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Nature of the Inorganic Carbon Species Actively Taken Up by the Cyanobacterium *Anabaena variabilis*[1]

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ABSTRACT

The nature of the inorganic carbon (C_i) species actively taken up by cyanobacteria CO_2 or HCO_3^- has been investigated. The kinetics of CO_2 uptake, as well as that of HCO_3^- uptake, indicated the involvement of a saturable process. The apparent affinity of the uptake mechanism for CO_2 was higher than that for HCO_3^-. Though the calculated V_{max} was the same in both cases, the maximum rate of uptake actually observed was higher when HCO_3^- was supplied. C_i uptake was far more sensitive to the carbonic anhydrase inhibitor ethoxyzolamide when CO_2 was the species supplied. Observations of photosynthetic rate as a function of intracellular C_i level (following supply of CO_2 or HCO_3^- for 5 seconds) led to the inference that HCO_3^- is the species which arrives at the inner membrane surface, regardless of the species supplied. When the two species were supplied simultaneously, mutual inhibition of uptake was observed.

On the basis of these and other results, a model is proposed postulating that a carboic anhydrase-like subunit of the C_i transport apparatus binds CO_2 and releases HCO_3^- at or near a membrane porter. The latter transports HCO_3^- ions to the cell interior.

The observed capability of green algae and cyanobacteria to accumulate C_i[2] within the cells has been attributed to the operation of a mechanism for active transport of HCO_3^- ions (for review, see Lucas [8]). However, the observation that supply of $^{14}CO_2$ to green algae (5, 9, 11) and cyanobacteria (2) leads to faster accumulation of C_i and acid-stable ^{14}C than does HCO_3^- supply has prompted the proposal that CO_2 may in fact be the species actively transported. In the present study we have investigated the uptake of each C_i species within a brief period of its supply to *Anabaena variabilis* cells and, further, have examined interactions between CO_2 and HCO_3^- during the uptake process. Data obtained bear on the molecular mechanism involved in C_i uptake in cyanobacteria.

MATERIALS AND METHODS

Cultures of *Anabaena variabilis* M-3 (from the collection of Tokyo University) were grown as described earlier (6). Accumulation of acid stable (photosynthetic products) and labile (inorganic) carbon were determined using the filtering centrifugation technique (6, 7). All experiments were conducted at pH 8.0. $^{14}CO_2$ or $H^{14}CO_3^-$ were prepared and supplied as described by Volokita *et al.* (12). The stock of $H^{14}CO_3^-$ in 100 mM BTP (pH 9.5) was freshly prepared under N_2 and was also used to prepare the $^{14}CO_2$ stock. Conversion of $H^{14}CO_3^-$ to $^{14}CO_2$ was carried out as follows: Aliquots of the $H^{14}CO_3^-$ solution were injected into closed microfuge tubes containing 100 mM Mes (pH 2.4). Following this injection the pH rose to 5.1 (maximum). The solution in the tube was mixed for 60 s with a syringe and the required volume withdrawn and injected into the cell suspension. Another sample was injected into 0.1 N NaOH. The radioactivity in the letter sample was assessed in order to correct for the loss of $^{14}CO_2$ from the microfuge tube. This procedure enabled accurate determination of the concentration of CO_2 supplied, and ensured that the specific activity of the C_i supplied was the same, whether presented as CO_2 or HCO_3^-. The injection of aliquots of the $H^{14}CO_3^-$ or $^{14}CO_2$ stocks changed the pH of the cell suspension (in 50 mM Hepes-NaOH, pH 8.0) to only a minor degree (maximum 0.1 pH units). This change was taken into account whenever the actual concentration of CO_2 or HCO_3^- present at a given time was calculated. EA was kindly donated by Professor S. Miyachi.

RESULTS

The cells of low CO_2 grown *Anabaena* accumulated acid-labile ^{14}C (C_i) more rapidly when the C_i supplied was in the form of $^{14}CO_2$ as compared with $H^{14}CO_3^-$ (Fig. 1). The discrepancy between CO_2 uptake and HCO_3^- uptake was larger in the case of high CO_2 adapted cells (not shown) than in that of low CO_2 grown cells.

Uptake of C_i from a range of CO_2 and HCO_3^- concentrations was followed with time, and initial rate of uptake estimated from the initial slope of the curves obtained; the initial rates are plotted as a function of external concentration in Figure 2. Interestingly, the CO_2 uptake curve shows saturation characteristics. Over the concentration range up to 150 μM, CO_2 was taken up faster than HCO_3^-. At external concentrations higher than 150 μM, on the other hand, HCO_3^- was taken up faster than CO_2. Kinetic analysis of the data (see Fig. 2, inset) indicated that the affinity of the transporting system for CO_2 was higher than that for HCO_3^- ($K_{1/2}$ = 17 μM and 60 μM, respectively). Apparent V_{max}, by contrast, was estimated to be the same (Fig. 2, inset) regardless of the form in which C_i was supplied. The fact that the experimentally obtained curve for CO_2 supply did not reach as high a value for velocity as did that for HCO_3^- supply (Fig. 2) suggests that at high CO_2 concentration CO_2 transport is limited by some other factor.

The discrepancy between the rates of CO_2 and HCO_3^- uptake at low external C_i concentrations might conceivably be partly due to different rates of diffusion from medium to cell membrane. Diffusional resistances in this pathway may be greater for HCO_3^- ions than for the electrically neutral CO_2 molecule. However, experiments comparing the uptake performance of

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[2] Abbreviations: C_i, inorganic carbon; EA, ethoxyzolamide; CA, carbonic anhydrase; Rubisco, ribulose 1,5-bisphosphate carboxylase; BTP, 1,3-bis(tris-[hydroxymethyl]methylamino)propane.

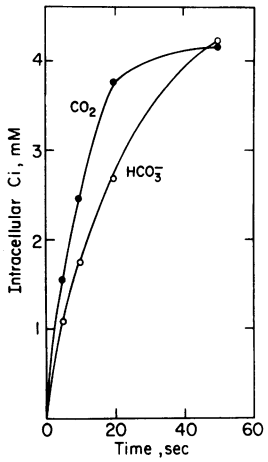

FIG. 1. The time course of accumulation of C_i within *A. variabilis* cells following the supply of C_i in the form of CO_2 or HCO_3^-. Low CO_2 grown cells. Light intensity was 6 mw·cm^{-2} (400–700 nM). Density of cell suspension corresponded to 11 μg Chl/ml, 30°C. C_i concentration was 10 μM (pH 8.0).

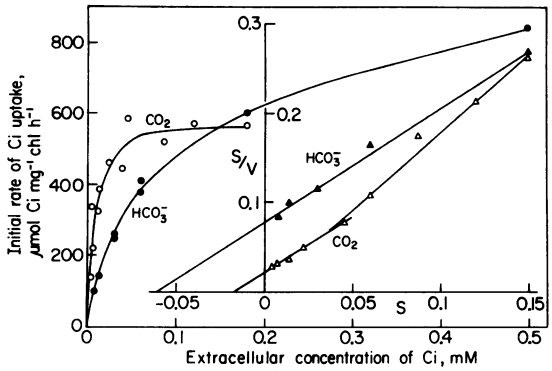

FIG. 2. Initial rate of uptake of C_i as a function of the concentration of CO_2 or HCO_3^- supplied. Data obtained from experiments in which the time course of uptake was determined. See Figure 1 for experimental conditions.

intact cells with that of spheroplasts prepared from the cells (13) have not yielded decisive enough evidence to assess the contribution of such diffusion resistances.

Nature of C_i Species Arriving at Inner Membrane Surface. The dependence of photosynthetic rate on the size of the intracellular C_i pool was analyzed using data obtained from experiments in which C_i was supplied for 5 s at various concentrations either in the form of CO_2 or of HCO_3^-, and the accumulation of acid stable (photosynthetic products) and labile ^{14}C (intracellular C_i) determined. Figure 3 shows that the points for both CO_2 and HCO_3^- supply lie on the same curve, *i.e.* the rate of photosynthesis at a given intracellular C_i level was not affected by the form in which C_i was supplied externally during the 5-s period. Since it is accepted that the substrate for Rubisco is CO_2 and not HCO_3^- ([4], confirmed also for *Anabaena* [1], one must conclude from Figure 3 that the ratio CO_2/HCO_3^- was the same at a given intracellular C_i concentration, regardless of whether CO_2 or HCO_3^- was the form of C_i supplied.

Figure 3 also shows that intracellular C_i pools as high as 15 mM (obtained following 5 s of CO_2 supply) were not saturating for photosynthesis. Since the $K_m(CO_2)$ for Rubisco isolated from *Anabaena* has been estimated as 0.2 to 0.3 mM (1; and A. Kaplan, unpublished data) this result indicates that the C_i in the internal pool after a 5-s supply was present mainly as HCO_3^-. The possibility might be considered that, owing to interconversion

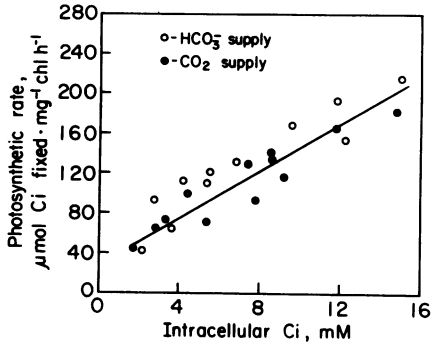

FIG. 3. Photosynthetic rate as a function of intracellular C_i concentration. Accumulation of acid stable ^{14}C and intracellular C_i were determined 5 s after the supply of C_i either as CO_2 or as HCO_3^- at concentrations ranging from 2 to 200 μM for CO_2 or 4 μM to 2 mM for HCO_3^-.

between the C_i species, CO_2 arriving at the transmembrane interface was rapidly converted to HCO_3^-. However, assuming a cytoplasmic pH of 7.5 to 7.8 (a minimum estimate at this external pH, [14]) even if interconversion had reached equilibrium, the intracellular CO_2 concentration when the total internal C_i level was 15 mM would still be 0.54 to 1.04 mM, *i.e.* 2 to 4 times the K_m for Rubisco. The curve in Figure 3 should therefore have deviated markedly from linearity. That this is not observed indicates that the CO_2 level must be lower even than that expected after interconversion to the CO_2/HCO_3^- equilibrium value. Moreover, the C_i species are not likely to have reached equilibrium in *Anabaena* cells for reasons pointed out earlier (6) including the lack of detectable carbonic anhydrase activity.

If the period of C_i supply is extended, the curve relating photosynthetic rate to the intracellular C_i level tends to deviate from linearity toward the shape of a saturation curve, but again the same curve is obtained regardless of whether CO_2 or HCO_3^- is supplied. The data are consistent with the suggestion that, whatever the form in which C_i is provided, it reaches the inner side of the plasmalemma as HCO_3^-. The latter is then converted to CO_2 and utilized in photosynthesis. If the relative rates of CO_2 formation and utilization are such that at high C_i concentrations CO_2 tends to accumulate within the cell, then with time it will reach levels which saturate photosynthesis, leading to a curvilinear relationship between photosynthetic rate and intracellular C_i concentration.

CO_2 and HCO_3^- Interaction. Possible interaction between the two species during uptake was investigated in competition experiments where the concentration of one species (labeled) was held constant while the concentration of the second (unlabeled) was varied. It was observed that the uptake of each species was suppressed in the presence of the other. Figure 4 presents a Dixon plot of the data. It indicates that the type of inhibition was competitive. The K_i values (200 μM CO_2 or HCO_3^-) however is larger than the corresponding K_m for each species (Fig. 2).

Interconversion between CO_2 and HCO_3^- in the external medium is unlikely to have affected the results appreciably. At the external pH used (8.0) the uncatalyzed rate of conversion of CO_2 to HCO_3^- is faster than the reaction in the reverse direction. While after 5 s up to 20% of the $^{14}CO_2$ supplied have been converted to $^{14}HCO_3^-$, only 0.3% of the $^{14}HCO_3^-$ supplied in the parallel experiment would have been converted to $^{14}CO_2$. Thus, the apparent competitive effects observed are unlikely to be attributable to conversion between CO_2 and HCO_3^-.

Effect of Carbonic Anhydrase Inhibitors. Sulfonamides such as acetazolamide (Diamox) EA, which are reputedly carbonic anhydrase inhibitors, have been observed to inhibit C_i uptake in cyanobacteria (6). Since CA activity is not detectable in *Anabaena*, it has been postulated (6) that the C_i transporter may

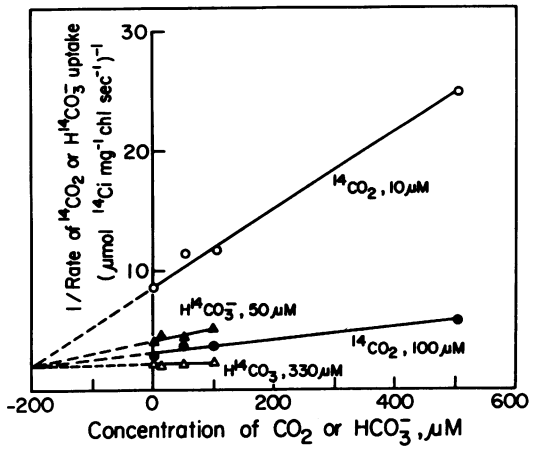

FIG. 4. Dixon plot showing reciprocal of the rate of $^{14}C_i$ ($^{14}CO_2$ or $H^{14}CO_3^-$) uptake as a function of the concentration of unlabeled C_i (HCO_3^- or CO_2, respectively). $^{14}CO_2$ and HCO_3^- were supplied simultaneously; alternatively, $H^{14}CO_3^-$ was supplied with CO_2. Uptake was terminated after 5 s by centrifugation.

Table I. *Intracellular C_i Pool Following the Supply of CO_2 or HCO_3^- to Cells of Anabaena as Affected by EA*

100 μM $^{14}C_i$ supplied for 5 s as $^{14}CO_2$ or as $H^{14}CO_3^-$. Other conditions as in Figure 1.

Treatment	Intracellular C_i	% of Control
	mM	
HCO_3^-	5.6 ± 0.5	100
HCO_3^- + 10 μM EA	4.8 ± 0.4	86
CO_2	14.4 ± 1.2	100
CO_2 + 10 μM EA	4.6 ± 0.6	32

possess a CA-like subunit which is sensitive to the sulfonamides. The results of experiments in which the effects of EA on CO_2 uptake and on HCO_3^- uptake were compared are given in Table I. Uptake of C_i was far more sensitive to EA when the C_i species supplied was CO_2.

DISCUSSION

Evidence collected in this investigation suggests that the C_i species arriving at the inner side of the membrane is HCO_3^-, regardless of whether HCO_3^- or CO_2 is the species supplied (deduced from Fig. 3). Further, the kinetics of CO_2 uptake (initial rate *versus* concentration, Fig. 2) indicate that a saturable process is involved. The possibility that the saturation kinetics reflect a process which consumes CO_2 after it has diffused into the cell, thus maintaining the inward diffusion gradient, can be rejected for the following reasons: First, after 5 s (the period of the experiment) C_i had been accumulated to a concentration 75 to 1000 (depending on the extracellular C_i concentration) times that in the external medium (Fig. 3), *i.e.* the diffusion gradient was very steep in the opposite, outward, direction. Second, during the experimental period only about 5 to 10% of the ^{14}C taken up was fixed; thus, CO_2 consumption in photosynthesis was almost negligible. Third, the reported K_m (CO_2) of carbonic anhydrase, which catalyzes the second major process in which CO_2 is consumed, differs from that indicated in Figure 2 by several orders of magnitude (10), and CA has in any case not been detected in *Anabaena* (6).

We conclude that the initial uptake of C_i when CO_2 is supplied is a saturatable, mediated transport process. This conclusion is supported by the following findings: First, there is mutual interaction between CO_2 uptake and HCO_3 uptake in competition experiments (Fig. 4). Second, CO_2 uptake as well as that of HCO_3^- is stimulated by light and inhibited by DCMU (D. Zenvirth, A. Kaplan, L. Reinhold, in preparation).

The evidence summarized above, together with the suggestive result that the uptake of CO_2 is depressed more drastically in the presence of a carbonic anhydrase inhibitor than is that of HCO_3^- (Table I) lead us to suggest a schematic model for C_i uptake. This model not only can account for the findings just referred to, but for a number of others as will be indicated below. We propose that a subunit of the C_i-transporting apparatus possesses CA-like activity (Fig. 5). CO_2 binds to this subunit and is converted to HCO_3^-. The nascent HCO_3^- generated in this process is immediately available, at or very near the site of its formation, to a HCO_3^- porter which transfers it to the inner membrane surface. The rate of conversion of CO_2 to HCO_3^- sets the upper limit for the rate of CO_2 uptake and is responsible for the lower maximum rate actually observed for CO_2 uptake as compared with HCO_3^- (Fig. 2). Linear conversion of uptake values at low external concentrations (Fig. 2) allows calculation of the V_{max} for the HCO_3^- porter, which would be expected to be the same for CO_2 and for HCO_3^-. Figure 2, inset, indicates that this is indeed the case. The apparently lower affinity of HCO_3^- for the porter as reflected in the relative K_m values for CO_2 and HCO_3^- may be attributable to the fact that the charged HCO_3^- ion encounters resistance in diffusing through the cell wall, and within the membrane, to the active site on the porter, whereas CO_2 meets with less resistance in the wall and may be very efficiently transferred to the porter within the membrane by the CA-like moiety. In other words, the 'nascent HCO_3^-' pool located within the membrane does not equilibrate very readily with the HCO_3^- in the medium.

The K_i for CO_2 inhibition of HCO_3^- uptake was observed to be conspicuously larger than the K_m for CO_2 uptake (Figs. 2, 4). Perhaps the weak performance of CO_2 as a competitor is due to strong product inhibition of the CA-like moiety by HCO_3^- when the latter is supplied together with CO_2. (Effective product inhibition has been reported for CA itself [10].)

The somewhat larger K_i for HCO_3^- (acting as an inhibitor of CO_2 uptake) as compared with its K_m remains perplexing. Perhaps the presence of an intermediate intracellular C_i pool, the nascent HCO_3^- pool, is responsible for the complexity of the interrelations between HCO_3^- and CO_2. On the other hand, each C_i species might be able to bind to the other's specific sites, but with relatively low affinity. At O_2 compensation point, the concentration of C_i in the bulk medium is very close to zero (A. Kaplan, unpublished data). It is possible, however, that the unstirred layer contains some HCO_3^-. This may have led to

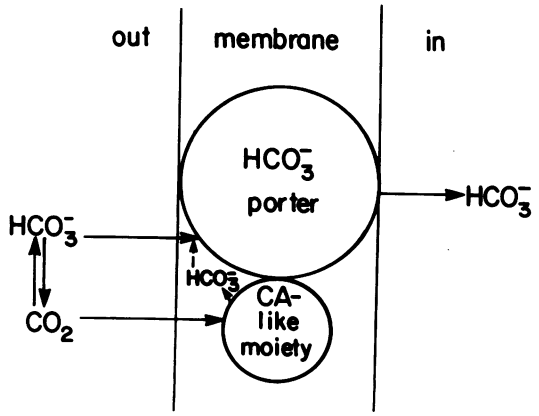

FIG. 5. Schematic model for HCO_3^- and CO_2 uptake in *Anabaena*. In and Out refer to the inner and outer interfaces of the plasmalemma.

underestimation of the kinetic parameters determined for HCO_3^- uptake. Those for CO_2 uptake would be far less affected because at the alkaline pH the concentration of this species would be very low.

An alternative scheme for uptake might postulate two distinct transport mechanisms, for HCO_3^- and CO_2, respectively. The CO_2 porter might resemble a CA molecule vectorially placed across the membrane so as to liberate HCO_3^- at its inner surface. The competitive effects observed might be due, as suggested above, to the ability of each species to bind to the other's porter with a relatively high K_m.

It has recently been proposed that the activity of CA in green algae (which, in the case of *Chlamydomonas* is located in the periplasmic space) enables fast interconversion of the C_i species (3, 9). We could not detect CA activity in *Anabaena*. The postulated CA-like moiety of the transport system, therefore, does not exhibit CA activity.

Both schemes would account for the hyperpolarization of membrane potential observed on addition of HCO_3^- to C_i-depleted cells. Similarly, both would explain the high sensitivity of CO_2 uptake to CA inhibitors.

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