COMMENTARY

Sensing inorganic carbon: CO_2 and HCO_3^-

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Enzymes and transporters that catalyse reactions involving inorganic carbon are well characterized with respect to the species of inorganic carbon (CO_2 or HCO_3^-) with which they interact. There is less information on the species recognized by proteins that sense inorganic carbon. In this issue of the *Biochemical Journal*, Hammer and colleagues show conclusively that cyanobacterial adenylyl cyclases are activated by CO_2 and not HCO_3^-, as was believed previously. While in some circumstances a similar *in vivo* regulatory outcome is achieved from sensing HCO_3^- as from sensing CO_2, there are cases in which the outcomes are significantly different. The most striking example is where a compartment lacks carbonic anhydrase yet supports large metabolic fluxes of inorganic carbon species so that CO_2 and HCO_3^- are not at equilibrium. Other examples involve changes in pH, or temperature, of a compartment containing an equilibrium mixture of CO_2 and HCO_3^-.

Key words: adenylyl cyclase (adenyl cyclase, adenylate cyclase), carbon-concentrating mechanism, cyanobacterium, inorganic carbon, pH, temperature.

Life as we know it is crucially dependent on the properties of carbon, and especially those of the carbon–carbon bond in organic molecules. However, the inorganic carbon species CO_2, H_2CO_3, HCO_3^- and CO_3^{2-} are ubiquitous within organisms and their environments, and it is prudent to assume that all organisms are guilty of carrying out carboxylation and decarboxylation reactions interconverting inorganic and organic carbon unless they have been proven innocent. CO_2 is the product of all decarboxylase reactions examined so far, including those which operate *in vivo* close to equilibrium and which can also function as carboxylases. Those carboxylases which are essentially irreversible *in vivo* can, depending on the enzyme involved, use either CO_2 or HCO_3^- [1]. These carboxylation and decarboxylation reactions are major links in the biogeochemical cycle of carbon, each accounting for a global flux of at least 10 Pmol (10^{15} mol) of carbon each year [1]. A group of enzyme which can react with both CO_2 and HCO_3^- are the polyphyletic carbonic anhydrases (EC 4.2.1.1) which catalyse the interconversion of CO_2 and HCO_3^-; the uncatalysed reaction is frequently slow relative to metabolic fluxes of the inorganic carbon species [1].

Inorganic carbon species are also involved in the regulation of enzyme activities. An example of activation of an enzyme is Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase; EC 4.1.1.39), the globally predominant carboxylase of autotrophs [1,2]. CO_2 is the species of inorganic carbon used by the carboxylase activity of Rubisco, and also activates the enzyme by formation of a carbamate at a lysine residue in the active site [2]. There are also developmental consequences of changed inorganic carbon concentrations. An example from autotrophs is the repression and derepression of CCMs (inorganic carbon-concentrating mechanisms) of many aquatic photosynthetic organisms [1,3,4]. However, the nature of the receptor for inorganic carbon, and of the downstream signal transduction pathway, is not well understood. A possible candidate for an inorganic carbon receptor in cyanobacteria, which have CCMs whose expression is regulated by inorganic carbon availability (with a subsidiary effect of oxygen) [3,4], is adenylyl cyclase (EC 4.6.1.1), which generates the signalling molecule cAMP [5]. As in mammals, the enzyme from cyanobacteria is activated by inorganic carbon, and the predominance of HCO_3^- at the *in vivo* pH at which the enzyme functions led to the assumption that this is the form of inorganic carbon that is involved in the enzyme activation [5].

In this issue of the *Biochemical Journal*, Hammer et al. [6] report definitive experiments showing that the cyanobacterial enzyme uses CO_2, and not HCO_3^-, as the inorganic carbon species involved in the activation of adenylyl cyclase. This specificity was determined using a technique first employed to determine the inorganic carbon species used in carboxylation by Rubisco, and subsequently used to identify the inorganic carbon species that is used by other carboxylases as well as the species that enters whole cells [1]. The method relies on the slow equilibration between CO_2 and HCO_3^- in the absence of carbonic anhydrase; a further decrease in the equilibration rate relative to the catalysed process under investigation is achieved by working at low temperatures. A solution of either CO_2 or HCO_3^- is added to an appropriately pH-buffered solution of the enzyme, and the time course of enzyme activity is measured during the slow conversion of the added CO_2 to HCO_3^- or vice versa. Hammer et al. [6] found that stimulation of enzyme activity occurred within the 10 s assay period when the inorganic carbon was supplied as CO_2 but that there was no significant stimulation when the inorganic carbon was supplied as HCO_3^-.

Why does it matter whether CO_2 or HCO_3^- is the inorganic carbon species used by adenylyl cyclase, or indeed any enzyme or transporter with inorganic carbon as a substrate? An obvious 'biochemical' answer is that the catalytic properties of a protein cannot be regarded as known when there is doubt as to the nature of one of the substrates. For the functioning of the catalyst *in vivo*, there is clearly a significant difference in the capacity to regulate the pH of cellular compartments if a transporter moves CO_2 rather than HCO_3^- across the membrane surrounding the compartment. For a carboxylase *in vivo*, the inorganic carbon species used has consequences for acid–base regulation of the compartment containing the enzyme, e.g. comparing phosphoenolpyruvate carboxylase (EC 4.1.1.31) (which uses HCO_3^-) and phosphoenolpyruvate carboxykinase (EC 4.1.1.32) (which

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uses CO_2), both of which consume phosphoenolpyruvate and produce oxaloacetate [1].

However, for an enzyme such as adenylyl cyclase, in which the inorganic carbon substrate is not consumed, considerations of acid–base regulation resulting from consumption of an inorganic carbon species during enzyme activity do not apply. The cyanobacterial enzyme is soluble, and presumably occurs in an aqueous compartment. A rather weak, teleological, argument for an intracellular rather than a periplasmic location is that if the enzyme's inorganic carbon stimulation is to be used in signalling it would be best located in an intracellular compartment where the inorganic carbon concentration is higher than that in the medium (at least in the light) as a result of the occurrence of the CCM. This argument is based on the relatively low CO_2 affinity of the cyclase: the half-maximal stimulation of activity requires 20.4 mM CO_2 [6], some three orders of magnitude greater than air-equilibrium CO_2 concentrations in the natural habitats of free-living cyanobacteria. A related argument is the relatively small (not more than 3-fold) stimulation of the cyanobacterial cyclase by saturating concentrations of CO_2 [6], which means that air-equilibrium concentrations of CO_2 are even more unlikely to cause a significant increase in cyclase activity.

An intracellular location of adenylyl cyclase in cyanobacteria means that the enzyme would be, within certain constraints, exposed to a constant ratio of CO_2 to HCO_3^- as a result of the cell's homoeostatic mechanisms. If this was the case, then it would not matter for signalling whether CO_2 or HCO_3^- was the species sensed by the cyclase. What are the constraints on the assumption of constant intracellular inorganic carbon speciation for any signalling role of the enzyme? The major constraint is the implicit assumption of equilibration between CO_2 and HCO_3^- in the compartment containing the cyclase. Carbonic anhydrase activity is required in view of the very large metabolic flux of CO_2 consumption by Rubisco, and the effective entry of the inorganic carbon used in photosynthesis from the medium as HCO_3^- [1,3,4]. Some of the inorganic carbon enters cyanobacteria via energized transporters that are specific for HCO_3^- in the plasmalemma [3,4]. The rest enters across the plasmalemma, probably involving proteinaceous channels, as CO_2 by diffusion, and is then converted into HCO_3^- by an energized reaction catalysed by a thylakoid-located NADH dehydrogenase (EC 1.6.99.3) subunit [3,4]. This reaction is, as a result of energization, effectively a unidirectional carbonic anhydrase-like reaction. The HCO_3^- then enters the carboxysome compartment where all of the cellular pool of Rubisco is located. The carboxysome compartment has (non-energized) carbonic anhydrase activity which converts HCO_3^- into CO_2; the CO_2 is then fixed by Rubisco [3,4,7].

There is no non-energized carbonic anhydrase, catalysing conversion of HCO_3^- into CO_2, in the cytosol, and engineered expression of soluble carbonic anhydrase in the cytosol inhibits the CCM very significantly [3,4]. The energized conversion into HCO_3^- of CO_2 entering across the plasmalemma, and any CO_2 leaking from the carboxysomes, keeps the steady-state concentration of CO_2 in the cytosol below the concentration in the medium. This means that the CO_2-sensing function of adenylyl cyclase could not function in the cytosol for the reasons explained earlier, and could only function in the carboxysome. Even here the steady-state CO_2 concentration would be much less than the half-saturating value for activation of adenylyl cyclase, with 20 mM intracellular (cytosol and carboxysome) HCO_3^- and a cytosol pH of 7.6 in the illuminated cyanobacterial cell [8], giving a carboxysomal CO_2 concentration at equilibrium with HCO_3^- of some 0.5 mM. This is almost sufficient to saturate the carboxylase activity of the low-CO_2-affinity cyanobacterial Form 1B Rubiscos [2–4], but would still not have a large stimulatory effect on cyanobacterial adenylyl cyclase [6]. However, the carboxysome is the most likely compartment in the cyanobacterial cell in which adenylyl kinase could function as a CO_2 sensor in any cAMP-dependent signalling pathway related to CCM expression.

Calculation of the equilibrium ratio of CO_2/HCO_3^- in the carboxysomes requires knowledge of the local pH, the temperature and the ionic strength [9]. Estimation of the intracellular (cytosolic) pH of cyanobacteria, determined by weak anion distribution or by NMR, does not refer directly to the contents of the carboxysome. The soluble contents of the carboxysome are linked to the cytosol by anion-selective channels in the proteinaceous carboxysome shell [7], and there may be different ion-exchange properties of non-diffusible macromolecules in the cytosol and the carboxysomes, with the possibility of a Donnan potential across the carboxysome shell. The ionic strength of the cyanobacterial cell contents is important in terms of the equilibrium CO_2/HCO_3^- ratio because the pK_{a1} of the inorganic carbon system, which determines the CO_2/HCO_3^- ratio, is dependent on the ionic strength and the temperature. The ionic strength of the cell contents is relatively constant despite changes in external osmolarity, with increments in internal osmolarity with turgor regulation as external osmolarity increases, largely accounted for by organic compatible solutes which do not change the pK_{a1}.

Temperature is a determinant of pK_{a1} [9], and temperature variations on a diel basis are significant in some cyanobacterial environments, especially cyanobacterial mats in shallow waters [4]. Higher temperatures during the middle of the day decrease pK_{a1} and thus, for a given intracellular pH, decrease the CO_2/HCO_3^- ratio [1,2]. Furthermore, higher temperatures decrease the affinity of Rubisco for CO_2 and increase the competitive inhibition of carboxylation by the oxygenase activity [1,2]. These considerations make the sensing of CO_2 rather than HCO_3^- more selectively useful for cyanobacteria.

There is also a temperature effect on the regulated cytosolic pH of organisms, another determinant of the equilibrium CO_2/HCO_3^- ratio at a given pK_{a1} [9]. The α-stat hypothesis for regulation of cytosolic pH with variations in temperature involves an increase in pH with decreasing temperature of some 0.016 pH units per degree Celsius of temperature decrease [9,10]. This would maintain the H^+/OH^- ratio of water constant, and a constant ionization state of histidine residues which have a pK_{a1} that is close to cytoplasmic pH values. While many data on poikilothermic animals agree with the α-stat hypothesis, other data do not [10], and the only datum available for photosynthetic cells (giant cells of the green alga *Chara corallina*) shows that the change in cytoplasmic pH with temperature is less than that required by the α-stat hypothesis, but maintains approximate constancy of the cytoplasmic ratio of CO_2/HCO_3^- with changing temperature [9]. While more data for photosynthetic cells, and especially for cyanobacteria, are needed, the datum available suggests that the CO_2/HCO_3^- ratio is temperature-independent and that there is no superiority, on this basis, of sensing CO_2 rather than HCO_3^-.

A final determinant of the intracellular pH at a given temperature is the external pH [8,9]. The external pH can increase significantly in the light in cyanobacterial mats as inorganic carbon is consumed faster than it can be replaced from the overlying water [4]. Homoeostasis of intracellular pH is not complete, and an increase in external pH of 2 units can involve an increase in cytosolic pH of up to 0.2 units (see [8] and references therein), with a corresponding decrease in the equilibrium CO_2/HCO_3^- ratio to 0.58 of its initial value. This constitutes another reason for sensing of CO_2 rather than HCO_3^- being appropriate in the regulation of the cyanobacterial CCM.

This discussion of the variations in the intracellular equilibrium CO_2/HCO_3^- ratio with environmental changes suggests that CO_2,

as sensed by adenylyl cyclase, is a more appropriate indicator for regulation of the CCM in cyanobacteria than is HCO_3^-. As relevant to the possible role of adenylyl cyclase sensing CO_2 in the regulation of CCMs is the need for the location of adenylyl cyclase in the carboxysome: there is as yet no evidence for the location of adenylyl cyclase of cyanobacteria. Regardless of this lack of evidence, the work of Hammer et al. [6] in showing that cyanobacterial adenylyl cyclase senses CO_2 rather than HCO_3^- is a crucial first step in investigating the role(s) for adenylyl cyclase in the regulation of the CCM and other systems in cyanobacteria.

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