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

Martin J. Cann¹,², Arne Hammer³, Jie Zhou², and Tobias Kanacher³

¹Corresponding author. E-mail: m.j.cann@durham.ac.uk
²Department of Biological and Biomedical Sciences, University of Durham, South Road, Durham, DH1 3LE, United Kingdom
³Pharmazeutische Biochemie, Pharmazeutisches Institut, Morgenstelle 8, D-72076 Tübingen, Germany

Proofs addressed to Martin J. Cann

Fax number: (+44) 191 334 1201
Telephone number: (+44) 191 334 1313

Running Title: Bicarbonate ion regulated adenylyl cyclases
Summary

The molecular basis by which organisms detect and respond to fluctuations in inorganic carbon is not known. The cyaB 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. \textit{Escherichia coli}; class II ACs are exclusive to certain toxin-producing bacteria e.g. \textit{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. \textit{Aeromonas hydrophila}; class V consists of a single member from the obligate anaerobe \textit{Prevotella ruminicola}; and the recently described class VI ACs found in the genomes of the \textit{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 \textit{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
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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 BamHI and HindIII site were added at the 5' and 3' end, respectively. The cyaB fragment was cloned between the BamHI and HindIII sites of pQE30. The resulting open reading frame codes for amino acids 160-353 of the cyaB adenylyl cyclase with an MRGSH6GS 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 Rv12641-397 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 OD600 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°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 $[^{32}\text{P}]$ATP (25 kBq) and 2 mM [2,8-3H]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 HCO₃⁻ 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 HCO₃⁻. We expressed the catalytic domain of cyaB1 (cyAB1595-859) 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 cyaB1595-859 was measured in the presence or absence of various salts (Figure 1B). Specific activity was unchanged in the presence of NaCl and KCl while NaHCO₃ and KHCO₃ both gave an approximately two-fold increase of cyaB1595-859 specific activity demonstrating that HCO₃⁻ activation of cyaB1595-859 was independent of the associated cation. We measured the specific activity of cyaB1595-859 over a range of HCO₃⁻ concentrations with Cl⁻ as a control for non-specific ionic effects (Figure 2A). A maximal two-fold stimulation was seen in the presence of HCO₃⁻ with an EC⁵₀ of 9.6 mM. The GAF-B domain of cyaB1 binds cAMP and activates the AC catalytic domain (14).
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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.

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°C-47°C) 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 $\Delta$H. The $K_m$ value for ATP at pH 8.5 and 45°C
was approximately three-fold greater with HCO₃⁻ (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 HCO₃⁻ (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 HCO₃⁻ (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 HCO₃⁻ 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 IIC2 [17]) with a threonine (T721 in cyaB1). D1018 is involved in substrate definition in AC by forming a hydrogen bond with N⁶ 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_{595-859} specific activity was stimulated approximately three-fold relative to the Cl⁻ activity over the tested range (0-60 mM HCO₃⁻) (Figure 4A). We investigated the involvement of the canonical active site residues of a class III AC in HCO₃⁻ stimulation using point mutations. Although the basal specific activities of cyaB1_{595-859}R732A (transition state stabilization), cyaB1_{595-859}N728A (transition state stabilization), and cyaB1_{595-859}D719A (a residue examined for possible functional homology to D1018 of AC IIC2) were significantly reduced compared to wild type enzyme their fold stimulation by HCO₃⁻ was equivalent (Supplemental Data Figure 1). A key difference between T721 of cyaB1 and D1018 of AC IIC2 is the loss of the aspartate carboxy group. We reasoned that HCO₃⁻ possibly mimics the carboxy group within the active site but, interestingly,
HCO$_3^-$ mediated up regulation of cyaB$_{595-859}$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 cyaB$_{595-859}$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 cyaB$_{595-859}$T721D mutant protein was refractory to HCO$_3^-$ stimulation and had an enhanced basal specific activity relative to cyaB$_{595-859}$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$_{160-353}$ 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$_{356-535}$ specific activity was up regulated approximately three-fold in the presence of HCO$_3^-$ over the concentration range tested (Figure 5B) while Rv1264$_{1-397}$ 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 HCO₃⁻ extending the number of identified class III ACs that are stimulated by HCO₃⁻ and stimulation was cation independent and anion dependent. The measured EC₅₀ of 9.6 mM is well within the range of calculated intracellular HCO₃⁻ 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 HCO₃⁻. Intracellular cAMP has previously been correlated with the rate of HCO₃⁻ uptake in the cyanobacterium *Anabaena flos-aquae* (25) indicating that the protein chemistry we describe is functional *in vivo*. HCO₃⁻ 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.

HCO₃⁻ 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 Kₘ for ATP and Vₘₐₓ. The cyanobacterium *Synechococcus* PCC6301 (*Anacystis nidulans*) has an intracellular ATP concentration of approximately 1 mM (value calculated from data in [26]). As the Kₘ (ATP) for both cyaB1₅₉₅-₈₅₉ and holoenzyme is of the order of <50 µM it is likely that the effect of HCO₃⁻ on Kₘ 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$_{595-859}$ HCO$_3^-$ responsiveness. We demonstrated, however, that loss of K646 (equivalent to K938 of AC IIIC$_2$) 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


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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$_{595-859}$ catalyst (assayed at pH 8.5 and 45°C using 53 nM enzyme). Salt concentrations are 20 mM.

**Figure 2 (A).** Dose response of cyaB1$_{595-859}$ AC specific activity in the presence of NaHCO$_3$ (squares) or NaCl (triangles) (assayed at pH 8.5 and 45°C with 53 nM enzyme). (B) Time dependence of cyaB1$_{1-859}$ 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°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$_{595-859}$ catalyst (assayed at pH 8.5 and 45°C) in the presence of 20 mM NaHCO$_3$ (squares) or NaCl (triangles).
**Figure 4.** (A) Dose response of cyaB<sub>1595-859</sub> specific activity in the presence of KHCO<sub>3</sub> (squares) or KCl (triangles) (assayed at pH 7.5 and 45°C with 53 nM enzyme). (B) Dose response of cyaB<sub>1595-859</sub>T721A specific activity (662 nM enzyme). (C) Dose response of cyaB<sub>1595-859</sub>K646A specific activity (662 nM enzyme). (D) Dose response of cyaB<sub>1595-859</sub>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<sub>3</sub><sup>-</sup> 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<sub>160-353</sub> specific activity in the presence of NaHCO<sub>3</sub> (squares) or NaCl (triangles) (assayed at pH 7.5 and 45°C with 90 nM enzyme). (B) Dose response of *Mycobacterium tuberculosis* H37Rv Rv1319c<sub>356-535</sub> specific activity in the presence of KHCO<sub>3</sub> (squares) or KCl (triangles) (assayed at pH 7.5 and 37°C with 1.5 μM enzyme and 1 mM ATP as substrate). (C) Dose response of *Mycobacterium tuberculosis* H37Rv Rv1264<sub>1-397</sub> specific activity in the presence of KHCO<sub>3</sub> (squares) or KCl (triangles) (assayed at pH 7.5 and 37°C with 1.5 μM enzyme and 0.5 mM ATP as substrate).

**Supplemental Data Figure 1.** (A) Dose response of cyaB<sub>1595-859</sub>D719A specific activity in the presence of KHCO<sub>3</sub> (squares) or KCl (triangles) (assayed at pH 7.5 and 45°C with 53 nM enzyme). (B) Dose response of cyaB<sub>1595-859</sub>R732A specific activity (assayed at pH 7.5 and 45°C with 53 nM enzyme). (C) Dose response of cyaB<sub>1595-859</sub>N728A specific activity (assayed at pH 8.5 and 45°C with 331 nM enzyme). Symbols for (B) and (C) are as for (A) above.
Figure 1A

Anabaena cyaB1  638 FNYEGTLDKFIGDALM (59) GSHKRMDYTVIGDGVN---LSHRLETV  736
Rattus mA C1       87 LIFGGDLIKFAGDALL (55) GDETRMYFLVIGDVMVQAQLQNMQ  184
Rattus mA C2       336 FMFD------KGCSPFL (51) GNTVRHEYTIGGWVN---IAAMMMY  420
Spirulina CyaC     1049 FENGGTVDKFGVDAIM (66) GSQERSDFTAIGPSVN---IAARLQEA  1154
Stigmella CyaB     203 LTCGGTLIKFLGDLGM (66) GSSMRTEYTCIGDADVN---VAARLCEL  308
Mycobacterium Rv1319c399 DRHGLINKFKFGDAAL (50) GAKQRFEYTVGKPVPN---QAARLCEL  488
Mycobacterium Rv1264 253 TAPPWFIKTIGDAVN (40) ------PAGDWPFGSVPN---VASRUTGV  327
Bos tmAC1 C1       345 HCR---RIKIGDCYY (54) GLR--KWQYDWSNDVT----LANYMEA  434
Bos tmAC1 C2       915 FKYDEKIKTIGSTYM (62) GAR--RPQYDIWGNTVN---VASRMIST  1015
Rattus tmAC3 C1     359 HQL---RIKIGDCYY (54) GQK--RWQYDWSNDVT----VANKMEAG  448
Rattus tmAC3 C2     967 KFRVITIKTIGSTYM (72) GAR--KPHYDIWGNTVN---VASRMEST  1077
Mus tmAC9 C1        434 KCE---KISTIGDCYY (54) GMR--RPQYDWSNDVN---LANLMEQL  519
Mus tmAC9 C2        1096 DYNSEIKITIGATYM (62) GTG--KLLYDIWGDTVM---IASRMIDT  1196
Figure 1B

![Bar chart showing specific activity concentration of cAMP in different conditions.](chart.png)

**X-axis:** Basal, NaCl, KCl, NaHCO₃, KHCO₃

**Y-axis:** Specific Activity [nmol cAMP/mg/min]
Figure 2A

A graph showing the relationship between Specific Activity [nmol cAMP/mg/min] and Salt [log mM]. The graph displays a curve where the Specific Activity increases with increasing Salt concentration, reaching a peak and then decreasing again.
Figure 2B
Figure 3
Figure 4A
Figure 4B

[Graph showing the relationship between salt concentration (log mM) and specific activity (pmol cAMP/mg/min). The graph has a logarithmic scale on the x-axis and linear scale on the y-axis.]
Figure 4C

Specific Activity [nmol cAMP/mg/min]

Salt [log mM]
Figure 4D

- **Specific Activity [pmol cAMP/mg/min]** vs. **Salt [log mM]**

The graph shows the relationship between specific activity and salt concentration (on a logarithmic scale). The data points are connected by lines, indicating a decreasing trend as salt concentration increases.
Figure 5A

Salt [log mM]

Specific Activity [nmol cAMP/mg/min]
Figure 5B

Salt [log mM] vs. Specific Activity [nmol cAMP/mg/min]
Figure 5C

![Graph showing specific activity vs. salt concentration](image-url)
Supplemental Data Figure 1A.
Supplemental Data Figure 1B.
Supplemental Data Figure 1C.

The graph shows the specific activity (µmol cAMP/mg/min) of adenylyl cyclases as a function of salt concentration (log mM). The activity increases with higher salt concentration, indicating a regulated response by bicarbonate.