A defined subset of adenylyl cyclases is regulated by bicarbonate ion.

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Summary

The molecular basis by which organisms detect and respond to fluctuations in inorganic carbon is not known. The *cyaB1* gene of the cyanobacterium *Anabaena* sp. PCC7120 codes for a multi-domain protein with a C-terminal class III adenylyl cyclase catalyst that was specifically stimulated by bicarbonate ion (EC_{50} 9.6 mM). Bicarbonate lowered substrate affinity, but increased reaction velocity. A point mutation in the active site (K646) reduced activity by 95% and was refractory to bicarbonate activation. We propose that K646 specifically co-ordinates bicarbonate in the active site in conjunction with an aspartate to threonine polymorphism (T721) conserved in class III adenylyl cyclases from diverse eukaryotes and prokaryotes. Using recombinant proteins we demonstrated that adenylyl cyclases that contain the active site threonine (cyaB of *Stigmatella aurantiaca* and Rv1319c of *Mycobacterium tuberculosis*) are bicarbonate responsive while adenylyl cyclases with a corresponding aspartate (Rv1264 of *Mycobacterium*) are bicarbonate insensitive. Large numbers of class III adenylyl cyclases may therefore be activated by bicarbonate. This represents a novel mechanism by which diverse organisms can detect bicarbonate ion.

Introduction

cAMP is one of the most prevalent signaling molecules among prokaryotes and eukaryotes, modulating the responses of an organism to diverse environmental stimuli. The enzyme adenylyl cyclase (AC)[1] synthesizes cAMP and belongs to a large gene family consisting of six phylogenetically defined classes (1-4). Class I ACs are found in the Enterobacteria e.g. *Escherichia coli*; class II ACs are exclusive to certain toxin-producing bacteria e.g. *Bacillus anthracis*; class III (the universal class) ACs are the only class found among higher eukaryotes and also includes the mammalian guanylyl cyclases and prokaryotic members; class IV enzymes are found in certain prokaryotic thermophiles e.g. *Aeromonas hydrophila*; class V consists of a single member from the obligate anaerobe *Prevotella ruminicola*; and the recently described class VI ACs found in the genomes of the *Rhizobiaceae*.

cAMP is synthesized in mammals by a seemingly ubiquitous family of class III plasma membrane spanning ACs (transmembrane adenylyl cyclase; tmAC), which mediates cellular responses to extracellular signals. Additionally, a cytosolic form of AC (soluble adenylyl cyclase; sAC) has been identified in mammals that was demonstrated to be molecularly and biochemically distinct from the tmACs (5). Although most abundantly expressed in testis, sAC is expressed ubiquitously (6,7) and is directly activated by bicarbonate ion in a pH independent manner (8).

The HCO_3^- regulated mammalian sAC is more closely related to other prokaryotic class III ACs than to other mammalian tmACs (5,9) Consistent with this phylogenetic relationship, it was demonstrated that a single cyanobacterial class III AC, cyaC of *Spirulina platensis*, was also stimulated by HCO_3^- (8). If HCO_3^- stimulation were a general feature of at least a subset of class III ACs they would represent the first family

of HCO_3^- responsive signaling molecules. HCO_3^- is fundamental to prokaryotic biology; accumulated cytoplasmic HCO_3^- is the primary source of inorganic carbon transported to the cyanobacterial carboxysome for photosynthesis (10) and is also hypothesized to have been the predominant carbon source utilized by oxygenic phototrophs in the generation of Earth's oxygen atmosphere (11).

To define the extent to which class III ACs may be stimulated by HCO_3^- we have utilized the *cyaB1* AC gene of the nitrogen fixing freshwater cyanobacterium *Anabaena* sp. PCC7120 as a model system. Cyanobacteria are dependent upon the accumulation of intracellular HCO_3^- for growth but the mechanism by which they detect HCO_3^- is unknown and a major stumbling block in the study of this environmentally important class of organisms. The genome of *Anabaena* sp. PCC7120 encodes six AC genes (12,13) and *cyaB1* codes for a protein that has an N-terminal auto-regulatory GAF (found in cGMP-phosphodiesterases, adenylyl cyclases, and FhlA [formate hydrogen lyase transcriptional activator]) domain that binds cAMP and up regulates catalytic activity (14). Biochemical analysis of the catalytic center of cyaB1 revealed that HCO_3^- stimulates the catalytic activity of AC by an increase in reaction velocity. In addition we have defined a residue (K646) essential for HCO_3^- action within the catalytic center. We have examined the catalytic centers of a number of other prokaryotic class III ACs and demonstrated that an active site lysine co-ordinates HCO_3^- in the catalytic cleft of the subset of ACs which contain an aspartate to threonine active site polymorphism. On the basis of this hypothesis, we propose that a large number of prokaryotic class III AC catalytic domains are HCO_3^- responsive. HCO_3^- signaling through cAMP synthesis is established as a mechanism by which a variety of eukaryotic and prokaryotic organisms can respond to environmental carbon. This knowledge is of fundamental importance in understanding the global impact of bicarbonate on organismal biology.

Experimental Procedures

Recombinant DNAs

The *cyaB1* gene of *Anabaena* sp. PCC7120 with associated single amino acid point mutations and the *Mycobacterium tuberculosis* H37Rv Rv1264 gene were assembled as previously described (14,15). Full details of the *Mycobacterium* Rv1319c gene will be reported elsewhere.

Nucleotides 1349-1930 of the *Stigmatella aurantiaca* B17R20 *cyaB* gene (Genbank Accession number AJ223795; gift of Dr. O. Sismeiro, Institut Pasteur) were amplified by PCR and cloning was performed using standard molecular biology techniques. A discrepancy from the published sequence was noted that gave an amino acid change (P163R). A *Bam*H I and *Hin*d III site were added at the 5' and 3' end, respectively. The *cyaB* fragment was cloned between the *Bam*H I and *Hin*d III sites of pQE30. The resulting open reading frame codes for amino acids 160-353 of the cyaB adenylyl cyclase with an MRGSH_6GS metal-affinity tag at the N-terminal end. Primer sequences are available on request.

Expression and purification of bacterially expressed proteins

Anabaena cyaB1 wild type and mutant proteins and *Mycobacterium* $Rv1264_{1-397}$ protein were expressed and purified as previously described (14,15). Full details of the *Mycobacterium* Rv1319c protein will be reported elsewhere.

The *Stigmatella* pQE30-*cyaB* construct was transformed into *E. coli* BL21(DE3)[pREP4]. A culture was grown in LB broth medium containing 100 mg/L ampicillin and 25 mg/L kanamycin at 30°C to an OD_{600} of 0.5. 60 μM isopropyl-β-D-thiogalactopyranoside was added and the culture kept at room temperature for 3 hours. Cells were harvested by centrifugation at 4000 g and washed once with 10 mM Tris-HCl pH 7.5. The cell pellet was resuspended in 20 ml buffer A (50 mM Tris-HCl pH 8.0, 2.5

mM 1-thioglycerol, 50 mM NaCl) and disrupted by two treatments in a French Press at 1000 psi. Particulate material was removed at 31 000 g for 30 min. The supernatant was supplemented with 250 mM NaCl, 15 mM imidazole, and 200 µl Ni^{2+}-nitrilotriacetic acid slurry (Qiagen) for 30 min. The resin was washed with 3 mls each of buffer B (Tris-HCl pH 8.0, 2.5 mM 1-thioglycerol, 2 mM $MgCl_2$, 400 mM NaCl, 5 mM imidazole), buffer C (buffer B with 15 mM imidazole) and buffer D (buffer C with 10 mM NaCl). The enzyme was eluted with 0.4 ml buffer E (buffer B with 10 mM NaCl and 150 mM imidazole). The preparation was stabilized with 20% glycerol and stored at 4^{o}C.

AC assay

The AC activity of cyaB1 wild type protein, cyaB1 mutant proteins, and other prokaryotic AC recombinant proteins was assessed in a final volume of 100 µL (16). Reactions typically contained 22% glycerol, 50 mM MOPS-Na as buffer, 2 mM $MnCl_2$ as divalent metal ion co-factor, and 75 µM [α-^{32}P]ATP (25 kBq) and 2 mM [2,8-^{3}H]cAMP (150 Bq) to determine yield during production isolation (cAMP was not added to assays for cyaB1 holoenzyme). Details of pH, temperature, and enzyme concentration are provided in the figure legends. Differences in buffer or co-factor usage are also indicated in the text. Protein concentration was adjusted to keep substrate conversion at <10%. Kinetic constants were determined over a concentration range of substrate of 1-100 µM. The data represents the means of several independent experiments and error bars represent the standard error.

Results

The *cyaB1* (alr2266; http://www.kazusa.or.jp/cyano/Anabaena/) gene of *Anabaena* sp. PCC7120 codes for a protein consisting of two tandem GAF (GAF-A and GAF-B) domains, a PAS domain (found in periodic clock protein, aryl hydrocarbon receptor, and single-minded protein), and a C-terminal AC catalytic domain. A CLUSTALW alignment of the AC catalytic domain of cyaB1 with those of a number of prokaryotic and eukaryotic ACs showed that the active site amino acids involved in divalent metal ion coordination (D650; numbering as for cyaB1), transition state stabilization (N728, R732), and substrate definition (K646) were conserved between all the ACs (Figure 1A). T721, a residue essential for full catalysis in cyaB1 (14) was conserved among several of the ACs including HCO_3^- responsive sAC and *Spirulina* cyaC, while the remainder expressed a D residue essential for substrate definition in the corresponding position. Given the conservation of the active site T polymorphism between cyaB1, sAC, and *Spirulina* cyaC we investigated whether cyaB1 was also stimulated by HCO_3^-. We expressed the catalytic domain of cyaB1 ($cyaB1_{595-859}$) to include a region of the C-terminus (amino acids 795-828) that had some similarity to a tetratricopeptide repeat and is essential for production of functional soluble protein in *Escherichia coli* (14).

The activity of $cyaB1_{595-859}$ was measured in the presence or absence of various salts (Figure 1B). Specific activity was unchanged in the presence of NaCl and KCl while $NaHCO_3$ and $KHCO_3$ both gave an approximately two-fold increase of $cyaB1_{595-859}$ specific activity demonstrating that HCO_3^- activation of $cyaB1_{595-859}$ was independent of the associated cation. We measured the specific activity of $cyaB1_{595-859}$ over a range of HCO_3^- concentrations with Cl^- as a control for non-specific ionic effects (Figure 2A). A maximal two-fold stimulation was seen in the presence of HCO_3^- with an EC_{50} of 9.6 mM. The GAF-B domain of cyaB1 binds cAMP and activates the AC catalytic domain (14).

cyaB1 therefore acts as a self-activating switch. We asked whether the behavior of this switch is affected by HCO_3^- and expressed recombinant protein corresponding to the cyaB1 holoenzyme ($cyaB1_{1-859}$) that contains the GAF domains and examined its specific activity in the presence or absence of HCO_3^-. $cyaB1_{1-859}$ specific activity showed a non-linear time dependence as previously reported (14) and the rate of cAMP formation was significantly accelerated in the presence of 10 mM $KHCO_3$ indicating that HCO_3^- activated the GAF-B mediated positive feedback mechanism of cyaB1 (Figure 2B). The rate of cAMP formation was also stimulated in the presence of 10 mM $NaHCO_3$, but inhibited in the presence of higher concentrations of $NaHCO_3$ indicating that Na^+ may block GAF-B binding of cAMP or intramolecular signalling[2].

$cyaB1_{595-859}$ specific activity showed a non-linear protein dependence (Figure 3) indicating that homodimerization was necessary for formation of the active site. This has been independently confirmed by titration of complementary mutant $cyaB1_{595-859}$ proteins that are inactive as homodimers, but restored catalytic activity as heterodimers (14). To determine whether HCO_3^- up regulated $cyaB1_{595-859}$ specific activity by increasing homodimer formation we examined the ratio of the HCO_3^- and Cl^- specific activities as a function of protein concentration. Interestingly, this ratio remained constant over the range of protein concentrations tested indicating that HCO_3^- did not affect homodimer formation. The protein concentration independence of HCO_3^- up regulation of specific activity allowed us to make comparisons between experiments in which different concentrations of protein were assayed (see Figure 4).

We examined the kinetic properties of $cyaB1_{595-859}$ to determine if HCO_3^- altered the behavior of the active site. The activation energy (E_a) was derived from the linear arm of an Arrhenius plot (tested range 4^oC-47^oC) and was similar in the presence of either Cl^- (91.6±4.9 kJ/mol) or HCO_3^- (97.7±3.7 kJ/mol) indicating that HCO_3^- did not fulfill the criteria for a true catalyst in lowering ΔH. The K_M value for ATP at pH 8.5 and 45^oC

was approximately three-fold greater with HCO_3^- (33.8±2.8 μM) than with Cl^- (11.8±0.8 μM) indicating a higher requisite substrate concentration to achieve a given reaction velocity. The corresponding V_{max} values were 2.5-fold greater with HCO_3^- (238.0±36.3 nmol/mg/min) than with Cl^- (93.5±8.2 nmol/mg/min). A consequence of this is that enzyme efficiency (k_{cat}/K_M) was similar for both ions but substrate turnover rate (k_{cat}) was approximately 2.5-fold greater for HCO_3^- (7.0 min^{-1}) than for Cl^- (2.6 min^{-1}). A Hill coefficient of 1.1 indicated that neither ion stimulated significant co-operativity of the two catalytic sites

The kinetic data implied that HCO_3^- interacts with the catalytic center to alter substrate-binding kinetics. The catalytic center is in close agreement with a canonical class III catalytic cleft (17,18) except for the replacement of an aspartate (D1018 in AC IIC_2 [17]) with a threonine (T721 in cyaB1). D1018 is involved in substrate definition in AC by forming a hydrogen bond with N^6 of the adenine ring of ATP (17). T721 functionally replaced this aspartate and may act as a hydrogen acceptor from the purine ring (14). When assayed at pH 7.5 to eliminate problems with divalent metal ion depletion, $cyaB1_{595\text{-}859}$ specific activity was stimulated approximately three-fold relative to the Cl^- activity over the tested range (0-60 mM HCO_3^-) (Figure 4A). We investigated the involvement of the canonical active site residues of a class III AC in HCO_3^- stimulation using point mutations. Although the basal specific activities of $cyaB1_{595\text{-}859}$R732A (transition state stabilization), $cyaB1_{595\text{-}859}$N728A (transition state stabilization), and $cyaB1_{595\text{-}859}$D719A (a residue examined for possible functional homology to D1018 of AC IIC_2) were significantly reduced compared to wild type enzyme their fold stimulation by HCO_3^- was equivalent (Supplemental Data Figure 1). A key difference between T721 of cyaB1 and D1018 of AC IIC_2 is the loss of the aspartate carboxy group. We reasoned that HCO_3^- possibly mimics the carboxy group within the active site but, interestingly,

HCO_3^- mediated up regulation of cyaB1$_{595-859}$T721A specific activity was equivalent to wild type despite a >99% reduction in basal activity (Figure 4B). We noted that K938 of AC IIC_2 (substrate definition and equivalent to K646 of cyaB1; [17]) was proposed to act not only as a hydrogen acceptor for the N^1 of the ATP purine ring but also as a hydrogen donor to the carboxy group of the adjacent D1018 residue (19). Thus K646 may form a stabilizing hydrogen bond with HCO_3^- at a position equivalent to the carboxy group of AC IIC_2. Although basal activity was reduced by approximately 95%, HCO_3^- activation was completely abolished in cyaB1$_{595-859}$K646A in support of this hypothesis (Figure 4C). If HCO_3^- mimics a carboxy group within the active site reintroduction of this carboxy group should ablate HCO_3^- responsiveness. A cyaB1$_{595-859}$T721D mutant protein was refractory to HCO_3^- stimulation and had an enhanced basal specific activity relative to cyaB1$_{595-859}$T721A (Figure 4D) lending positive support to this hypothesis. This represents the first description of a site for HCO_3^- action within a signaling molecule.

Although the amino acid equivalent to K646 of cyaB1 and K938 of AC IIC_2 is conserved in all the ACs examined (Figure 1A) we reasoned that an adjacent threonine or aspartate within the catalytic cleft of a class III enzyme (i.e. at the position corresponding to T721) could be a marker for HCO_3^- AC responsiveness or non-responsiveness, respectively. To test this hypothesis we generated recombinant proteins corresponding to diverse prokaryotic class III ACs with either a threonine or aspartate at the position equivalent to cyaB1 T721 (Figure 1A) and examined them for their response to HCO_3^-.

Stigmatella aurantiaca B17R20 is a myxobacterium from which two ACs have been identified (20). We expressed amino acids 160 to 353 of cyaB as a recombinant protein (cyaB$_{160-353}$) that contained a threonine residue (T293) at the position corresponding to cyaB1 T721 (Figure 1A). cyaB$_{160-353}$ specific activity was up regulated by HCO_3^- approximately two-fold relative to the Cl^- dependent activity (EC_{50} 8.6 mM)

(Figure 5A) consistent with the hypothesis that the threonine at amino acid 293 is a marker for HCO_3^- responsiveness. This stimulation was maintained in the presence of alternative anions to Cl^- indicating that $cyaB_{160\text{-}353}$ was most likely stimulated by HCO_3^- rather than inhibited by Cl^- [2].

Mycobacterium tuberculosis H37Rv is a gram-negative bacterium and important human pathogen for which the genome has revealed a number of putative class III ACs (15,21,22). We expressed two ACs that contain either a threonine (amino acids 356-535 of Rv1319c) or an aspartate (Rv1264 holoenzyme) at the position corresponding to T721 of cyaB1 (Figure 1A). Consistent with our hypothesis that the threonine residue is a marker for AC HCO_3^- responsiveness $Rv1319c_{356\text{-}535}$ specific activity was up regulated approximately three-fold in the presence of HCO_3^- over the concentration range tested (Figure 5B) while $Rv1264_{1\text{-}397}$ specific activity did not respond to HCO_3^- over an identical concentration range (Figure 5C).

The data of Figure 5 supports the hypothesis posited in Figure 4 and indicates that HCO_3^- responsive class III AC domains are widespread in biology and represents the sole candidate mechanism for HCO_3^- detection in an organism.

Discussion

cyaB1 of *Anabaena* sp. PCC7120 is a class III AC whose catalytic center is functionally equivalent to that identified for the mammalian tmACs (17,18) except for a threonine residue (T721) which replaces an aspartate highly conserved among the tmACs. T721 functionally replaces aspartate and is suggested to act as a hydrogen acceptor from the purine ring (14). cyaB1 catalytic activity was demonstrated to be responsive to HCO_3^- extending the number of identified class III ACs that are stimulated by HCO_3^- and stimulation was cation independent and anion dependent. The measured EC_{50} of 9.6 mM is well within the range of calculated intracellular HCO_3^- concentrations for cyanobacteria (23). Although the inorganic carbon pool for *Anabaena* sp. PCC7120 has not been measured, the related heterocyst forming species *Anabaena variabilis* M3 can accumulate up to 50 mM internal inorganic carbon depending upon the growth conditions (24). cAMP production through cyaB1 is therefore likely to be responsive to variations in intracellular HCO_3^-. Intracellular cAMP has previously been correlated with the rate of HCO_3^- uptake in the cyanobacterium *Anabaena flos-aquae* (25) indicating that the protein chemistry we describe is functional *in vivo*. HCO_3^- was able to functionally activate not only the catalytic domains but also the entire holoenzyme with its associated GAF and PAS domains. The GAF-B mediated positive feedback loop created by cyaB1 may therefore be accelerated by the availability of a fixable carbon source in *Anabaena* sp. PCC7120.

HCO_3^- did not affect cyaB1 homodimer formation or lower the activation energy for transition state formation but did significantly alter substrate binding kinetics by increasing the K_M for ATP and V_{max}. The cyanobacterium *Synechococcus* PCC6301 (*Anacystis nidulans*) has an intracellular ATP concentration of approximately 1 mM (value calculated from data in [26]). As the $K_{M\ (ATP)}$ for both $cyaB1_{595\text{-}859}$ and holoenzyme is of the order of <50 μM it is likely that the effect of HCO_3^- on K_M is biologically irrelevant

and that cyaB1 is activated by HCO_3^- in the intracellular environment by an increase in reaction velocity. Point mutations revealed that loss of T721 did not affect $cyaB1_{595\text{-}859}$ HCO_3^- responsiveness. We demonstrated, however, that loss of K646 (equivalent to K938 of AC IIC_2) ablated HCO_3^- stimulation of specific activity. In class III ACs that contain an aspartate residue corresponding to the position of T721, the adjacent lysine in the catalytic center has been proposed to form a hydrogen bond with the aspartate carboxy group (19). We hypothesize that in cyaB1 HCO_3^- can functionally replace this carboxy group and is co-ordinated within the catalytic cleft by K646. A T721D point mutation was refractory to HCO_3^- in support of this hypothesis. The enhanced basal activity of T721D relative to T721A may represent an enzyme mimicking HCO_3^- activation. If HCO_3^- does functionally replace the carboxy group of an aspartate, it is surprising that HCO_3^- increases $K_{M\ (ATP)}$ given that a logical extension of our hypothesis would be that HCO_3^- forms a hydrogen bond with N^6 of the adenine ring and increase affinity for substrate. It is possible that HCO_3^- binding results in subtle changes in the structure of the substrate-binding pocket that lowers affinity, but optimizes orientation for catalysis. As there is no effect on E_a in the presence of HCO_3^- it is unlikely that this effect is on the acquisition of the transition state. The increase in k_{cat} demonstrates that there is an increase in catalytic activity on formation of the enzyme-substrate complex and this may therefore occur after formation of the transition state. The exact mechanism of HCO_3^- activation of AC is an interesting question that requires further investigation.

Independent support for the proposed site of action of HCO_3^- came from studies with recombinant class III AC domains from other prokaryotic species that contained either a T or a D residue corresponding to the position of cyaB1 T721. To date, all ACs that are responsive to HCO_3^- contain a threonine residue (*Anabaena* cyaB1, *Stigmatella* cyaB, *Mycobacterium* Rv1319c [this study], mammalian sAC, and *Spirulina* cyaC [8]) and those that are unresponsive contain an aspartate residue (mammalian tmACs [8],

Mycobacterium Rv1264 [this study], and Rv1625c [M.J.C., unpublished data]). In addition, mammalian soluble and receptor-type guanylyl cyclases (GC) have also been demonstrated to be HCO_3^- non-responsive[3]. Presumably the change in the binding pocket of GC relative to AC that allows a glutamate residue essential for substrate specificity to interact with N^1 and N^2 of the guanine ring (19) would not permit HCO_3^- at the active site.

HCO_3^- is ubiquitous in the intracellular and extracellular aqueous environment. HCO_3^- has a huge impact on the biology of multiple eukaryotic and prokaryotic systems but the mechanism by which organisms detect and respond to fluctuating HCO_3^- is unknown. The expression of HCO_3^- regulated class III AC domains among diverse prokaryotes and eukaryotes represents the sole mechanism by which organisms may respond to environmental carbon.

References

1. Barzu, O., and Danchin, A. (1994) *Prog Nucleic Acid Res Mol Biol* 49, 241-83.
2. Cotta, M. A., Whitehead, T. R., and Wheeler, M. B. (1998) *FEMS Microbiol Lett* 164(2), 257-60.
3. Sismeiro, O., Trotot, P., Biville, F., Vivares, C., and Danchin, A. (1998) *Journal of Bacteriology* 180(13), 3339-3344
4. Tellez-Sosa, J., Soberon, N., Vega-Segura, A., Torres-Marquez, M. E., and Cevallos, M. A. (2002) *J Bacteriol* 184(13), 3560-8.
5. Buck, J., Sinclair, M. L., Schapal, L., Cann, M. J., and Levin, L. R. (1999) *Proc Natl Acad Sci U S A* 96(1), 79-84.
6. Sinclair, M. L., Wang, X. Y., Mattia, M., Conti, M., Buck, J., Wolgemuth, D. J., and Levin, L. R. (2000) *Mol Reprod Dev* 56(1), 6-11.
7. Zippin, J. H., Chen, Y., Nahirney, P., Kamenetsky, M., Wuttke, M. S., Fischman, D. A., Levin, L. R., and Buck, J. (2003) *Faseb J* 17(1), 82-4.
8. Chen, Y., Cann, M. J., Litvin, T. N., Iourgenko, V., Sinclair, M. L., Levin, L. R., and Buck, J. (2000) *Science* 289(5479), 625-8.
9. Roelofs, J., Meima, M., Schaap, P., and Van Haastert, P. J. (2001) *Embo J* 20(16), 4341-8.
10. Bhaya, D., Schwarz, R., and Grossman, A. (2000) in *The ecology of cyanobacteria.* (Whitton, B. A., and Potts, M., eds), pp. 398-442, Kluwer Academic Publishers, Dordrecht
11. Dismukes, G. C., Klimov, V. V., Baranov, S. V., Kozlov, Y. N., DasGupta, J., and Tyryshkin, A. (2001) *Proc Natl Acad Sci U S A* 98(5), 2170-5.
12. Katayama, M., and Ohmori, M. (1997) *J Bacteriol* 179(11), 3588-93.
13. Ohmori, M., Ikeuchi, M., Sato, N., Wolk, P., Kaneko, T., Ogawa, T., Kanehisa, M., Goto, S., Kawashima, S., Okamoto, S., Yoshimura, H., Katoh, H., Fujisawa,

T., Ehira, S., Kamei, A., Yoshihara, S., Narikawa, R., and Tabat, S. (2001) *DNA Res* 8(6), 271-84.

14. Kanacher, T., Schultz, A., Linder, J. U., and Schultz, J. E. (2002) *Embo J* 21(14), 3672-80.
15. Linder, J. U., Schultz, A., and Schultz, J. E. (2002) *J Biol Chem* 277(18), 15271-6.
16. Salomon, Y., Londos, C., and Rodbell, M. (1974) *Anal Biochem* 58(2), 541-8.
17. Tesmer, J. J., Sunahara, R. K., Gilman, A. G., and Sprang, S. R. (1997) *Science* 278(5345), 1907-16.
18. Zhang, G., Liu, Y., Ruoho, A. E., and Hurley, J. H. (1997) *Nature* 386(6622), 247-53.
19. Tucker, C. L., Hurley, J. H., Miller, T. R., and Hurley, J. B. (1998) *Proc Natl Acad Sci U S A* 95(11), 5993-7.
20. Coudart-Cavalli, M. P., Sismeiro, O., and Danchin, A. (1997) *Biochimie* 79(12), 757-67.
21. Guo, Y. L., Seebacher, T., Kurz, U., Linder, J. U., and Schultz, J. E. (2001) *Embo J* 20(14), 3667-75.
22. Reddy, S. K., Kamireddi, M., Dhanireddy, K., Young, L., Davis, A., and Reddy, P. T. (2001) *J Biol Chem* 276(37), 35141-9.
23. Price, G., Sultemeyer, D., Klughammer, B., Ludwig, M., and Badger, M. (1998) *Can J Bot* 76, 973-1002
24. Kaplan, A., Badger, M., and Berry, J. (1980) *Planta* 149, 219-226
25. Francko, D., and Wetzel, R. (1981) *J Phycol* 17, 129-134
26. Ihlenfeldt, M., and Gibson, J. (1975) *Arch Microbiol* 102(1), 13-21

Footnotes

1 The abbreviations used are AC-adenylyl cyclase, GC – guanylyl cyclase, sAC-soluble adenylyl cyclase, tmAC-transmembrane adenylyl cyclase.

2 Unpublished observations.

3 Martin J. Cann and David L. Garbers, unpublished observations.

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Figure Legends

Figure 1. (A) Sequence alignment of a portion of the catalytic domain of *Anabaena* cyaB1 with the homologous region of a number of adenylyl cyclases. Arrowheads indicate the residues mutated in this study for determining the basis of AC HCO_3^- responsiveness. Amino acids that contribute to the active site are indicated in bold type. Numbers correspond to amino acid residue from the accession numbers (below). Bracketed number corresponds to the number of amino acids not represented in the figure for clarity. Accession numbers for the aligned amino acid sequences are as follows: *Stigmatella* cyaB [P40138] *Mycobacterium* Rv1264 [Z77137], *Mycobacterium* Rv1319c [Q10632], *Rattus* sAC [AAD04035], *Anabaena* cyaB1 [BAA13998], *Spirulina* cyaC [BAA22997], *Mus* tmAC9 [CAA03415], *Bos* tmAC1 [AAA79957], and *Rattus* tmAC3 [M55075]. **(B)** Cation independence of the HCO_3^- up regulated specific activity of the cyanobacterial $AC_{595\text{-}859}$ catalyst (assayed at pH 8.5 and 45^{o}C using 53 nM enzyme). Salt concentrations are 20 mM.

Figure 2 (A). Dose response of $cyaB1_{595\text{-}859}$ AC specific activity in the presence of $NaHCO_3$ (squares) or NaCl (triangles) (assayed at pH 8.5 and 45^{o}C with 53 nM enzyme). **(B)** Time dependence of $cyaB1_{1\text{-}859}$ AC specific activity in the presence (squares) or absence (triangles) of 10 mM $KHCO_3$ (assayed at pH 7.5 [Tris-HCl buffered] and 37^{o}C with 7.8 nM enzyme and 75 μM Mg-ATP as substrate). Note that the time dependent increase in cAMP formation is accelerated in the presence of $KHCO_3$.

Figure 3. Protein dependence of the specific activity of the cyanobacterial $AC_{595\text{-}859}$ catalyst (assayed at pH 8.5 and 45^{o}C) in the presence of 20 mM $NaHCO_3$ (squares) or NaCl (triangles).

Figure 4. (A) Dose response of cyaB1$_{595-859}$ specific activity in the presence of $KHCO_3$ (squares) or KCl (triangles) (assayed at pH 7.5 and 45^{o}C with 53 nM enzyme). (**B)** Dose response of cyaB1$_{595-859}$T721A specific activity (662 nM enzyme). (**C)** Dose response of cyaB1$_{595-859}$ K646A specific activity (662 nM enzyme). **(D)** Dose response of cyaB1$_{595-859}$T721D specific activity (662 nM enzyme). Symbols and assay conditions for (B), (C), and (D) are as for (A) above. Specific activities dropped at HCO_3^- concentrations above the tested range due to depletion of divalent metal ion co-factor (unpublished data).

Figure 5. (A) Dose response of *Stigmatella aurantiaca* B17R20 CyaB AC$_{160-353}$ specific activity in the presence of $NaHCO_3$ (squares) or NaCl (triangles) (assayed at pH 7.5 and 45^{o}C with 90 nM enzyme). **(B)** Dose response of *Mycobacterium tuberculosis* H37Rv Rv1319c$_{356-535}$ specific activity in the presence of $KHCO_3$ (squares) or KCl (triangles) (assayed at pH 7.5 and 37^{o}C with 1.5 μM enzyme and 1 mM ATP as substrate). **(C)** Dose response of *Mycobacterium tuberculosis* H37Rv Rv1264$_{1-397}$ specific activity in the presence of $KHCO_3$ (squares) or KCl (triangles) (assayed at pH 7.5 and 37^{o}C with 1.5 μM enzyme and 0.5 mM ATP as substrate).

Supplemental Data Figure 1. (A) Dose response of cyaB1$_{595-859}$D719A specific activity in the presence of $KHCO_3$ (squares) or KCl (triangles) (assayed at pH 7.5 and 45^{o}C with 53 nM enzyme). **(B)** Dose response of cyaB1$_{595-859}$R732A specific activity (assayed at pH 7.5 and 45^{o}C with 53 nM enzyme). **(C)** Dose response of cyaB1$_{595-859}$N728A specific activity (assayed at pH 8.5 and 45^{o}C with 331 nM enzyme). Symbols for (B) and (C) are as for (A) above

Figure 1A

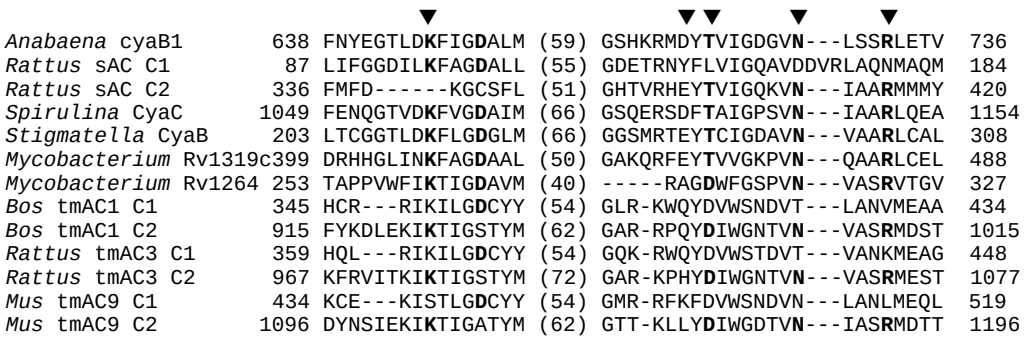

Anabaena cyaB1 638 FNYEGTLDKFIGDALM (59) GSHKRMDYTVIGDGVN---LSSRLETV 736
Rattus sAC C1 87 LIFGGDILKFAGDALL (55) GDETRNYFLVIGQAVDDVRLAQNMAQM 184
Rattus sAC C2 336 FMFD------KGCSFL (51) GHTVRHEYTVIGQKVN---IAARMMMY 420
Spirulina CyaC 1049 FENQGTVDKFVGDAIM (66) GSQERSDFTAIGPSVN---IAARLQEA 1154
Stigmatella CyaB 203 LTCGGTLDKFLGDGLM (66) GGSMRTEYTCIGDAVN---VAARLCAL 308
Mycobacterium Rv1319c 399 DRHHGLINKFAGDAAL (50) GAKQRFEYTVVGKPVN---QAARLCEL 488
Mycobacterium Rv1264 253 TAPPVWFIKTIGDAVM (40) -----RAGDWFGSPVN---VASRVTGV 327
Bos tmAC1 C1 345 HCR---RIKILGDCYY (54) GLR-KWQYDVWSNDVT---LANVMEAA 434
Bos tmAC1 C2 915 FYKDLEKIKTIGSTYM (62) GAR-RPQYDIWGNTVN---VASRMDST 1015
Rattus tmAC3 C1 359 HQL---RIKILGDCYY (54) GQK-RWQYDVWSTDVT---VANKMEAG 448
Rattus tmAC3 C2 967 KFRVITKIKTIGSTYM (72) GAR-KPHYDIWGNTVN---VASRMEST 1077
Mus tmAC9 C1 434 KCE---KISTLGDCYY (54) GMR-RFKFDVWSNDVN---LANLMEQL 519
Mus tmAC9 C2 1096 DYNSIEKIKTIGATYM (62) GTT-KLLYDIWGDTVN---IASRMDTT 1196

Figure 1B

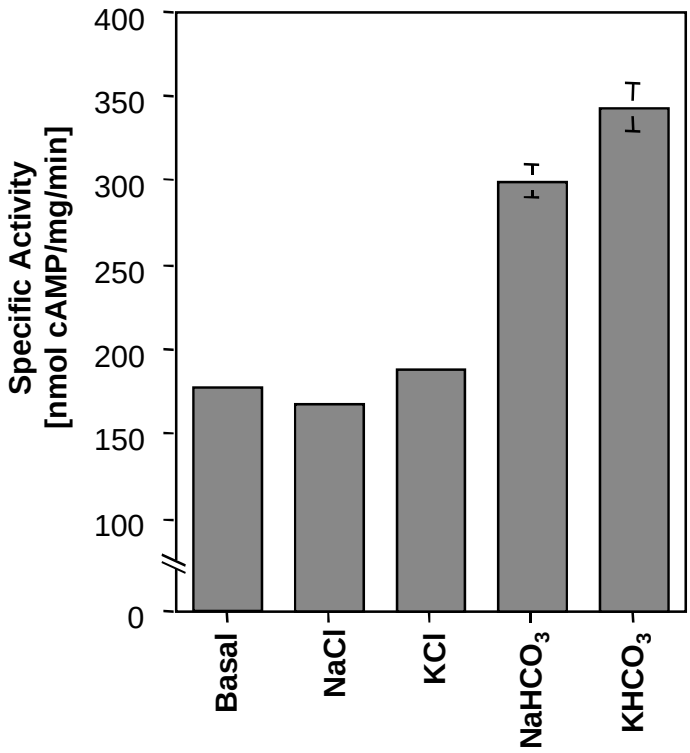

Specific Activity
[nmol cAMP/mg/min]
400
350
300
250
200
150
100
0
Basal
NaCl
KCl
NaHCO3
KHCO3

Figure 2A

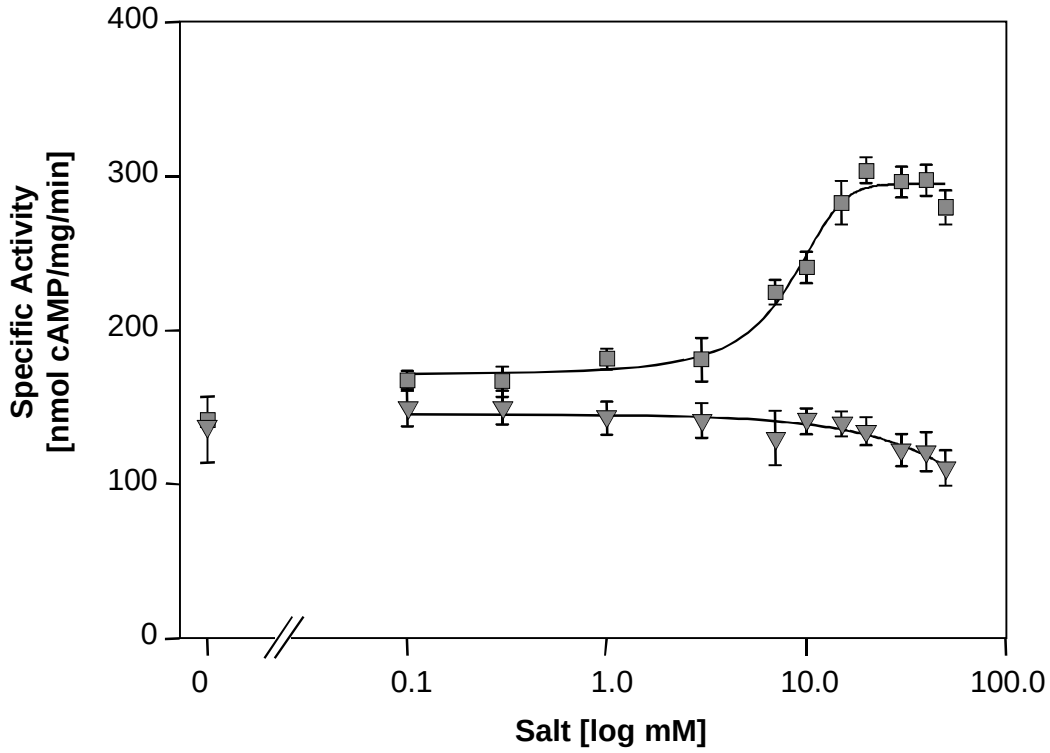

400
300
200
100
0
Specific Activity
[nmol cAMP/mg/min]
0
0.1
1.0
10.0
100.0
Salt [log mM]

Figure 2B

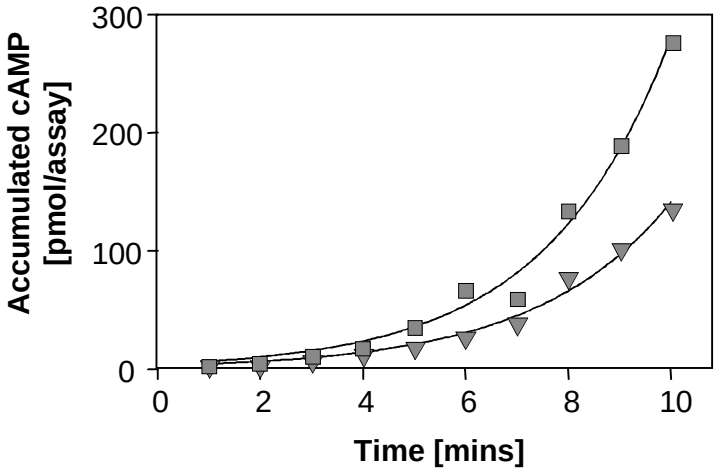


Figure 3

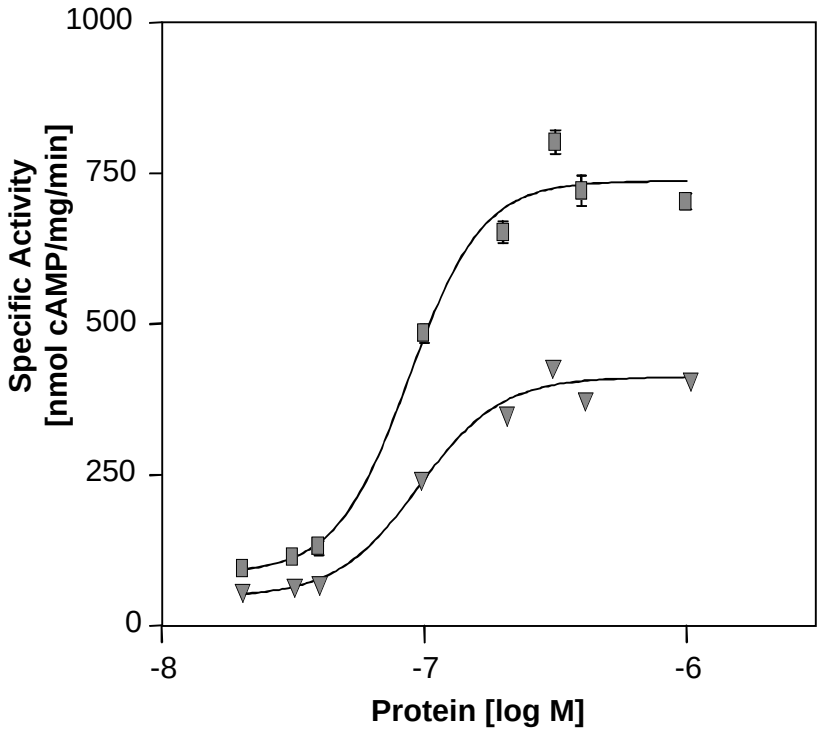

1000
750
500
250
0
Specific Activity
[nmol cAMP/mg/min]
-8
-7
-6
Protein [log M]

Figure 4A

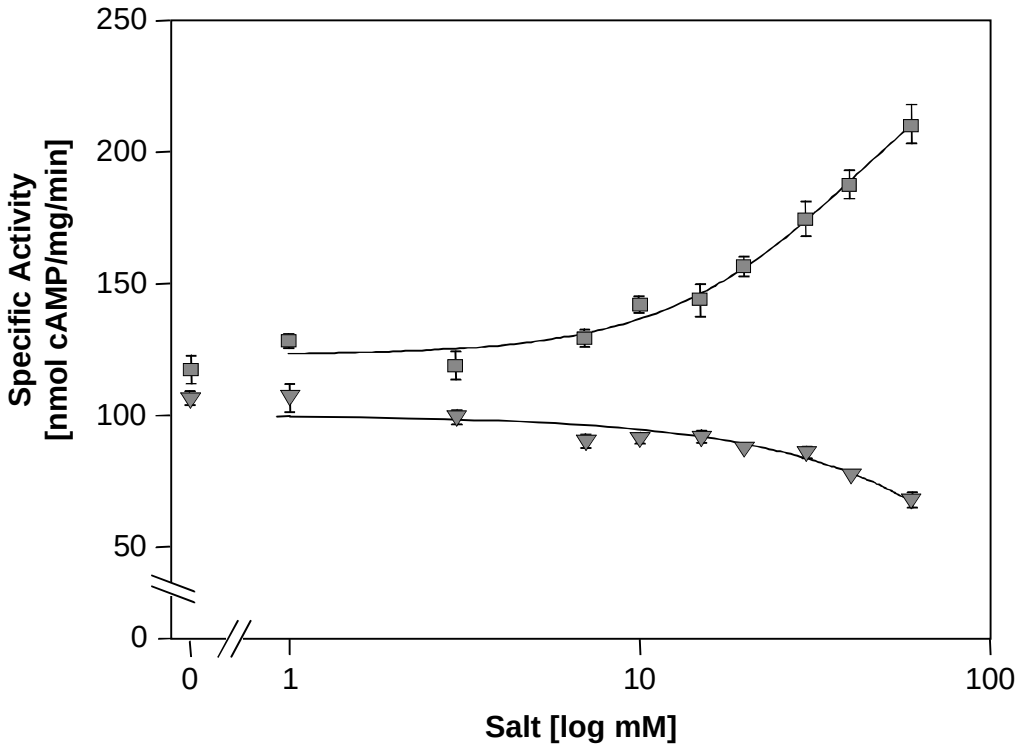

250
200
150
100
50
0
Specific Activity
[nmol cAMP/mg/min]
0
1
10
100
Salt [log mM]

Figure 4B

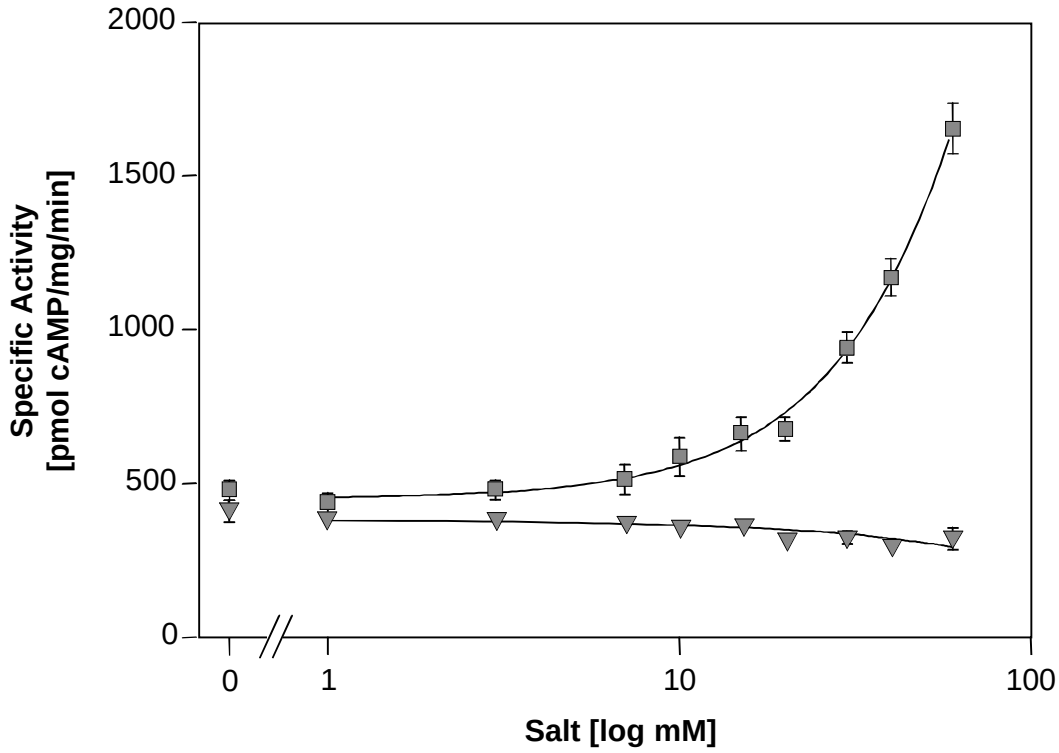

2000
1500
1000
500
0
Specific Activity
[pmol cAMP/mg/min]
0
1
10
100
Salt [log mM]

Figure 4C

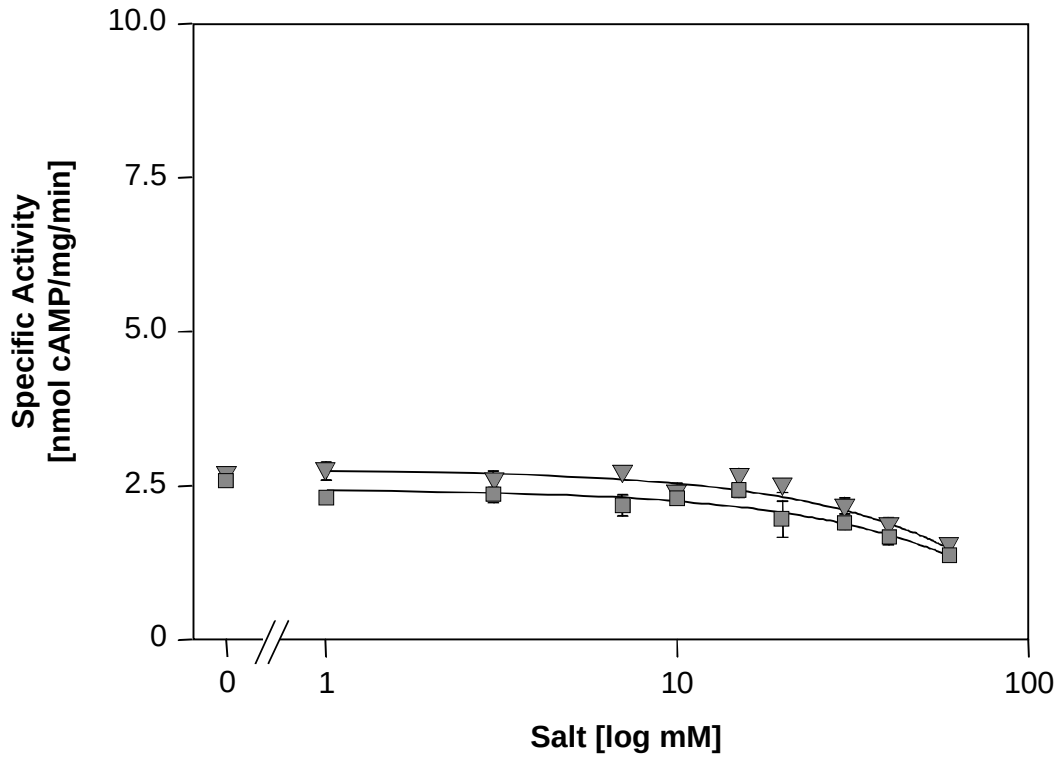

10.0
7.5
5.0
2.5
0
Specific Activity
[nmol cAMP/mg/min]
0
1
10
100
Salt [log mM]

Figure 4D

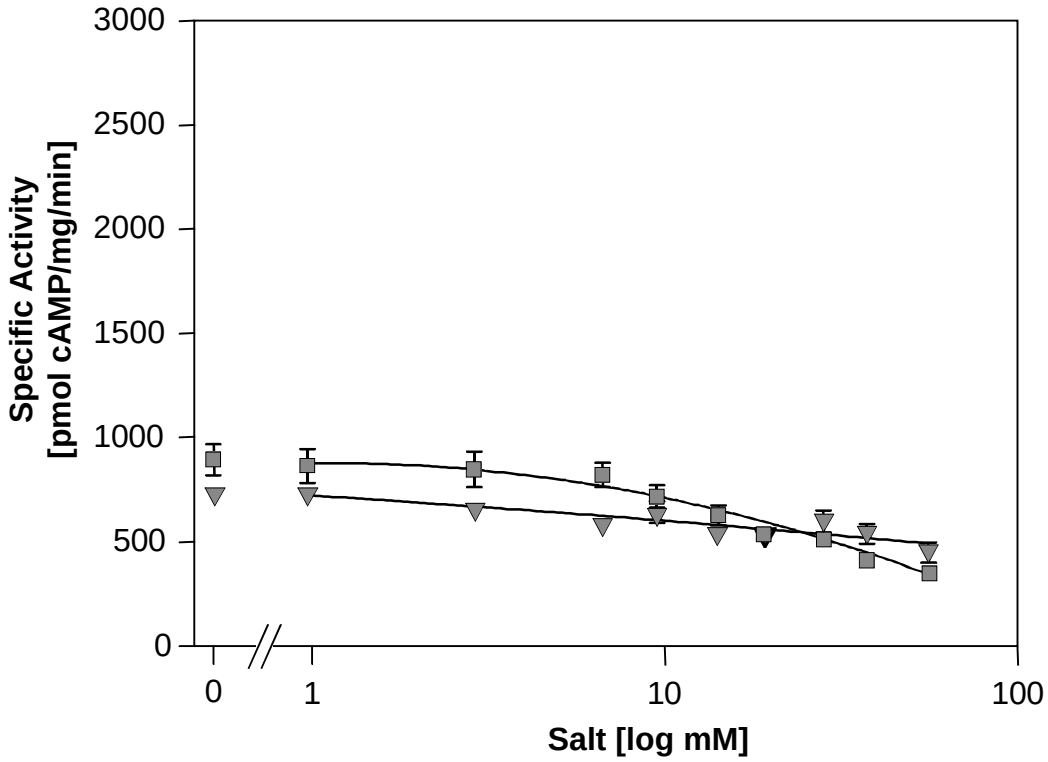

3000
2500
2000
1500
1000
500
0
Specific Activity
[pmol cAMP/mg/min]
0
1
10
100
Salt [log mM]

Figure 5A

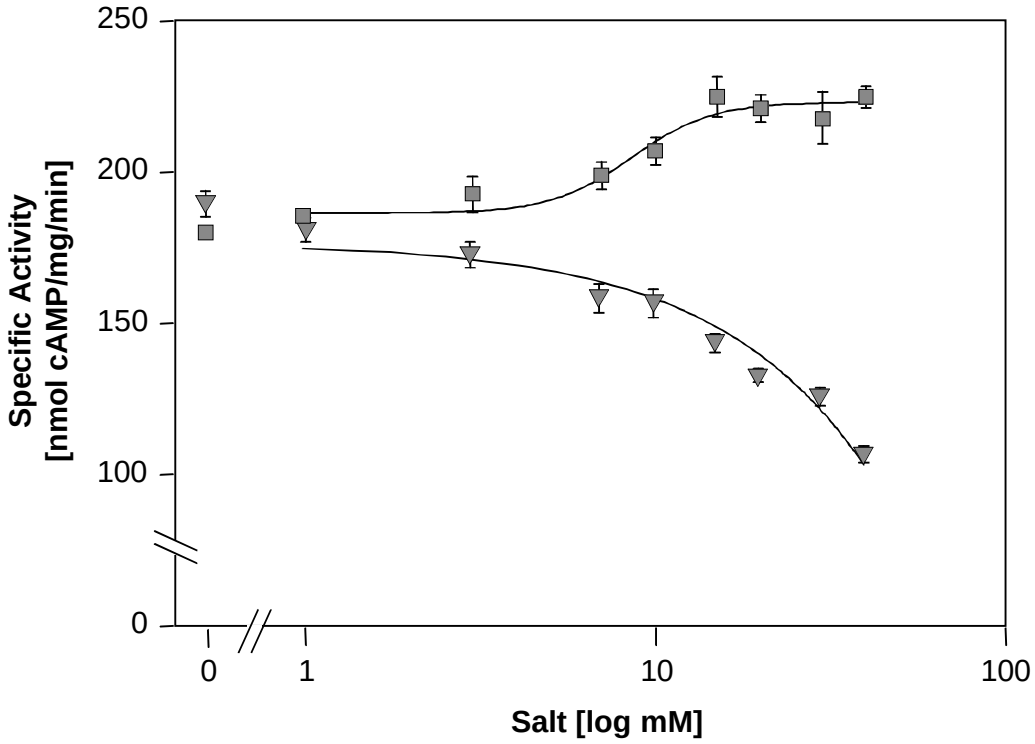

250
200
150
100
0
Specific Activity
[nmol cAMP/mg/min]
0
1
10
100
Salt [log mM]

Figure 5B

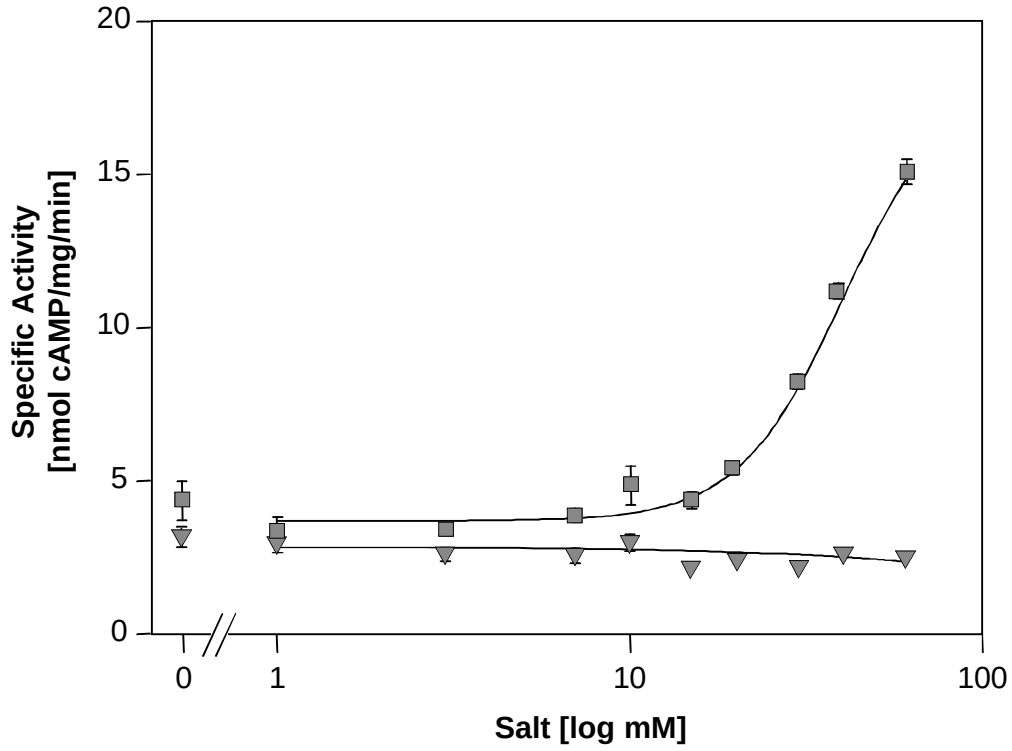
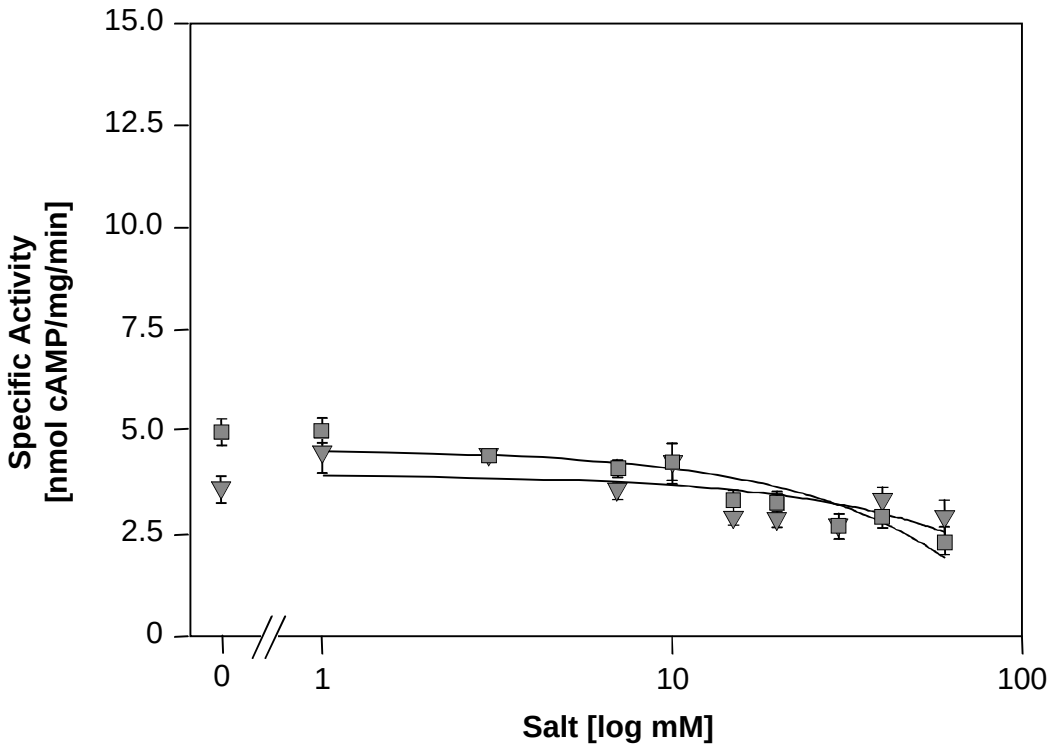
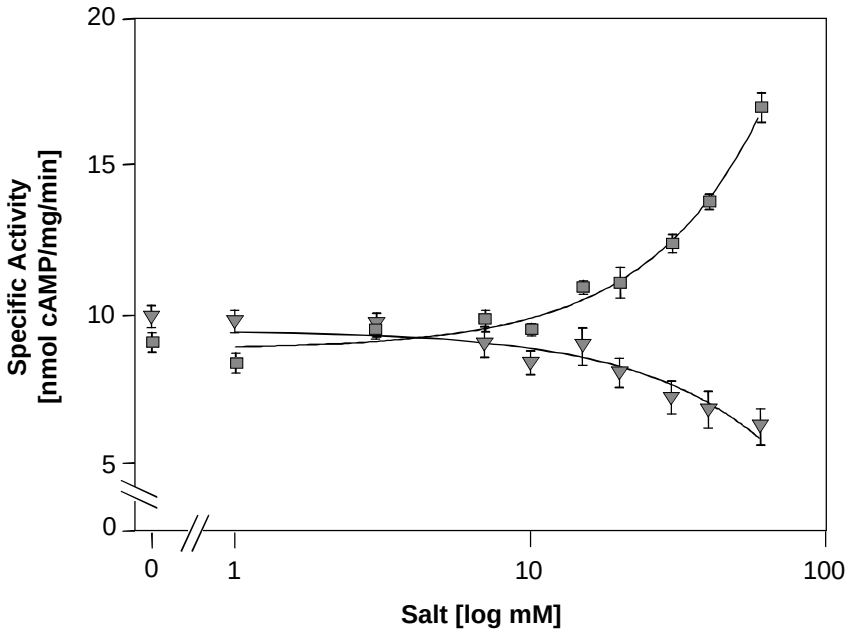

Specific Activity
[nmol cAMP/mg/min]
20
15
10
5
0
0
1
10
100
Salt [log mM]

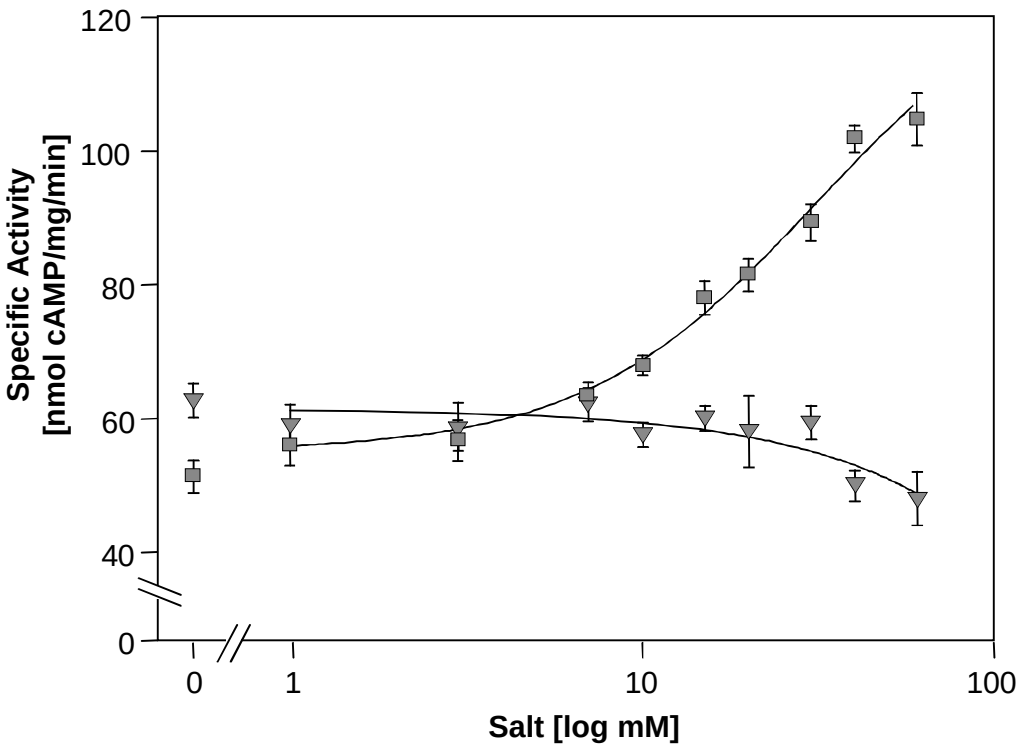
Figure 5C


15.0
12.5
10.0
7.5
5.0
2.5
0
Specific Activity
[nmol cAMP/mg/min]
0
1
10
100
Salt [log mM]

Supplemental Data Figure 1A.



Supplemental Data Figure 1B.



Supplemental Data Figure 1C.