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REGULATION OF PROKARYOTIC ADENYLYL CYCLASES BY CARBON DIOXIDE

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Running title: Adenylyl Cyclase and Carbon Dioxide.

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SYNOPSIS.

The Slr1991 adenylyl cyclase of the model prokaroyte *Synechocystis* PCC 6803 was stimulated two-fold at 20 mM total inorganic carbon at pH 7.5 through an increase in $k_{cat}$. A dose response demonstrated an E.C.50 of 52.7 mM total inorganic carbon at pH 6.5. Slr1991 was activated by CO$_2$ but not 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.

Keywords: adenylyl cyclase, cAMP, carbon dioxide, bicarbonate, *Synechocystis*
INTRODUCTION.

Inorganic carbon (Ci) is fundamental to the physiology of all organisms. Carbon dioxide and bicarbonate ions exist in a pH dependent equilibrium and are the major biologically active forms of Ci. CO$_2$ and HCO$_3^-$ are vital to such diverse physiological processes as photosynthetic carbon fixation [1], pH homeostasis [2], and carbon metabolism [3]. Study of Ci 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 Ci activated signalling molecules and their role in physiology is fundamental to understanding the diverse roles of Ci in biology. Currently, no signalling enzymes directly activated by CO$_2$ are known.

The mammalian soluble adenylyl cyclase (sAC) synthesizes the second messenger adenosine 3', 5'-cyclic monophosphate and is stimulated by HCO$_3^-$ [5, 6]. It was observed that HCO$_3^-$ regulation of adenylyl cyclase (AC) was conserved in a cyanobacterial AC, CyaC of *Spirulina (Arthrospira) platensis*, which had significant sequence homology 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, Ci 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 Ci 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 Ci signalling through AC as hypothesized HCO$_3^-$ responsive ACs are widespread in these organisms and Ci 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 Ci. 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 proven to respond to CO$_2$ rather than HCO$_3^-$, This 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 genomic DNA of Synechocystis PCC 6803, subcloned into pQE30, and fitted with an N-terminal MRGSH6GS affinity tag. Constructs were confirmed by double-stranded sequencing. Slr1991\textsubscript{120-337} protein was expressed in *Escherichia coli* M15 [pREP4] cells at 25 °C, for 3 h with 300 µM isopropyl β-D-1-thiogalacto-pyranoside. Pelleted cells were washed (50 mM Tris-HCl 8.5, 1 mM EDTA), resuspended (50 mM Tris-HCl pH 8.5, 250 mM NaCl, 10 mM 1-thioglycerol), lysed by sonication (1 x 150 s), and protein purified from the supernatant with Ni\textsuperscript{2+}-nitrilotriacetic acid (NTA, Qiagen) as previously described [10]. CyaB\textsubscript{595-859} protein was generated as previously described [10]. Primer sequences are available on request.

Adenylyl cyclase assays - AC assays were performed at 40 °C in a final volume of 100 µl and typically contained 50 mM buffer, 2 mM MnCl\textsubscript{2}, 2 mM [2,8-\textsuperscript{3}H]-cAMP (150 Bq), and \([α\textsuperscript{32}P]\text{ATP (25 kBq)}\) 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 of 1-100 µM (Mn\textsuperscript{2+}-ATP). The following buffers were used at pH 6.5 (2-[N-morpholino]ethanesulfonic acid; MES), pH 7.0-7.5 (3-[N-morpholino]propanesulfonic acid), pH 8.0-8.5 (Tris-[hydroxymethyl]aminoomethane hydrochloride, and pH 9.0 (2-[N-cyclohexylamino]ethanesulfonic acid). Enzyme, buffer, and substrate were all prepared at the appropriate pH for the required assay. CO\textsubscript{2} was quantitated by titration against NaOH. Assay pH was stable over a period of at least 40 minutes. All errors correspond to the standard error of the mean. 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 (Forkhead Associated) 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 cAMP mg$^{-1}$ min$^{-1}$, n=8) and Mn$^{2+}$-ATP (5816 ± 87 pmol cAMP mg$^{-1}$ min$^{-1}$, n=8) under optimal conditions (pH 9.5, 40 °C, 0.3 mM ATP, 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 Ci with a view to determining the identity of the activating species, CO$_2$ or HCO$_3^-$. Slr1991$_{120-337}$ specific activity was stimulated two-fold by 20 mM total Ci (1.2 mM CO$_2$/18.8 mM HCO$_3^-$) at pH 7.5 compared to Cl$^-$. Stimulation was independent of cation and robust to 95% confidence intervals (Figure 1b). A previous report had not observed stimulation of Slr1991 by Ci at pH 7.5 [13]. We noted that an extended assay period (40 mins) was required to observe robust Ci activation of Slr1991 at pH 7.5. Although Masuda and Ono do not report assay time, this is the most likely cause of the discrepancy.

We determined the kinetics of activation of Slr1991$_{120-337}$ by Ci (Table I). 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 Ci than Cl$^-$ but $V_{max}$ values were proportionately greater for Ci than Cl$^-$. The overall result was that Ci increased turnover rate ($k_{cat}$). A dose response curve with increasing Ci was performed at a reduced pH 6.5 to eliminate problems with enzyme inhibition at >20 mM total Ci at pH 7.5 in the presence of Mn$^{2+}$-ATP (Figure 2). The experiment revealed a maximum eight-fold stimulation with an apparent E.C.$_{50}$ for Ci 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 Ci at varying pH to gain insight into whether the enzyme is responsive to CO$_2$ and/or HCO$_3^-$. The experiment was performed using Mg$^{2+}$-ATP as substrate as Mg$^{2+}$ co-factor is more soluble than Mn$^{2+}$ in the presence of Ci at alkaline pH. Intriguingly, relative stimulation (Ci: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 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 Ci 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 Ci 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 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 this data we defined conditions for assaying AC under conditions of disequilibrium using 20 mM CO\(_2\) or HCO\(_3^-\) as a 10 second assay period at 0°C after addition of Ci. Under these conditions, Ci 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 (clamped with 100 mM MES).

Control experiments demonstrated that under the conditions used for the assay final pH was equivalent when either CO\(_2\), HCO\(_3^-\), or Cl\(^-\) were added demonstrating that any observed stimulation was due to addition of Ci and not a change in assay pH (data not shown).

We assayed Slr1991_{120-337} under conditions of Ci 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 not proven [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 Ci disequilibrium (Figure 3d).

This data demonstrates 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 Spirulina platensis, Cryptococcus neoformans, Candida albicans, 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.
REFERENCES


ACKNOWLEDGEMENTS.

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FIGURE LEGENDS.

**Figure 1.** Adenylyl cyclase activity of purified recombinant Slr1991_{120-337}. **A,** Purification of recombinant Slr1991_{120-337} (SDS-PAGE analysis, Coomassie Blue staining). 1.5 µg of protein was applied and molecular mass standards (in kDa) are indicated. **B,** Slr1991_{120-337} specific activity (n=8) in presence of 20 mM total Ci/salt (0.6 µM protein, 20 µM Mn^{2+}-ATP, pH 7.5).

**Figure 2.** Response of wild Slr1991_{120-337} to Ci. Slr1991_{120-337} specific activity (n=6) at increasing total Ci (1.5 µM protein, 20 µM Mn^{2+}-ATP, pH 6.5, Na^+ as cation, total salt made up to 200 mM with NaCl).

**Figure 3.** Activation of adenylyl cyclase by CO\(_2\). **A,** The ratio of the specific activities of Slr1991_{120-337} when assayed in the presence of 40 mM total Ci or NaCl at varying pH (8 µM protein, 1 mM Mg^{2+}-ATP, 20 mM Mg^{2+}). The inset shows the percentage of total Ci 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\(_3\) (arrow) in the presence (squares) or absence (triangles) of 132 U carbonic anhydrase at 0°C. **C,** cAMP produced by Slr1991_{120-337} under conditions of Ci disequilibrium (50 µM Slr1991_{120-337} protein, 0°C, 10 secs, 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 Ci disequilibrium (38 µM CyaB1_{595-859} protein, 0°C, 10 secs, 20 mM CO\(_2\)/NaHCO\(_3\)/NaCl, 100 mM MES pH 6.5, 150 µM Mn^{2+}-ATP, n=6).
Table I. Kinetic parameters for Slr1991_{120-337}. 0.6 µM protein was assayed at pH 7.5 in the presence of 20 mM salt (n=6).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Cl⁻</th>
<th>HCO₃⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{\text{max}}$ (nmol cAMP mg⁻¹ min⁻¹)</td>
<td>0.74 ± 0.01</td>
<td>1.13 ± 0.03</td>
</tr>
<tr>
<td>$K_{M\text{[ATP]}}$ (µM)</td>
<td>11.4 ± 0.7</td>
<td>16.2 ± 1.3</td>
</tr>
<tr>
<td>Hill Slope</td>
<td>1.01 ± 0.01</td>
<td>1.03 ± 0.03</td>
</tr>
<tr>
<td>$k_{\text{cat}}$ (min⁻¹)</td>
<td>0.018</td>
<td>0.027</td>
</tr>
</tbody>
</table>
Figure 1a.
Figure 1b.
Figure 2.
Figure 3a.
Hammer et al., 2006

Adenylyl cyclase and carbon dioxide

Figure 3b.
Hammer et al., 2006

Adenylyl cyclase and carbon dioxide

**Figure 3c**

![Bar graph showing pmol cAMP/assay for CO₂, HCO₃⁻, and Cl⁻]
Figure 3d