ACCELERATED PUBLICATION

Regulation of prokaryotic adenylyl cyclases by CO_2

Arne HAMMER*, David R. W. HODGSON† and Martin J. CANN*[1]

*School of Biological and Biomedical Sciences, Durham University, South Road, Durham DH1 3LE, U.K., and †Department of Chemistry, Durham University, South Road, Durham DH1 3LE, U.K.

The Slr1991 adenylyl cyclase of the model prokaroyte *Synechocystis* PCC 6803 was stimulated 2-fold at 20 mM total C_i (inorganic carbon) at pH 7.5 through an increase in k_{cat}. A dose response demonstrated an EC_{50} of 52.7 mM total C_i at pH 6.5. Slr1991 adenylyl cyclase was activated by CO_2, but not by HCO_3^-. CO_2 regulation of adenylyl cyclase was conserved in the CyaB1 adenylyl cyclase of *Anabaena* PCC 7120. These adenylyl cyclases represent the only identified signalling enzymes directly activated by CO_2. The findings prompt an urgent reassessment of the activating carbon species for proposed HCO_3^--activated adenylyl cyclases.

Key words: adenylyl cyclase (adenyl cyclase, adenylate cyclase), bicarbonate (HCO_3^-), cAMP, carbon dioxide (CO_2), *Synechocystis*.

INTRODUCTION

Inorganic carbon (C_i) is fundamental to the physiology of all organisms. CO_2 and HCO_3^- exist in a pH-dependent equilibrium and are the major biologically active forms of C_i. CO_2 and HCO_3^- are vital to such diverse physiological processes as photosynthetic carbon fixation [1], pH homoeostasis [2] and carbon metabolism [3]. Study of C_i biology is essential to understand these vital physiological processes. Relatively little is known of the signalling mechanisms through which prokaryotic and eukaryotic cells directly detect CO_2/HCO_3^- fluctuations [4]. The identification of C_i-activated signalling molecules and their role in physiology is fundamental to understanding the diverse roles of C_i in biology. Currently, no signalling enzymes directly activated by CO_2 are known.

The mammalian sAC (soluble adenylyl cyclase) synthesizes the second messenger 3′,5′-cAMP and is stimulated by HCO_3^- [5,6]. It was observed that HCO_3^- regulation of AC (adenylyl cyclase) was conserved in a cyanobacterial AC, CyaC of *Spirulina* (*Arthrospira*) *platensis*, which had significant sequence similarity in the AC domain to sAC [6]. More recently, an active-site Asp → Thr polymorphism in the Class III AC family has been proposed as a marker for HCO_3^--responsiveness [7]. On this basis, proposed HCO_3^--responsive ACs are predicted to be widespread among the genomes of prokaryotes and eukaryotes [8]. To date, C_i regulation of AC has been confirmed in prokaryotes as diverse as *Anabaena* PCC 7120, *Mycobacterium tuberculosis*, *Stigmatella aurantiaca* and *Chloroflexus aurantiacus* [7,9]. An implicit assumption is made in the literature that the activating C_i ligand for AC is HCO_3^-, on the basis that the ionic form is more likely to bind in the active site than CO_2. Identification of the activating carbon ligand for AC is essential to validate or question the relevance of significant recent literature in the field.

The photosynthetic cyanobacteria are an excellent model for investigating C_i signalling through AC, since hypothesized HCO_3^--responsive ACs are widespread in these organisms and C_i has clearly defined roles in their physiology. Here we demonstrate that the single Class III AC, Slr1991 (Cya1), of the unicellular cyanobacterium *Synechocystis* PCC 6803, is activated by C_i. Furthermore, we demonstrate, surprisingly, that the activating ligand for this enzyme is CO_2 and not HCO_3^-. A previously characterized proposed HCO_3^- regulated AC, CyaB1 of *Anabaena* PCC 7120, is also shown to respond to CO_2 rather than HCO_3^-. The present work provides the first evidence for AC as a CO_2-activated signalling molecule. This original finding prompts an immediate reassessment of the true activating carbon species in reported HCO_3^--responsive ACs.

MATERIALS AND METHODS

Recombinant proteins

DNA corresponding to amino acids 120–337 of slr1991 was isolated by PCR from the genomic DNA of *Synechocystis* PCC 6803, subcloned into pQE30, and fitted with an N-terminal $MRGSH_6GS$ dodecapeptide affinity tag. Constructs were confirmed by double-stranded sequencing. $Slr1991_{120-337}$ protein was expressed in *Escherichia coli* M15 [pREP4] cells at 25 °C, for 3 h with 300 μM isopropyl β-D-thiogalactoside. Pelleted cells were washed with 50 mM Tris/HCl (pH 8.5)/1 mM EDTA, resuspended in 50 mM Tris/HCl (pH 8.5)/250 mM NaCl/10 mM 1-thioglycerol, lysed by sonication (1 × 150 s) and protein was purified from the supernatant with Ni^{2+}-nitrilotriacetic acid (Qiagen) as previously described [10]. $CyaB1_{595-859}$ protein was generated as previously described [10]. Primer sequences are available on request from M. J. C.

AC assays

AC assays were performed at 40 °C in a final volume of 100 μl and typically contained 50 mM buffer, 2 mM $MnCl_2$, 2 mM [2,8-^{3}H]cAMP (150 Bq) and [α-^{32}P]ATP (25 kBq) as substrate, if not stated otherwise [11]. Protein concentrations were adjusted to maintain substrate utilization at less than 10 %. Kinetic constants were determined over a concentration range of substrate (Mn^{2+}-ATP) of 1–100 μM. The following buffers were used: pH 6.5, Mes; pH 7.0–7.5, Mops; pH 8.0–8.5, Tris/HCl; and pH 9.0, Ches [2-(*N*-cyclohexylamino)ethanesulphonic acid]. Enzyme, buffer and substrate were all prepared at the appropriate pH for the required assay. CO_2 was quantified by titration against NaOH. The

Abbreviations used: (s)AC, (soluble) adenylyl cyclase; C_i, inorganic carbon.

[1] To whom correspondence should be addressed (email m.j.cann@durham.ac.uk).

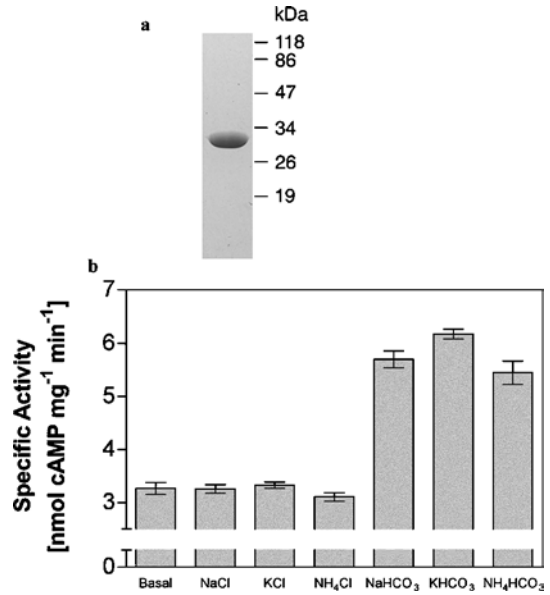

Figure 1 AC activity of purified recombinant Slr1991$_{120-337}$

(**a**) Purification of recombinant Slr1991$_{120-337}$ (SDS/PAGE analysis and Coomassie Blue staining). A 1.5 μg portion of protein was applied and molecular-mass standards (in kDa) are indicated. (**b**) Slr1991$_{120-337}$ specific activity ($n = 8$) in the presence of 20 mM total C_i/salt (0.6 μM protein and 20 μM Mn^{2+}-ATP, pH 7.5).

assay pH was stable over a period of at least 40 min. All errors correspond to the S.E.M. If absent, errors are smaller than the symbol used to depict the data point.

RESULTS AND DISCUSSION

The *cya1* (slr1991; http://www.kazusa.or.jp/cyano/Synechocystis) gene of the unicellular cyanobacterium *Synechocystis* PCC 6803 encodes an enzyme consisting of a single FHA (**f**ork**h**ead **a**ssociated) domain and a Class III AC domain that contains an Asp → Thr polymorphism associated with a putative HCO_3^- responsiveness [7,12]. We expressed the AC domain of Slr1991 as a purified recombinant protein (Figure 1a). The purified wild-type protein had a significant AC specific activity in the presence of both Mg^{2+}-ATP (154 ± 2.0 pmol of cAMP · $min^{-1} \cdot mg^{-1}$, $n = 8$) and Mn^{2+}-ATP (5816 ± 87 pmol of $cAMP \cdot mg^{-1} \cdot min^{-1}$, $n = 8$) under optimal conditions (pH 9.5, 40 °C, 0.3 mM ATP and 8 μM protein).

The Slr1991$_{120-337}$ protein had a pH optimum of 9.5 and a temperature optimum of 40 °C. The enthalpy of activation (E_A) derived from the linear arm of an Arrhenius plot using Mn^{2+}-ATP was 33.5 ± 1.4 kJ · mol^{-1} ($n = 6$). We investigated whether Slr1991$_{120-337}$ was regulated by C_i with a view to determining the identity of the activating species, CO_2 or HCO_3^-. Slr1991$_{120-337}$ specific activity was stimulated 2-fold by 20 mM total C_i (1.2 mM CO_2/18.8 mM HCO_3^-) at pH 7.5 compared with Cl^-. Stimulation was independent of cation and robust to 95 % confidence intervals (Figure 1b). A previous report by Masuda and Ono [13] had not observed stimulation of Slr1991 by C_i at pH 7.5. We noted that an extended assay period (40 min) was required to observe robust C_i activation of Slr1991 at pH 7.5. Although Masuda and Ono [13] did not report the assay time, this is the most likely cause of the discrepancy.

We determined the kinetics of activation of Slr1991$_{120-337}$ by C_i (Table 1). Slr1991$_{120-337}$ showed Michaelis–Menten kinetics in the presence of both Cl^- and Ci. The K_m value for Mn^{2+}-ATP was greater in the presence of C_i than Cl^-, but V_{max} values were proportionately greater for C_i than Cl^-. The overall result was that C_i increased turnover rate (k_{cat}). A dose–response curve with increasing C_i was performed at a reduced pH (6.5) to eliminate problems with enzyme inhibition at > 20 mM total C_i at pH 7.5 in the presence of Mn^{2+}-ATP (Figure 2). The experiment revealed a maximum 8-fold stimulation with an apparent EC_{50} for C_i of 52.7 ± 1.0 mM ($n = 6$) (20.4 mM CO_2/32.3 mM HCO_3^-).

We investigated the response of Slr1991$_{120-337}$ to total C_i at various pH values to gain an insight into whether the enzyme is responsive to CO_2 and/or HCO_3^-. The experiment was performed using Mg^{2+}-ATP as substrate since Mg^{2+} cofactor is more soluble than Mn^{2+} in the presence of C_i at alkaline pH. Intriguingly, relative stimulation (C_i/NaCl) varied from 1.1 at pH 8.5 (0.3 mM CO_2/39.1 mM HCO_3^-/0.6 mM CO_3^{2-}) to 2.4 at pH 6.5 (15.5 mM CO_2/24.5 mM HCO_3^-) (Figure 3a). This is consistent with a role for CO_2 as opposed to HCO_3^- as the activating carbon species, but may also be due to the altered protonation status of the enzyme limiting the ability of Slr1991 to respond to HCO_3^- at elevated pH. We therefore sought direct evidence for regulation of Slr1991$_{120-337}$ by CO_2 and/or HCO_3^- by analysis under conditions of C_i disequilibrium when a single predominant carbon species, CO_2 or HCO_3^-, is present at a defined pH. We exploited the fact that acquisition of the equilibrium between CO_2 and HCO_3^- is significantly lowered at reduced temperature in the absence of carbonic anhydrase and is a well established method for identifying the C_i substrate for CO_2/HCO_3^- -fixing enzymes [14]. We followed the acquisition of the CO_2/HCO_3^- equilibrium by measuring the pH of a weakly buffered (5 mM) Mes solution on

Table 1 Kinetic parameters for Slr1991$_{120-337}$

Protein at 0.6 μM was assayed at pH 7.5 in the presence of 20 mM salt ($n = 6$).

Parameter	Addition ...	Value	
		Cl^-	HCO_3^-
V_{max} (nmol of cAMP · $min^{-1} \cdot mg^{-1}$)		0.74 ± 0.01	1.13 ± 0.03
$K_{m,ATP}$ (μM)		11.4 ± 0.7	16.2 ± 1.3
Hill slope		1.01 ± 0.01	1.03 ± 0.03
k_{cat} (min^{-1})		0.018	0.027

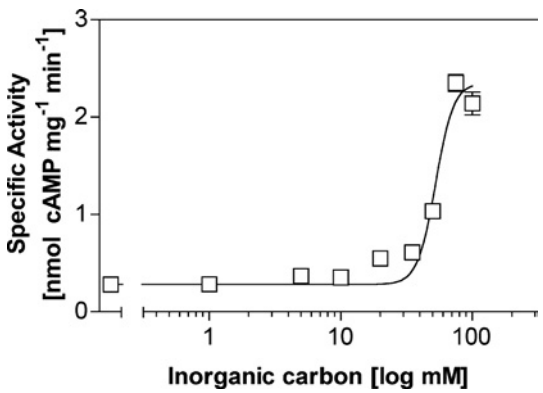

Figure 2 Response of wild-type Slr1991$_{120-337}$ to C_i

Slr1991$_{120-337}$ specific activity ($n = 6$) was plotted against increasing total C_i ('inorganic carbon'). The assay mixture contained 1.5 μM protein and 20 μM Mn^{2+}-ATP, pH 6.5, with Na^+ as cation. The total salt concentration was adjusted to 200 mM with NaCl.

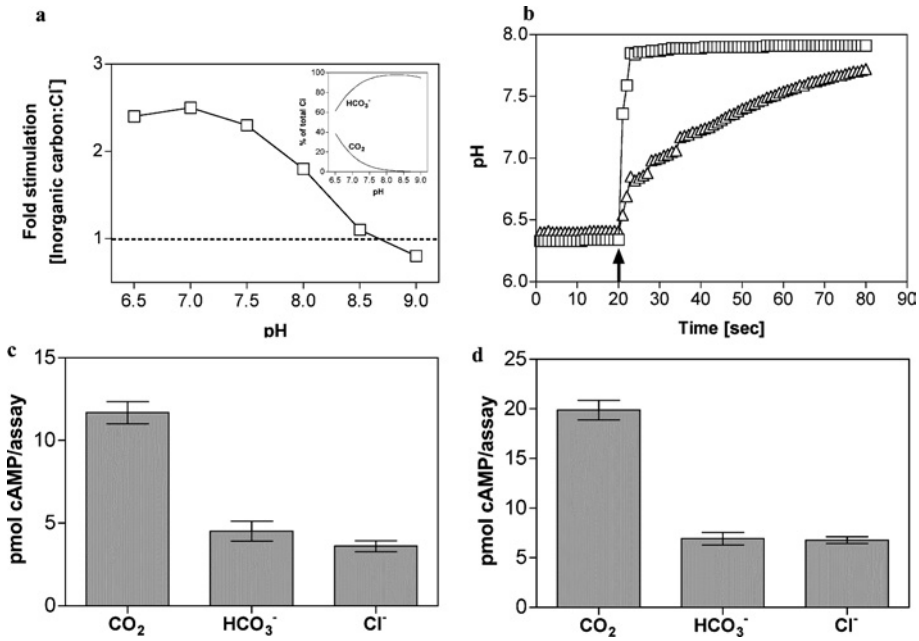

Figure 3 Activation of AC by CO_2

(**a**) Ratio of the specific activities of $Slr1991_{120-337}$ when assayed in the presence of 40 mM total C_i or NaCl at various pH values (8 μM protein, 1 mM Mg^{2+}-ATP and 20 mM Mg^{2+}). The inset shows the percentage of total C_i made up by CO_2 and HCO_3^- over the pH range tested. (**b**) Change in pH of a 5 mM Mes solution (starting pH 6.4) on addition of 20 mM $NaHCO_3$ (↑) in the presence (□) or absence (△) of 132 units of carbonic anhydrase at 0 °C. (**c**) cAMP produced by $Slr1991_{120-337}$ under conditions of C_i disequilibrium (50 μM $Slr1991_{120-337}$ protein, 0 °C, 10 s, 20 mM CO_2/$NaHCO_3$/NaCl, 100 mM Mes, pH 6.5, 150 μM Mn^{2+}-ATP). (**d**), cAMP produced by $CyaB1_{595-859}$ under conditions of C_i disequilibrium (38 μM $CyaB1_{595-859}$ protein, 0 °C, 10 s, 20 mM CO_2/$NaHCO_3$/NaCl, 100 mM Mes, pH 6.5, 150 μM Mn^{2+}-ATP, $n = 6$).

addition of 20 mM CO_2 or 20 mM $NaHCO_3$ in the presence or absence of carbonic anhydrase at 0 °C (Figure 3b). On the basis of these results we defined conditions for assaying AC under conditions of disequilibrium using 20 mM CO_2 or HCO_3^- as a 10 s assay period at 0 °C after addition of C_i. Under these conditions, C_i is predominantly in the form added to the assay (CO_2 or HCO_3^-) and has not significantly advanced toward the equilibrium determined by assay pH (held with 100 mM Mes). Control experiments demonstrated that, under the conditions used for the assay, the final pH was equivalent when CO_2, HCO_3^- or Cl^- were added, demonstrating that any observed stimulation was due to addition of C_i and not a change in assay pH (results not shown).

We assayed $Slr1991_{120-337}$ under conditions of C_i disequilibrium and observed, surprisingly, that CO_2, but not HCO_3^-, stimulated the enzyme (Figure 3c). We investigated whether this highly significant result was unique to Slr1991 or of more general significance. The $CyaB1_{595-859}$ protein of *Anabaena* PCC 7120 was previously shown to respond to HCO_3^-/CO_2, but the activating species was not demonstrated [7]. Consistent with the findings for $Slr1991_{120-337}$, $CyaB1_{595-859}$ was also stimulated by CO_2, but not HCO_3^-, under conditions of C_i disequilibrium (Figure 3d).

These results demonstrate that, for at least two randomly selected prokaryotic ACs, Slr1991 and CyaB1, the activating carbon species is dissolved CO_2 and not the more ably binding HCO_3^- species. These enzymes therefore represent the first identified signalling molecules demonstrated to respond directly to CO_2. HCO_3^- regulation of AC has been proposed, but not proven, for enzymes from species as diverse as the cyanobacterium *Spirulina platensis*, the encapsulated yeast-like fungus *Cryptococcus neoformans*, the yeast *Candida albicans*, the photosynthetic bacterium *Chloroflexus aurantiacus* and mammals [6,9,15,16]. An urgent examination of these systems is required to prove whether the ACs defined from these species respond to HCO_3^- or to CO_2 as described here.

We thank Professor Alistair Hetherington, Department of Biological Sciences, Lancaster University, Lancaster, U.K., for helpful discussions. The BBSRC (Biotechnology and Biological Sciences Research Council) supported this work.

REFERENCES

1 Badger, M. R. and Price, G. D. (2003) CO_2 concentrating mechanisms in cyanobacteria: molecular components, their diversity and evolution. J. Exp. Bot. **54**, 609–622
2 Adrogue, H. E. and Adrogue, H. J. (2001) Acid–base physiology. Respir. Care **46**, 328–341
3 Smith, K. S. and Ferry, J. G. (2000) Prokaryotic carbonic anhydrases. FEMS Microbiol. Rev. **24**, 335–366
4 Hetherington, A. M. and Raven, J. A. (2005) The biology of carbon dioxide. Curr. Biol. **15**, R406–R410
5 Buck, J., Sinclair, M. L., Schapal, L., Cann, M. J. and Levin, L. R. (1999) Cytosolic adenylyl cyclase defines a unique signaling molecule in mammals. Proc. Natl. Acad. Sci. U.S.A. **96**, 79–84
6 Chen, Y., Cann, M. J., Litvin, T. N., Iourgenko, V., Sinclair, M. L., Levin, L. R. and Buck, J. (2000) Soluble adenylyl cyclase as an evolutionarily conserved bicarbonate sensor. Science **289**, 625–628
7 Cann, M. J., Hammer, A., Zhou, J. and Kanacher, T. (2003) A defined subset of adenylyl cyclases is regulated by bicarbonate ion. J. Biol. Chem. **278**, 35033–35038
8 Cann, M. (2004) Bicarbonate stimulated adenylyl cyclases. IUBMB Life **56**, 529–534
9 Kobayashi, M., Buck, J. and Levin, L. R. (2004) Conservation of functional domain structure in bicarbonate-regulated "soluble" adenylyl cyclases in bacteria and eukaryotes. Dev. Genes Evol. **214**, 503–509
10 Kanacher, T., Schultz, A., Linder, J. U. and Schultz, J. E. (2002) A GAF-domain-regulated adenylyl cyclase from *Anabaena* is a self-activating cAMP switch. EMBO J. **21**, 3672–3680
11 Salomon, Y., Londos, C. and Rodbell, M. (1974) A highly sensitive adenylate cyclase assay. Anal. Biochem. **58**, 541–548

12 Kaneko, T., Sato, S., Kotani, H., Tanaka, A., Asamizu, E., Nakamura, Y., Miyajima, N., Hirosawa, M., Sugiura, M., Sasamoto, S. et al. (1996) Sequence analysis of the genome of the unicellular cyanobacterium *Synechocystis* sp. strain PCC6803. II. Sequence determination of the entire genome and assignment of potential protein-coding regions. DNA Res. **3**, 109–136
13 Masuda, S. and Ono, T. A. (2005) Adenylyl cyclase activity of Cya1 from the cyanobacterium *Synechocystis* sp. strain PCC 6803 is inhibited by bicarbonate. J. Bacteriol. **187**, 5032–5035
14 Cooper, T. G. and Filmer, D. (1969) The active species of "CO_2" utilized by ribulose diphosphate carboxylase. J. Biol. Chem. **244**, 1081–1083
15 Klengel, T., Liang, W. J., Chaloupka, J., Ruoff, C., Schroppel, K., Naglik, J. R., Eckert, S. E., Mogensen, E. G., Haynes, K. and Tuite, M. F. (2005) Fungal adenylyl cyclase integrates CO_2 sensing with cAMP signaling and virulence. Curr. Biol. **15**, 2021–2026
16 Steegborn, C., Litvin, T. N., Levin, L. R., Buck, J. and Wu, H. (2005) Bicarbonate activation of adenylyl cyclase via promotion of catalytic active site closure and metal recruitment. Nat. Struct. Mol. Biol. **12**, 32–37

Received 8 March 2006/28 March 2006; accepted 30 March 2006
Published as BJ Immediate Publication 30 March 2006, doi:10.1042/BJ20060372