97 specific activity was not affected by Li⁺ indicating that removal of N-terminal regulatory
98 domains from CyaB1 revealed a small cation-binding pocket in the catalytic domain. We
99 determined the dose response of CyaB1₁₋₈₅₉ inhibition by Na⁺ with K⁺ as control and
100 confirmed that the reduction in specific activity occurred over a biologically relevant
101 concentration range with an apparent I.C.₅₀ of 14.3±2.2 mM (S.D.) for Na⁺ (Figure 1C).
102 As Na⁺ is acting on recombinant protein the data is unambiguous and CyaB1₁₋₈₅₉
103 represents the first identified directly and specifically Na⁺ regulated signalling molecule.

104 As the AC domain is not the site of action of Na⁺ in the holoenzyme we
105 investigated the role of the GAF domains in the Na⁺ response. The GAF-B domain of
106 CyaB1₁₋₈₅₉ is an autoregulatory domain that binds cAMP and up regulates AC activity by
107 an unidentified mechanism while the GAF-A domain is of unknown function (Kanacher et
108 al., 2002). We hypothesized that Na⁺ inhibited AC activity by suppression of the cAMP-

109 mediated positive feedback loop and we reasoned that exogenous cAMP would
110 compensate for this inhibition. In support of this reasoning Na^+ increased the $E.C_{.50}$ for
111 cAMP activation of the AC domain to $0.4 \mu\text{M}$ from $0.17 \mu\text{M}$ in the presence of K^+ and
112 Na^+ inhibition was not observed at $1 \mu\text{M}$ cAMP and above (95% C.I.) (Figure 2A). More
113 specifically, Na^+ did not permit cAMP mediated activation of the AC domain at 30 nM
114 exogenous cAMP and below (95% C.I.) indicating that Na^+ likely represents a mechanism
115 to block uncontrolled activation of GAF domain positive feedback by low levels of
116 cAMP. As CyaB1_{1-859} autoregulation is mediated through the GAF-B domain we
117 investigated Na^+ inhibition of recombinant proteins in which either the GAF-A (CyaB1_{1-}
118 $_{859}\text{D190A}$) or GAF-B ($\text{CyaB1}_{1-859}\text{D360A}$) domains were functionally compromised. The
119 mutated aspartate forms part of a conserved $\text{NKX}_n\text{FX}_3\text{DE}$ motif in mammalian PDEs
120 essential for GAF domain functional integrity, assessed through cGMP binding, but does
121 not bind cGMP (Martinez et al., 2002b; McAllister-Lucas et al., 1995). Na^+ down
122 regulated $\text{CyaB1}_{1-859}\text{D190A}$ specific activity ($E.C_{.50}$ for cAMP= $1.48 \mu\text{M}$) compared to K^+
123 ($E.C_{.50}$ for cAMP= $0.67 \mu\text{M}$). Similar to wild type enzyme Na^+ did not permit cAMP
124 mediated activation of the AC domain at 30 nM and below and Na^+ inhibition was not
125 observed at $1 \mu\text{M}$ cAMP and above (95% C.I.) (Figure 2B). At any given concentration of
126 exogenous cAMP, fold inhibition of $\text{CyaB1}_{1-859}\text{D190A}$ specific activity is reduced relative
127 to wild type indicating that the GAF-A domain may have a subtle role in the inhibitory
128 mechanism. $\text{CyaB1}_{1-859}\text{D360A}$ specific activity was not responsive to Na^+ consistent with
129 a role for Na^+ in the inhibition of cAMP mediated autoregulation (Figure 2C). As CyaB1_{1-}
130 $_{859}\text{D360A}$ specific activity was maintained similar to the basal activity of the wild type
131 enzyme (Figure 2A), the loss of inhibition is not an artefact of altered enzyme activity.
132
133 **cAMP dependent Na^+ binding to the GAF domains of the CyaB2 adenylyl cyclase.**

134 The *cyaB2* gene (all1904) of *Anabaena* PCC 7120 codes for a protein with an
135 identical domain structure to CyaB1 (Katayama and Ohmori, 1997). In contrast to CyaB1,
136 however, cAMP binds to and signals through both the GAF-A and GAF-B domains
137 (Bruder et al., 2005; Martinez et al., 2005). The CyaB2 holoenzyme cannot be expressed
138 as a recombinant protein but the tandem GAF domains of CyaB2 can be fused to the PAS
139 and AC domains of CyaB1 to generate a chimeric recombinant protein (CyaB2-GAF-
140 CyaB1) that enables study of the CyaB2 GAF domains (Bruder et al., 2005). Similar to
141 CyaB1, the AC specific activity of the CyaB2 chimera is down regulated by Na⁺
142 compared to other monovalent cations (Figure 3A). It is important to note that the specific
143 inhibition of CyaB1₅₉₅₋₈₅₉ by Li⁺ is not observed in CyaB1₁₋₈₅₉ or CyaB2-GAF-CyaB1
144 indicating that loss of the GAF/PAS domain region exposed an additional cation binding
145 site but is most likely not of relevance *in vivo*. A dose response with Na⁺ gives an apparent
146 I.C.₅₀ of 24.5±0.9 mM (S.D.) (Figure 3B). The specific inhibitory effect of Na⁺ is also
147 compensated for by exogenous cAMP demonstrating that Na⁺ has a similar activity in
148 both CyaB1 and CyaB2 of inhibiting cAMP mediated autoregulation (Figure 3C).

149 To support a role for Na⁺ binding to and modulation of GAF domain activity in
150 CyaB1 and CyaB2 we sought evidence for a direct Na⁺-GAF domain interaction.
151 Unfortunately, the tandem GAF domain motif of CyaB1 cannot be expressed as a
152 recombinant protein for these studies but the tandem GAF domain of the CyaB2 AC of
153 *Anabaena* expresses at a high level (CyaB2 GAFA/B₅₈₋₄₄₅) (Martinez et al., 2005). As Na⁺
154 inhibits the specific activity of the CyaB1 AC domain when expressed with either the
155 CyaB1 or CyaB2 tandem GAF domain motif we reasoned that an analysis of CyaB2
156 GAFA/B₅₈₋₄₄₅ would inform our study of both the CyaB1 and CyaB2 enzymes.

157 SBFI is a fluorescent indicator specific for Na⁺ and has a K_D for Na⁺ (3.8 mM in
158 the absence of K⁺) of a similar order of magnitude as the I.C.₅₀ for CyaB2-GAF-CyaB1.
159 We reasoned that adding a protein that specifically binds Na⁺ to a SBFI/Na⁺ mix would

160 manifest as a decrease in SBF1 fluorescence emission. CyaB2 GAFA/B₅₈₋₄₄₅ specifically
161 bound Na⁺ to a substantially greater degree than K⁺ with a Hill Slope of 0.94±0.05 (S.D.)
162 (Figure 4A). Na⁺ therefore binds and functions specifically at the GAF domains and not an
163 alternative region of the enzyme. A thermodynamic analysis gave a binding constant for
164 Na⁺ (K_i) of 302.3±21.1 μM Na⁺ (S.D.), a value significantly lower than the I.C.₅₀ values
165 obtained by enzymology. Addition of cAMP to the quenching assay at an elevated
166 concentration sufficient to saturate the necessarily high protein concentration in the assay
167 blocked Na⁺ binding to CyaB2 GAFA/B₅₈₋₄₄₅ supporting the biochemistry and
168 demonstrating that cAMP compensates for Na⁺ inhibition by displacing Na⁺ from the
169 GAF domain (Figure 4B).

170 Given that Na⁺ bound to a model tandem GAF domain protein and was displaced
171 by cAMP we sought further evidence of the mechanism of Na⁺ inhibition of GAF domain
172 function. We used isothermal microcalorimetry to assess the effect of Na⁺ on cAMP
173 binding to CyaB2 GAFA/B₅₈₋₄₄₅. Microcalorimetry has been used successfully to
174 demonstrate cAMP binding to the GAF-A domain of the TcrPDEB2 PDE of *Trypanosoma*
175 *cruzi* and 2-oxoglutarate binding to the NifA GAF domain from *Azotobacter* (Diaz-
176 Benjumea et al., 2006; Martinez-Argudo et al., 2004). Remarkably, Na⁺ had no specific
177 effect on the affinity of CyaB2 GAFA/B₅₈₋₄₄₅ for cAMP with dissociation constants of
178 0.87±0.07 (basal conditions), 0.98±0.06 (50 mM Na⁺), and 1.04±0.01 (50 mM K⁺) μM
179 cAMP (S.D.).

180 As cAMP is able to displace Na⁺ but not vice versa we investigated the role of Na⁺
181 in an event associated with the tandem GAF domain but which is independent of cAMP
182 binding. Aromatic amino acids display chiroptical activity at near UV wavelengths.
183 Chiroptical activity between 250 and 300 nm is particularly sensitive to changes in protein
184 tertiary structure (Sreerama and Woody, 2000). Obtained circular dichroism spectra for
185 CyaB2 GAFA/B₅₈₋₄₄₅ with 50 mM K⁺ or in the absence of salt are essentially identical both

186 in the presence and absence of cAMP (Figure 5). In contrast spectra in the presence of 50
187 mM Na⁺ are significantly different. This demonstrated that Na⁺ effected a change in the
188 tertiary structure of CyaB2 GAFA/B₅₈₋₄₄₅ and is the likely cause of the disruption in GAF
189 domain activity.

190

191 **CyaB1 and CyaB2 are required for sodium homeostasis at limiting sodium in**
192 ***Anabaena*.**

193 To determine whether the Na⁺ inhibition of CyaB1₁₋₈₅₉ and CyaB2-GAF-CyaB1 *in*
194 *vitro* was of functional significance *in vivo*, we examined the Na⁺ homeostatic response of
195 *Anabaena* PCC 7120 strains ablated for *cyaB1* or *cyaB2* gene function (Δ *cyaB1* and
196 Δ *cyaB2* respectively) compared to wild type cells. The growth rates of wild type and
197 Δ *cyaB1* cells in a standard defined medium for *Anabaena* culture containing normal (4
198 mM) Na⁺ or elevated (40 mM) Na⁺ were indistinguishable (data not shown). We therefore
199 examined the homeostatic response of wild type, Δ *cyaB1*, and Δ *cyaB2* cells at limiting
200 Na⁺ concentrations. Significantly, Δ *cyaB1* and Δ *cyaB2* cells were unable to grow below
201 0.6 mM Na⁺ while wild type cells were able to grow at concentrations at least as low as
202 0.2 mM Na⁺ (Figure 6A). This effect is independent of osmolarity or ionic strength as
203 identical results are obtained when the removed Na⁺ is replaced with K⁺. The nitrogen
204 growth regime for Δ *cyaB1* cells did not affect the lethality at low Na⁺ (data not shown).
205 The *cyaA* gene (all1118) encodes a protein with an AC domain but no predicted GAF
206 domains. The growth of Δ *cyaA* cells was indistinguishable from wild type consistent with
207 a role for the GAF domain ensemble in Na⁺ signalling rather than a non-specific effect of
208 loss of an AC domain. N⁶, 2'-O-dibutyryladenine 3', 5'-cyclic monophosphate (db-
209 cAMP) is a cell-permeable cAMP analogue that can be used to effect entry of cAMP into
210 the cell. Inclusion of db-cAMP in the medium rescued growth of both Δ *cyaB1* and Δ *cyaB2*

211 cells at 0.2 mM Na⁺ after extended culture periods (Figure 6B). This demonstrated that the
212 inability to grow at limiting Na⁺ in the strains defective in AC expression could be
213 compensated for by cAMP.

214 Decreased environmental pH can compensate for a Na⁺ deficiency in the growth of
215 *Anabaena* (Abe et al., 1987). Consistent with this, growth of Δ *cyaB1* and Δ *cyaB2* cells
216 was partially rescued in 0.2 mM Na⁺ containing media at pH 7.0 while growth was not
217 supported at pH 8.0 (Figure 6C). The ability of an increased H⁺ concentration to
218 compensate for lowered environmental Na⁺ suggested that aspects of Na⁺ dependent H⁺
219 flux were abnormal in Δ *cyaB1* and Δ *cyaB2* cells grown at 0.2 mM Na⁺. We therefore
220 examined the phenotype of Δ *cyaB1* cells in more detail with respect to Na⁺/H⁺ antiporter
221 activity. Of the pH sensitive fluorescent probes assessed for use in cyanobacteria, acridine
222 orange has been demonstrated to be of general utility in monitoring intracellular Δ pH
223 (Teuber et al., 2001). Partitioning of acridine orange into an acidic intracellular space has
224 been used as a non-calibrated method to assess Na⁺/H⁺ antiporter activity in mammalian
225 cells and cyanobacteria (Blumwald et al., 1984; Elanskaya et al., 2002; Reenstra et al.,
226 1981; Wang et al., 2002). We therefore investigated Na⁺-dependent intracellular and
227 extracellular acidification in wild type and Δ *cyaB1* cells. Wild type cells pre-incubated for
228 24 hours at 0.2 mM Na⁺ showed a partitioning of acridine orange into an acidic
229 intracellular space when diluted into a Na⁺-free assay buffer (Figure 7, phase I) and this
230 partitioning was reversed on addition of 4 mM Na⁺ due to an alkalization of the
231 intracellular space (Figure 7, phase II). In contrast, Δ *cyaB1* cells showed an abnormal
232 partitioning of acridine orange after pre-incubation at 0.2 mM Na⁺ for 24 hours and this
233 was not fully reversed by addition of 4 mM Na⁺ (Figure 7) while Δ *cyaA* cells were
234 indistinguishable from wild type (data not shown). Acridine orange partitioning was
235 identical in wild type and Δ *cyaB1* cells grown at 4 mM Na⁺ and in cells exposed to a

236 transient downshift from 4 to 0.2 mM Na⁺ (data not shown). The requirement for
237 prolonged exposure to 0.2 mM Na⁺ to observe a phenotype in wild type versus Δ *cyaB1*
238 cells suggested a requirement for new protein synthesis in the homeostatic response to low
239 Na⁺. Indeed, wild type cells incubated for 24 hours at 0.2 mM Na⁺ in the presence of the
240 translational inhibitor chloramphenicol (400 μ g/ml) showed a defective phase I response
241 compared to cells in the absence of inhibitor (data not shown). Δ *cyaB1* cells grown at 0.2
242 mM Na⁺ therefore have a clear defect in Na⁺-dependent intracellular acidification and
243 alkalization. This indicated that Na⁺/H⁺ antiporter function is abnormal in Δ *cyaB1* cells
244 grown under conditions of low Na⁺.

245

246 **Na⁺ regulation is conserved in mammalian GAF domains.**

247 As we have demonstrated that Na⁺ specifically blocks cAMP mediated
248 autoregulation of a model cyanobacterial tandem GAF domain with functional
249 consequences for the Na⁺ stress response *in vivo* we asked whether Na⁺ regulation of GAF
250 domain function is conserved among other species. Select cyclic nucleotide regulated
251 GAF domains of the mammalian PDEs functionally couple to and regulate the activity of
252 the CyaB1 AC domain in chimeric molecules (Bruder et al., 2006; Gross-Langenhoff et
253 al., 2006; Kanacher et al., 2002). As we have defined the tandem GAF domain of the
254 *Anabaena* ACs as the Na⁺ responsive region of the protein we reasoned that introduction
255 of mammalian PDE GAF domains to generate chimeric CyaB1 proteins would enable us
256 to address specifically whether the mammalian GAF domains are able to respond to Na⁺.
257 A chimeric protein consisting of the rat PDE2 tandem GAF domain and CyaB1 PAS and
258 AC domains (PDE2-GAF-CyaB1) gives a protein with cGMP activated GAF domains that
259 in turn activate the CyaB1 AC domain (Kanacher et al., 2002). We examined the response
260 of PDE2-GAF-CyaB1 to Na⁺ with K⁺ as control over a range of cGMP concentrations and
261 found that Na⁺ inhibited AC activity relative to K⁺ under conditions of GAF domain

262 activation by cGMP (Figure 8A). Assay in the presence of a range of monovalent cations
263 demonstrated that this effect was specific for Na⁺ (Figure 8B). Inhibition was specific for
264 Na⁺ relative to K⁺ over a broad concentration range and gives an I.C.₅₀ of 47.4±10.0 mM
265 (S.D.) (Figure 8C). The principle of Na⁺ regulation of GAF domain function is therefore
266 conserved over a period of several billion years of evolution and can function in the
267 context of structurally diverse proteins.

268 **Discussion.**

269 The CyaB1 and CyaB2 ACs of *Anabaena* represent the first identified signal
270 transduction molecules whose activity is modulated specifically by Na⁺ and are
271 functionally required for sodium homeostasis. The dependence of Na⁺ inhibition in CyaB1
272 on GAF-B domain functional integrity and ablation of the Na⁺ response by exogenous
273 cAMP demonstrated that Na⁺ acts at the GAF domain to block cAMP mediated
274 autoregulation at low cAMP concentrations. The necessity for this process *in vivo* is clear;
275 without an inhibitor of autoregulation, activation of the AC domain would proceed
276 unchecked and uncontrolled. By demonstrating conservation of Na⁺ inhibition in a
277 CyaB2-CyaB1 chimeric molecule we were able to use the well characterized isolated
278 tandem GAF domain motif of CyaB2 to investigate the Na⁺ binding mechanism. Na⁺ but
279 not K⁺ bound effectively to the isolated GAF domains underpinning the results of the
280 biochemistry. The observation that cAMP blocked Na⁺ binding further supported the
281 biochemical finding that exogenous cAMP ablated Na⁺ inhibition of the holoenzyme.

282 Interestingly, Na⁺ had no effect on the affinity of the GAF domains for cAMP
283 demonstrating binding most likely occurred at distinct sites. A probable interpretation of
284 the data is that Na⁺ stabilized the GAF domain in a conformation that is unable to signal in
285 the presence of low levels of cAMP. Increased cAMP displaced Na⁺ and preferentially
286 altered the structure of the GAF domain to a new conformation capable of signalling.
287 Consistent with this hypothesis, non-cAMP bound Na⁺-free GAF domains have a distinct
288 tertiary structure compared to non-cAMP bound GAF domains in the presence of Na⁺ as
289 assessed by circular dichroism. Addition of cAMP to the Na⁺ free GAF domains affects a
290 distinct shift in tertiary structure that is consistent with signalling and most likely identical
291 to that of the recent crystal structure (Martinez et al., 2005). The spectrum of the GAF
292 domains in the presence of cAMP and Na⁺ is harder to interpret, as the cAMP
293 concentration used should mostly saturate the GAF domains. However, the fluorescence

294 quenching experiments demonstrate that a ten-fold molar excess of cAMP over protein
295 does not displace all of the bound Na^+ (Figure 4B). The GAF domain- Na^+ -cAMP
296 spectrum may therefore represent an average of a minor contribution of the GAF- Na^+
297 bound state to the Na^+ -free cAMP bound spectrum. Multiple conformational states have
298 also been proposed for the tandem GAF domain motif of mammalian PDE5 (Rybalkin et
299 al., 2003).

300 The I.C._{50} for Na^+ for both CyaB1 and CyaB2 are seemingly high for an enzyme
301 that must respond to much lower concentrations of Na^+ *in vivo*. Competition binding
302 analysis with SBFI, however, gave an affinity of the CyaB2 GAF domains (measured as
303 K_i) of $302.3 \pm 21.1 \mu\text{M Na}^+$. We speculate that cAMP levels in the AC assays are sufficient
304 to partially block Na^+ binding giving artificially raised values for the I.C._{50} that are not
305 reflective of the true affinity. The affinity of the GAF domains for Na^+ is well within the
306 range of Na^+ concentrations likely to exist in the intracellular environment.

307 Importantly, the observed biochemistry is not an *in vitro* artefact. Both ΔcyaB1 and
308 ΔcyaB2 cells show defects in Na^+ homeostasis at limiting Na^+ concentrations. A scenario
309 consistent with the biochemistry is that local intracellular Na^+ concentrations at 4 mM
310 extracellular Na^+ are sufficient to block cAMP mediated autoregulation of CyaB1 (Figure
311 9A). A drop in intracellular Na^+ precipitated by a fall in extracellular Na^+ then permits
312 autoregulatory activation of the AC by basal cAMP concentrations (Figure 9B). The
313 mechanism of cell death at limiting Na^+ is most likely due to defects in Na^+/H^+ antiporter
314 function. Na^+/H^+ antiporters essential for survival at low environmental Na^+ have been
315 characterized in the model cyanobacterium *Synechocystis* PCC 6803 (Mikkat *et al.*, 2000;
316 Wang *et al.*, 2002). Mutations in the *sll0273 (nhaS2)* and *slr1595 (nhaS4)* genes encoding
317 Na^+/H^+ antiporters have distinct defects in acridine orange partitioning. The observation
318 that ΔcyaB1 cells had a similar defect in an identical phenotypic assay argues persuasively
319 that CyaB1 is required to regulate the activity of Na^+/H^+ antiporters required for growth at

320 low Na^+ (Figure 9B). Unfortunately we were unable to detect an increase in cellular
321 cAMP in response to a drop in medium Na^+ . This finding is not entirely surprising as the
322 contribution of CyaB1 and CyaB2 to the cellular cAMP pool is not detectable (Katayama
323 and Ohmori, 1997). We were, however, able to rescue both mutant strains with exogenous
324 cAMP demonstrating that the Na^+ homeostasis defects were caused by an inability to
325 produce cAMP in response to a drop in extracellular Na^+ .

326 The mammalian PDEs catalyze the hydrolysis of cyclic nucleotides and are an
327 important mechanism for regulating cyclic nucleotide levels in the cell (Baillie et al.,
328 2005). The GAF domains of the mammalian PDEs bind cAMP or cGMP and regulate the
329 activity of the PDE catalytic domain (Martinez et al., 2002a; Zoraghi et al., 2004).
330 Replacement of the tandem GAF domain motif of CyaB1 with those of mammalian PDEs
331 permits activation of the AC domain by the specific cyclic nucleotide that binds the PDE
332 GAF domain motif. Such chimeric molecules are excellent tools to ask whether Na^+
333 regulates mammalian GAF domains by isolating them from the remainder of the PDE
334 molecule. Na^+ inhibited the function of the cGMP binding GAF domains of rat PDE type
335 2. Consistent with a role for Na^+ in blocking GAF domain activation, Na^+ had no effect on
336 AC activity in the absence of exogenous cGMP but inhibited AC specific activity in the
337 presence of cGMP. The difference in the requirements for cyclic nucleotide concentrations
338 for Na^+ inhibition between CyaB1₁₋₈₅₉/CyaB2-GAF-CyaB1 (low concentrations) and
339 PDE2-GAF-CyaB1 (elevated concentrations) indicates that the exact mechanism of Na^+
340 regulation may differ between enzymes. This may, however, be a reflection of the
341 differing topology of GAF domain structure between enzymes (Martinez et al., 2005) and
342 does not preclude the possibility of a similar site of action. Inhibitory Na^+ concentrations
343 for the PDE2 chimera are consistent with intracellular Na^+ concentrations of mammalian
344 cells exposed to high extracellular Na^+ (for example see (Efendiev et al., 2003; Komlosi et

345 al., 2003) indicating that regulation of cyclic nucleotide levels by PDEs can serve as a
346 mechanism to directly respond to changes in Na⁺ concentration.

347 The demonstration of functional regulation of GAF domain activity in *Anabaena*
348 by Na⁺ and the conservation of this biochemistry in mammalian PDEs demonstrates that a
349 subset of GAF domains represent a mechanism for Na⁺ detection and signalling conserved
350 over two billion years of evolution.

351 Experimental Procedures.

352 *Recombinant proteins:* The CyaB1₅₉₅₋₈₅₉, CyaB1₁₋₈₅₉ (wild type, D190A, and D360
353 mutations), CyaB2-GAF-CyaB1, PDE2-GAF-CyaB1, PDE10A1-GAF-CyaB1, and
354 CyaB2 GAFA/B₅₈₋₄₄₅ were expressed and purified as previously described except that
355 NaCl was omitted from all dialysis buffers (Bruder et al., 2005; Kanacher et al., 2002;
356 Martinez et al., 2005).

357

358 *Adenylyl cyclase assays:* The AC activity of all wild type and mutant AC proteins was
359 assessed in a final volume of 100 μ L (Salomon et al., 1974). Reactions contained 22%
360 glycerol, 50 mM Tris.HCl pH 7.5 as buffer, 10 mM MgCl₂ as divalent metal cofactor, and
361 75 μ M [α -³²P]ATP (25 kBq) as substrate unless otherwise indicated. 2 mM [2, 8-
362 ³H]cAMP was added to the terminated assays to determine yield during product isolation.
363 All assays were performed at 37°C. Assay conditions were adjusted to keep substrate
364 conversion <10% unless otherwise indicated. The data represent the means of independent
365 experiments and error bars represent the standard error. Absent error bars indicates that
366 the S.E.M. was smaller than the symbol used to indicate the data point.

367

368 *Sodium binding fluorescence-quenching assay:* Sodium fluorescence was measured using
369 a Jasco FP-6200 Spectrofluorimeter. Assays were performed in a final volume of 50 μ L
370 and contained 25% glycerol, 50 mM Tris.HCl pH 7.5 as buffer, 10 mM MgCl₂, and,
371 where required, 0.5 μ M sodium-binding benzofuran isophthalate or potassium-binding
372 benzofuran isophthalate (SBFI/PBFI; Molecular Probes) and 3.8 mM NaCl or KCl.
373 Fluorescence was excited at 340 nm and 380 nm and excitation measured at 505 nm using
374 a band width of 5 nm for both excitation and emission wavelengths. Emission in the
375 absence of indicator was subtracted from all data points. Error bars represent the S.E.M.

376

377 *Isothermal microcalorimetry:* Binding of cAMP to CyaB2 GAFA/B₅₈₋₄₄₅ was assessed by
378 titration isothermal calorimetry using a MicroCal VP-ITC and data analyzed using
379 dedicated Microcal/Origin® software. A 1.42 ml volume containing 50 mM Tris.HCl pH
380 7.5, 25% glycerol, 2 mM MgCl₂, and 30 μM CyaB2 GAFA/B₅₈₋₄₄₅ in the presence or
381 absence of 50 mM salt was titrated with cAMP in the same buffer. The heat of dilution of
382 cAMP into buffer alone was subtracted from all data.

383

384 *Circular dichroism:* Changes in the tertiary structure of CyaB2 GAFA/B₅₈₋₄₄₅ were
385 monitored using a Jasco J-810 Spectropolarimeter. Briefly, a 500 μL volume containing
386 50 mM Tris.HCl pH 7.5, 25% glycerol, 2 mM MgCl₂, and 27 μM CyaB2 GAFA/B₅₈₋₄₄₅
387 with or without salt and cAMP was scanned at 50 nm sec⁻¹ with a band pass of 2 nm and
388 response time of 4 sec. Each spectrum was acquired 8 times and corrected for activity in
389 the absence of protein. The data is representative of several individual experiments.

390

391 *Cyanobacterial strains and growth:* Wild type and mutant strains of *Anabaena* PCC 7120
392 were grown in standard BG11 medium supplemented with 20 mM TES-NaOH pH 8.0
393 without combined nitrogen at 30°C under a photosynthetically active light regime of ≈ 30
394 μmols m⁻² s⁻¹ (Katayama and Ohmori, 1997). Media containing defined amounts of
395 sodium were prepared by the addition of NaCl to BG11 and all other salts were added
396 with potassium as cation. Adjustments to medium pH were performed using 20 mM TES-
397 KOH (pH 7.0 or 8.0). For phenotypic assays, cells were grown to the mid log phase and
398 media replaced with BG11 20 mM TES-KOH pH 8.0 containing 0.2 mM NaCl for 24
399 hours. Growth experiments were transferred to 12-well plates for photography.
400 Chlorophyll measurements were performed as previously described (Arnon et al., 1974).

401

402 *Measurement of intracellular acidification:* Intracellular acidification was assessed using
403 the acridine orange fluorescence quenching technique (Blumwald et al., 1984). A 25 μg
404 protein equivalent in 6 μl of harvested cells pre-incubated at 0.2 mM Na^+ was diluted into
405 2 ml of Na^+ free assay buffer (0.8 M mannitol, 10 mM HEPES pH 7.5, 1 μM acridine
406 orange). 4 mM Na_2SO_4 was added to the assay where indicated. Fluorescence
407 spectroscopy was carried out using a Jobin-Yvon Fluorolog FL3-22 spectrofluorimeter.
408 Assays were performed in a 1 cm x 1 cm cross-section cuvette equipped with a magnetic
409 stirrer and held at 30°C. The samples were irradiated at 492 nm and the intensity at 530
410 nm was recorded using a time driven acquisition mode. Both monochromators were set to
411 a band pass of 2.5 nm. The observed intensity/time profiles were corrected for fluctuations
412 in the excitation source and for quenching of acridine orange in a mock assay. Results
413 shown are representative of several independent experiments.

414

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562
563

563 **Figure Legends.**

564 **Figure 1.** (A) Response of the CyaB1 catalytic domain to monovalent cations. 0.6 μM
565 CyaB1₅₉₅₋₈₅₉ was assayed with 1 mM Mg^{2+} -ATP and 20 mM salt (n=8). (B) Response of
566 the CyaB1 holoenzyme to monovalent cations. 25 nM CyaB1₁₋₈₅₉ was assayed with 75 μM
567 Mg^{2+} -ATP and 20 mM salt (n=8). (C) Dose response of the CyaB1 holoenzyme with salt.
568 25 nM CyaB1₁₋₈₅₉ was assayed with 75 μM Mg^{2+} -ATP at increasing NaCl (squares) or KCl
569 (triangles) concentrations (n=6). All differences except those of the basal activities were
570 significant (95% C.I.)

571

572 **Figure 2.** (A) Dose response of the CyaB1 holoenzyme with cAMP. 10 nM CyaB1₁₋₈₅₉
573 was assayed with 75 μM Mg^{2+} -ATP at increasing cAMP. All substrate conversion rates
574 are <12% (n=6). (B) Dose response of the CyaB1 GAF-A mutant with cAMP. 100 nM
575 CyaB1₁₋₈₅₉D190A was assayed with 75 μM Mg^{2+} -ATP at increasing cAMP (n=4). (C)
576 Dose response of the CyaB1 GAF-B mutant with cAMP. 100 nM CyaB1₁₋₈₅₉D360A was
577 assayed with 75 μM Mg^{2+} -ATP at increasing cAMP (n=6). All dose responses are
578 performed in the presence of 50 mM NaCl (squares) or KCl (triangles).

579

580 **Figure 3.** (A) Response of the CyaB2-GAF-CyaB1 chimera to monovalent cations. 480
581 nM CyaB2-GAF-CyaB1 was assayed with 75 μM Mg^{2+} -ATP, 0.7 μM cAMP, and 50 mM
582 salt (n=6). (B) Dose response of the CyaB2-GAF-CyaB1 chimera with salt. 480 nM
583 CyaB2-GAF-CyaB1 was assayed with 75 μM Mg^{2+} -ATP and 0.7 μM cAMP at increasing
584 NaCl (squares) or KCl (triangles) concentrations (n=6). (C) CyaB2-GAF-CyaB1 chimera
585 assayed with 75 μM Mg^{2+} -ATP, 50 mM salt, and cAMP as indicated (n=4).

586

587 **Figure 4.** (A) Cation binding to CyaB2 GAF domains. Fluorescence quenching of SBFI
588 (squares) or PBFI (triangles) was plotted against CyaB2 GAFA/B₅₈₋₄₄₅ concentration in the
589 presence of NaCl or KCl respectively (n=3). Inset; derived Hill Plot of the data for SBFI.
590 (B) cAMP blocks Na⁺ binding to GAF domains. Fluorescence quenching of SBFI with
591 500 μM CyaB2 GAFA/B₅₈₋₄₄₅ and 5 mM cAMP (n=4).

592

593 **Figure 5.** Circular dichroism spectropolarimetry of the CyaB2 tandem GAF domain.
594 Analysis of CyaB2 GAFA/B₅₈₋₄₄₅ without cAMP (dashed lines) or with 300 μM cAMP
595 (solid lines) with no salt (green), 50 mM NaCl (blue), or 50 mM KCl (red).

596

597 **Figure 6.** (A) The response of wild type, $\Delta cyaA$, $\Delta cyaB1$, and $\Delta cyaB2$ cells to limiting
598 Na⁺ *in vivo*. (B) Rescue of the limiting Na⁺ growth defect of $\Delta cyaB1$ and $\Delta cyaB2$ cells by
599 exogenous cAMP. (C) Rescue of the limiting Na⁺ growth defect of $\Delta cyaB1$ and $\Delta cyaB2$
600 cells by an increase in H⁺ concentration. 7 and 8 denote growth pH.

601

602 **Figure 7.** Intracellular acidification assessed by acridine orange quenching in wild type
603 and $\Delta cyaB1$ cells at limiting Na⁺. Change in acridine orange fluorescence intensity of cells
604 was plotted as a function of time. Cells and Na⁺ were added to the assay at the indicated
605 time point. I, H⁺ uptake. II, H⁺ efflux.

606

607 **Figure 8.** (A) Dose response of the Rat PDE2 CyaB1 chimera with cGMP. 0.44 μM
608 PDE2-GAF-CyaB1 was assayed with 75 μM Mg²⁺-ATP at increasing cGMP in the
609 presence of 50 mM NaCl (squares) or KCl (triangles) (n=8). (B) Response of the Rat
610 PDE2 GAF domains to monovalent cations. 0.44 μM PDE2-GAF-CyaB1 was assayed
611 with 75 μM Mg²⁺-ATP and 50 mM salt (n=4). (C) Dose response of the Rat PDE2 CyaB1

612 chimera with salt. 0.44 μM PDE2-GAF-CyaB1 was assayed with 75 μM Mg^{2+} -ATP with
613 increasing NaCl (squares) or KCl (triangles) (n=8).

614

615 **Figure 9.** Model for CyaB1 function *in vivo*. (A) Local concentrations of Na^+ at CyaB1 *in*
616 *vivo* are sufficient to block autoregulatory activation of CyaB1. (B) Reduced local
617 concentrations of Na^+ on lowering of extracellular Na^+ permit autoregulatory activation of
618 CyaB1 and regulation of Na^+/H^+ antiporter function. The dotted line between CyaB1 and
619 the Na^+/H^+ is not proven to be direct.

620

Figure 1A.

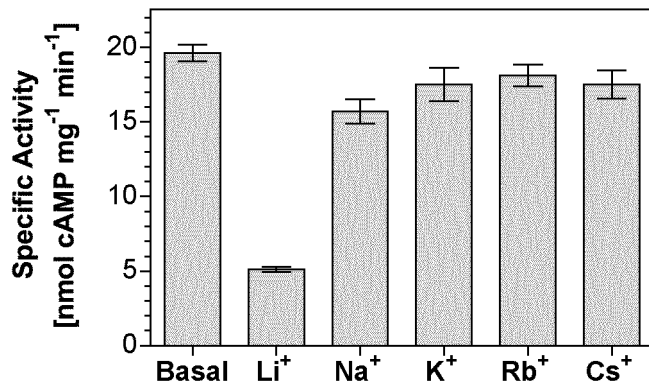


Figure 1B.

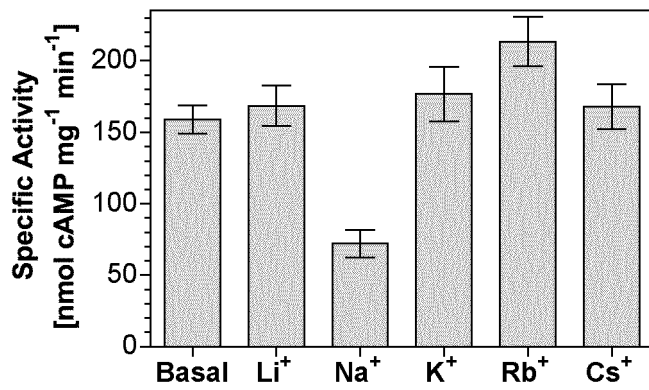


Figure 1C.

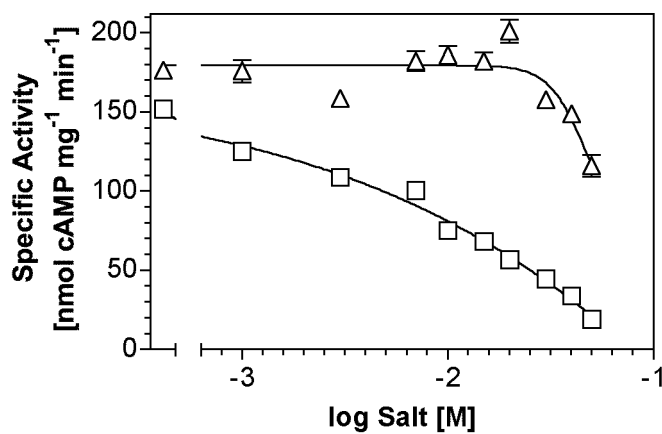


Figure 2A.

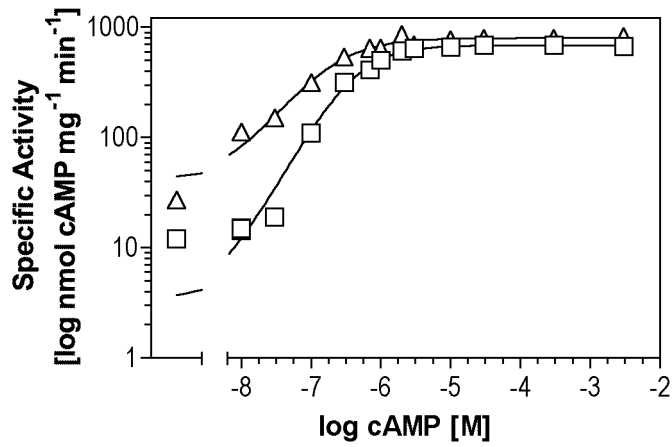


Figure 2B.

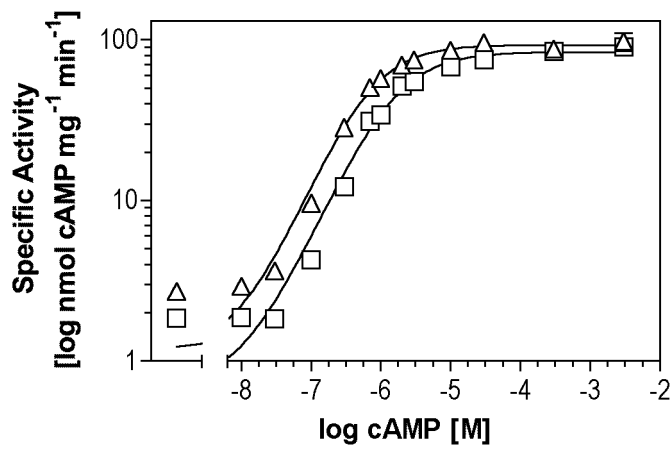


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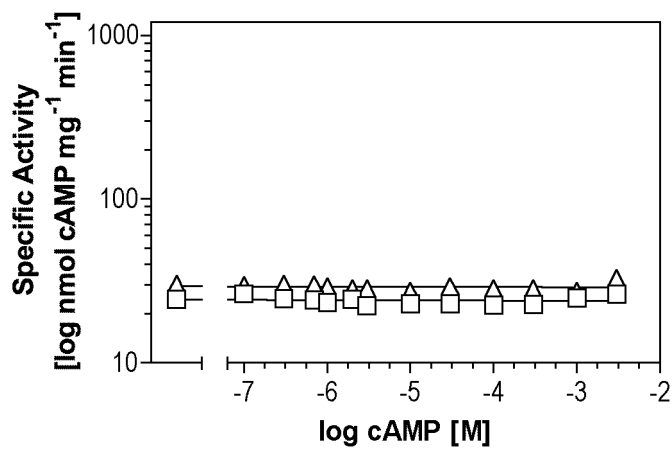


Figure 3A.

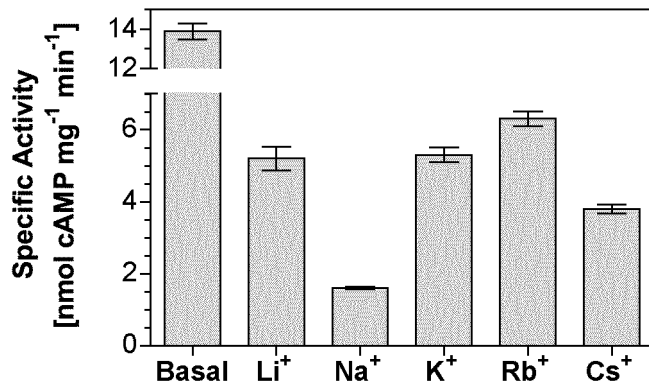


Figure 3B.

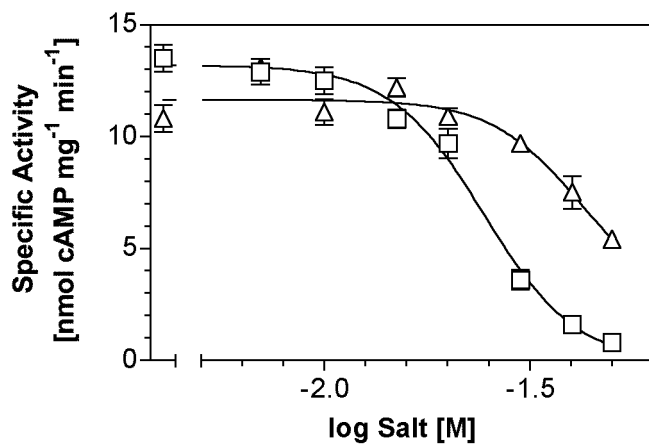


Figure 3C.

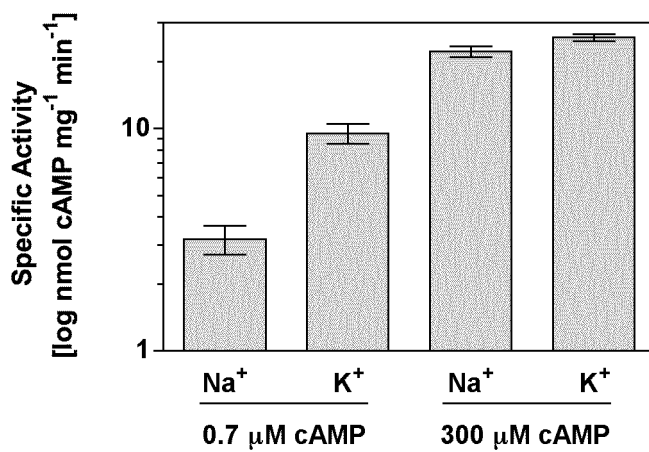


Figure 4A.

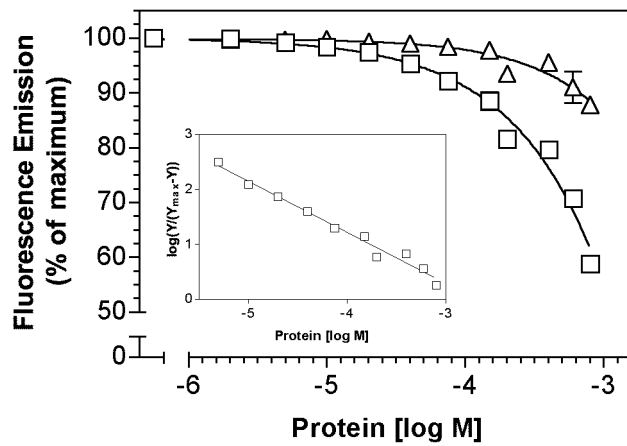


Figure 4B.

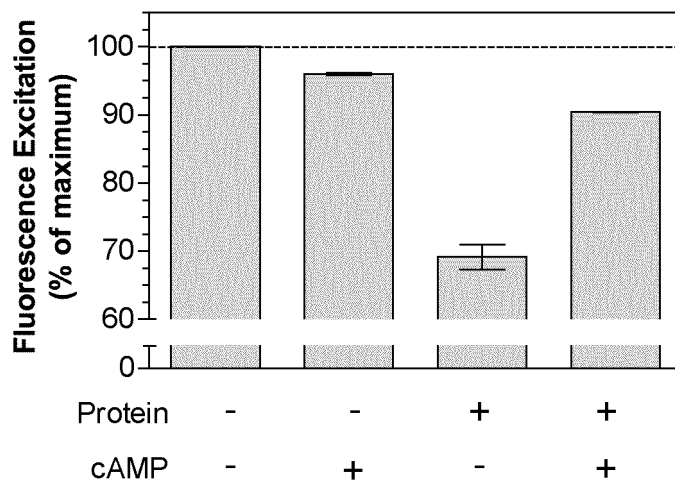


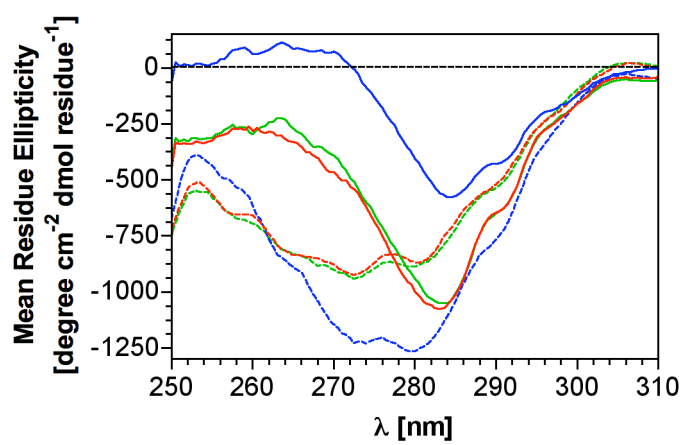
Figure 5.

Figure 6A.

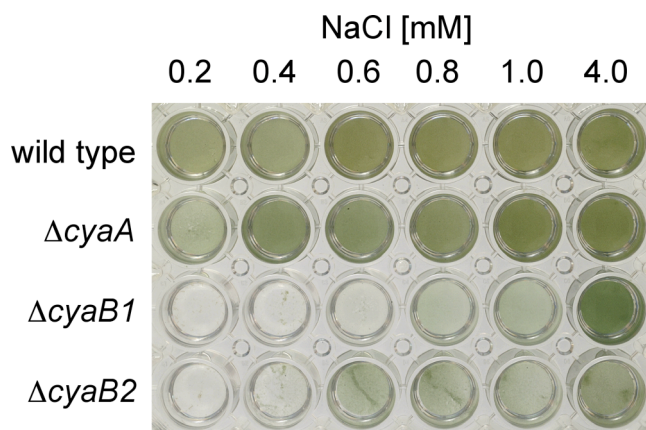


Figure 6B.

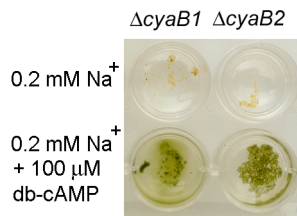


Figure 6C.

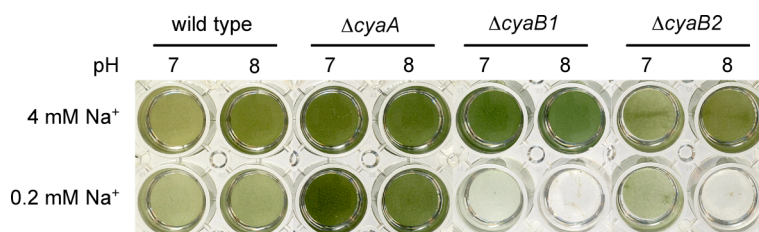


Figure 7.

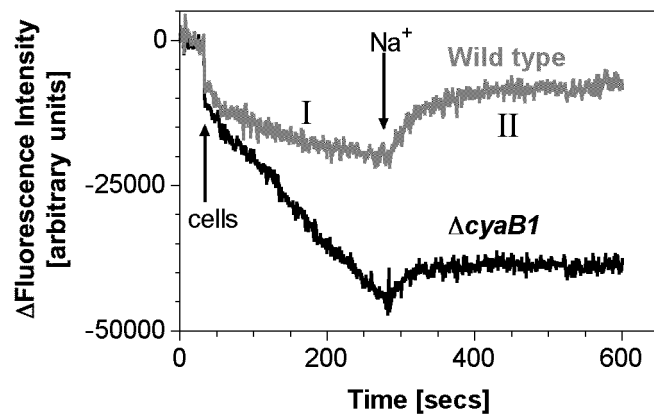


Figure 8A.

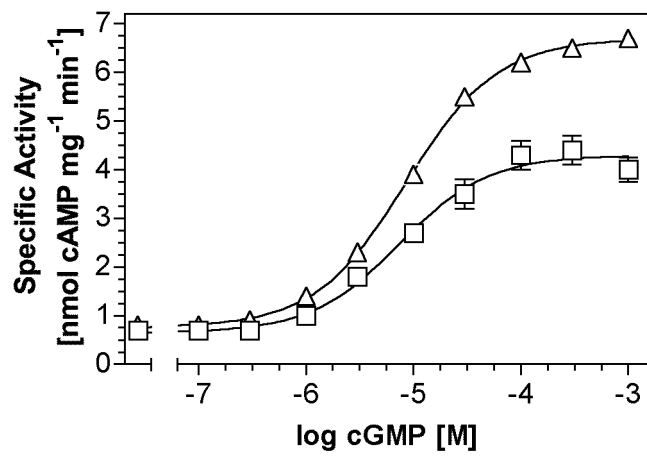


Figure 8B.

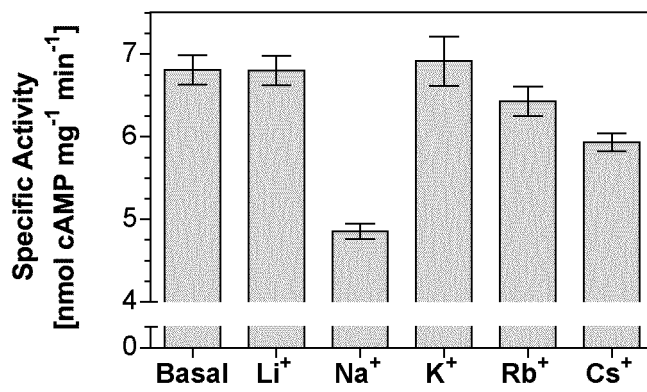


Figure 8C.

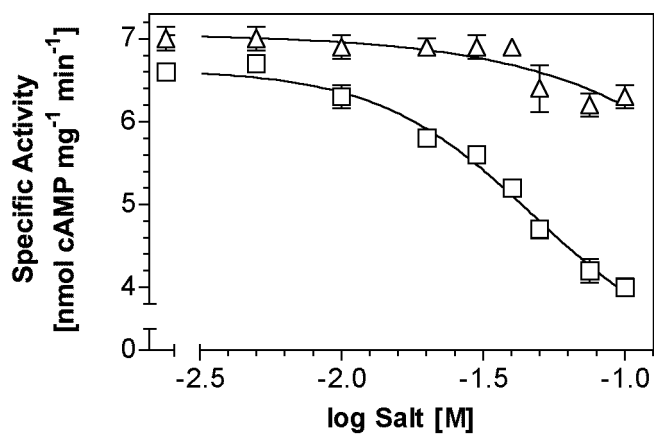


Figure 9.