Correlation between Carbonic Anhydrase Activity and Inorganic Carbon Internal Pool in Strain Synechocystis PCC 6174

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ABSTRACT

Existence of an internal carbonic anhydrase was demonstrated in the cyanobacterium Synechocystis PCC 6174. The enzyme, present at a low specific activity, was inducible by limitation in inorganic carbon and inhibited both in vivo and in vitro by acetazolamide. The internal inorganic carbon pool as determined by mass spectrometry, was similarly modulated by the actual inorganic carbon growth regime; its building up was also sensitive to acetazolamide. A possible role of carbonic anhydrase in inorganic carbon metabolism regulation through the control of the dimension and nature of the inorganic carbon pool is discussed.

In photosynthetic organisms, the steps preceding the assimilation of CO₂ by Rubisco, i.e. entry and accumulation of CO₂ and transformation of internal HCO₃⁻ to CO₂ are still poorly understood. The nature of the molecular species (CO₂/ HCO₃⁻) entering the cells seems to depend on the organisms. The available information, however, should be taken with caution since methodological biases may not be excluded (1). Except for one case (19), the presence of an active transport system seems to be accepted (8), though little information exists as to the identity of the transporter and the origin of the energy used (10).

One intracellular CA has been described in most species, located in the stroma of chloroplasts in green algae (9, 15) and in the cytoplasm for cyanobacteria (7). Chlamydomonas reinhardtii, in addition, possesses a very high, periplasmic CA activity (3). Association of CA with either the cytoplasmic or the thylakoidal membranes has been suggested, in view of its possible involvement in HCO₃⁻ transport or in regulation of PS II electron flow, respectively (16, 18).

No adaptation of Rubisco activity takes place as a response to variations of external Ci concentrations. In contrast, two events preceeding CO₂ incorporation by Rubisco may be affected. Thus, in Anacystis nidulans, depletion of external Ci induces the enhancement of a capacity to accumulate this substrate (10, 11). In addition, CA activity has been reported to vary with the Ci supply in a number of organisms, including cyanobacteria (4, 7–9). Which step is the initial target of the adaptation process has not been elucidated.

The present work gives evidence for a concomitant modification of these two latter functions, Ci accumulation and CA activity, in response to variations of external Ci concentrations, in the facultative phototrophic cyanobacterium Synechocystis PCC 6174. An implication of CA in the control of the intracellular pool is discussed.

MATERIALS AND METHODS

Strain and Growth Conditions

Synechocystis PCC 6174 was obtained from the Pasteur Institute (13). It was grown in Allen's minimal medium (5) modified by omitting the orthosilicate and doubling the NaNO₃ concentration. Standard growth conditions were 34°C and gentle agitation under 55 μE·m⁻²·s⁻¹ (3500 lux) provided by white light fluorescent tubes. 'High CO₂' (HC) conditions were obtained by blowing an air-CO₂ mixture (95:5) in the medium. 'Low CO₂' (LC) cells were grown in the above medium totally depleted of mineral carbon; atmospheric CO₂ (0.03%) on the surface of the liquid phase was the only carbon source. Generation times were 5 and 16 h for HC and LC cells, respectively.

Inorganic Carbon Uptake

Ci uptake was estimated by the silicone oil filtration technique, according to Miller and Colman (8). Assays were performed in the light at 28°C in 400 μL microfuge tubes. The tubes contained successive layers of NaOH 2 N (100 μL) and a mixture (1/1, v/v) of Wacker (Wacker Chemie GmbH, Munich) AR20 and AR200 silicone oils (50 μL). A 50 μL sample of an exponentially growing suspension (10⁴ cells/mL) previously depleted of CO₂ (checked by the disappearance of O₂ evolution) were added on top. NaH¹⁴CO₃ 1 mm final, corresponding to the saturating concentration, was added to the suspension. Incubation lasted 15 s under illumination (100 μE m⁻² s⁻¹ at the surface of the cell suspension). The reaction was stopped by centrifugation during 10 s in a Beckman Microfuge II. ¹⁴CO₂ incorporation in acid stable products was determined after acidification of an aliquot (40 μL) with 100 μL perchloric acid 2 N, and thorough agitation.
Radioactivity was determined using the Beckman MP liquid scintillation cocktail.

Mass Spectrometric Measurements of \(^{18}\)O Exchanges

The basis of the method is the measurement of the depletion in \(^{18}\)O from \(^{16}\)O\(_2\) resulting from the hydration-dehydration reaction between \(\text{CO}_2\) and water (17). The rate of \(^{18}\)O depletion was followed by mass spectrometry and a membrane inlet system. A temperature-controlled (25°C) glass reaction cell was connected to a three-collector mass spectrometer (type 14-80, VG Instruments) by means of a vacuum line. A polyethylene membrane at the bottom of the reaction vessel allowed dissolved gases to diffuse into the mass spectrometer. NaHC\(^{18}\)O\(_3\) (97.8% labeled) prepared by dissolving a saturating amount of NaHCO\(_3\) in H\(^{18}\)O, was injected in 10 mL Hepes buffer (20 mm, pH 7.5) previously bubbled with \(\text{CO}_2\)-free air, to obtain a final bicarbonate concentration of 1 mM. After 1 to 2 min, concentrated cells (3.10\(^6\) cells/mL final) were added. Light (100 \(\mu\)Em\(^2\) s\(^{-1}\) at the surface of the cell suspension) was provided by a projector (Oriel) equipped with a 1000 W quartz-halogen lamp. Evolution of the concentrations of C\(^{16}\)O\(^{18}\)O (mass 44), C\(^{16}\)O\(^{18}\)O (mass 46), C\(^{18}\)O\(^{18}\)O (mass 48) was simultaneously recorded.

Carbonic Anhydrase Measurement

Preparation of a soluble protein extract was adapted from Yagawa et al. (22). A 15 to 20 mL packed cells pellet was resuspended in a small volume of veronal buffer 25 mm (pH 8.5), containing 0.05% Triton X-100 and 1 mm PMSF. The cells were disrupted by two passages through a French press cell (12,000 p.s.i.) at 4°C. The broken cells were centrifuged 10 min at 10,000g and the supernatant centrifuged 90 min at 100,000g. The proteins of the supernatant fraction were concentrated by precipitation in 60% ammonium sulfate. The precipitate was collected by centrifugation, dissolved in a minimum volume of veronal buffer 25 mm (pH 8.5), and dialysed overnight against the same buffer. The proteins were then concentrated on Amicon membranes. Carbonic anhydrase was measured according to the Wilburg-Anderson electrochemical method (20). Assays were performed at 4°C in a glass vessel containing 5 mL veronal buffer 15 mm (pH 8.5) and 25 to 100 \(\mu\)L protein extract corresponding to 500 \(\mu\)g proteins. The reaction was started by injecting 2 mL of water saturated with \(\text{CO}_2\) at 4°C. The time necessary for the pH to drop from 8.4 to 7 was recorded. Enzyme activity was calculated using the formula:

\[
\text{Activity} = \frac{T_b}{T_c - 1}
\]

where \(T_b\) and \(T_c\) are the times for the uncatalyzed and catalyzed reactions, respectively (14).

RESULTS

Influence of Various \(\text{Ci}\) Concentration Regimes on Initial \(\text{Ci}\) Uptake Rate

\(\text{Ci}\) uptake was measured by the silicone oil technique in suspensions of cells adapted for several generations to HC or LC conditions. \(\text{Ci}\) uptake being linear for 30 s in our experimental conditions, incubations were carried out for 15 s, to obtain a correct measurement of initial rates. Using a saturating concentration of 1 mm of HCO\(_3\)-, rates of uptake obtained were 4.3 ± 0.5 and 5.4 ± 0.6 nmol \(\text{Ci}\)-min\(^{-1}\).mg Chl\(^{-1}\), for LC and HC cells, respectively.

Evaluation of the Internal \(\text{Ci}\) Pool of LC and HC Cells

As observed for other organisms (2, 17), \textit{Synechocystis} cells transferred from dark to light conditions showed an immediate uptake of external \(\text{Ci}\). The magnitude of this uptake can be considered as corresponding to the replenishment of their maximal reserve capacity. Quantitation of this value was determined by titration of \(\text{CO}_2\) depletion from the incubation medium. Comparisons were made for LC and HC cells. Typical results are presented in Figure 1A for HC cells. The cells were added in the dark to a reaction vessel prepared as described in “Materials and Methods” and the light turned on afterward, as indicated. Illumination induced a two-phase decrease of the \(\text{CO}_2\) concentration (\(\text{CO}_2\)) in the external medium: an immediate rapid one (c), followed by a slower steady one (d). Phase c corresponded to concomitant filling up of the internal \(\text{Ci}\) pool of the cells and \(\text{Ci}\) assimilation. \(\text{Ci}\) assimilation continued during phase d, when the cells had reached a steady state of photosynthetic activity (2).

Saturation of the pool was achieved after uptake of three times more \(\text{Ci}\) for LC than for HC cells (Table 1). The figure,

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**Figure 1.** Evolution of extracellular concentrations of \(\text{CO}_2\) upon illumination of HC cells. Mass spectrometric determination of \(^{18}\)O exchanges between C\(^{16}\)O\(_2\) and H\(_2\)O were performed as described in “Materials and Methods.” Arrows indicate addition of the cells and illumination. A, Evolution of the total amount of external \(\text{CO}_2\); B, evolution of the concentration of each molecular species, C\(^{16}\)O\(_2\) (\(\text{CO}_2\) – 48), C\(^{16}\)O\(^{18}\)O (\(\text{CO}_2\) – 46), and C\(^{18}\)O\(_2\) (\(\text{CO}_2\) – 44). Note the different ordinate scales between parts A and B.
Table I. Ci Pool Replenishment by LC and HC Cells

The amount of Ci taken up by the cells immediately after illumination was calculated from the CO₂ values as obtained in Figure 1. Treatment with AZA was for 2 h prior to the measure, the cells being grown under LC and HC conditions, respectively. One μg Chl represents 10⁷ cells.

<table>
<thead>
<tr>
<th>Type of Cells</th>
<th>Amount of Ci Taken Up</th>
<th>5 mM AZA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td></td>
</tr>
<tr>
<td>LC</td>
<td>266</td>
<td>109</td>
</tr>
<tr>
<td>HC</td>
<td>78</td>
<td>43</td>
</tr>
</tbody>
</table>

266 nmol·mg Chl⁻¹, obtained for LC cells which had been maintained in limiting Ci for several generations and thus were almost depleted of this substrate, reflected their maximal pool capacity. This value was only a partial measure of the total Ci pool since a corresponding proportion of the external HCO₃⁻ contributed to the buildup of the pool, either by direct entry or by external spontaneous interconversion. (This does not imply any presumption as to the nature of the carbon species entering the cells.) The fact that the slopes of the curves during phases c and d (Fig. 2) were larger than that of the spontaneous rate of interconversion CO₂⇌HCO₃⁻ (phase a), indicated that the rate of external interconversion, in our experimental conditions, was not a limiting factor.

Carbonic Anhydrase Activity in LC and HC Cells

As for other cyanobacteria (7, 8), no extracellular carbonic anhydrase activity could be detected in Synechocystis 6714. An intracellular activity was measured in a concentrated preparation of the soluble cytoplasmic proteins. CA activity was determined by the Wilburg-Anderson method, comparatively on LC and HC cells. The multistep procedure for the preparation of extracts resulted in important variations of absolute activities among independent experiments, as reported by Yagawa et al. (22). Values obtained from simultaneous preparations, however, always yielded a 7- to 10-fold increase for LC cells as compared to HC ones, as shown in Table II for two typical experiments.

CA activity was measured in vivo from the isotopic exchanges between HCl⁻⁴⁰O²⁻⁻⁻⁻, Cl⁻¹⁸O₂, and H₂O. These exchanges were determined by measuring the evolution of the concentrations of Cl⁻¹⁸O₂ (CO₂⁻⁴⁶), Cl⁻¹⁸O⁻⁴⁰O (CO₂⁻⁴⁶), and Cl⁻¹⁸O⁻⁴⁰O (CO₂⁻⁴⁴) in the incubation medium, the input of H⁺⁻¹⁸O²⁻⁻⁻⁻ being negligible in the reaction vessel (Fig. 1B). The relative decrease of ¹⁸O label in the external CO₂, calculated from the formula (17)

\[
\tau = \frac{[\text{CO}_2^-_{-46}] + 2[\text{CO}_2^-_{-48}]}{2[\text{CO}_2^-_{-44} + \text{CO}_2^-_{-46} + \text{CO}_2^-_{-48}]}
\]

was a measure of CA activity. Cell suspensions were introduced in the dark and illuminated as shown in Figure 1. Variations of log τ as a function of time (Fig. 2) showed a linear function, indicating first order kinetics. The rate constant, θ, defined as Shiraiwa and Miyachi (15), could be determined as the slope of the curves. Addition of the cells in the dark did not modify the uncatalysed exchange rate (Fig. 2, phases a and b). The ratio of the θ values for LC to HC cells (Table III), determined during the steady state of photosynthetic activity (phase d), was close to 5.

**Influence of AZA on the Initial Steps of Ci Metabolism**

AZA is an inhibitor of carbonic anhydrase. Although it has been reported as poorly permeant in species such as C. reinhardtii (9, 21), AZA did enter the cyanobacterial cells. This was shown by its inhibitory effect on cell growth. Suspensions of LC or HC cells treated with 5 mM AZA stopped growing after a lag equivalent to one generation time under the corresponding Ci growth regimes.

AZA showed differential effects on the early steps of Ci metabolism, but did not discriminate between LC and HC cells. Ci uptake rate was not or only slightly affected for up to 24 h of contact with the drug (Fig. 3, curves A). Longer treatments led to cell bleaching and parallel loss of all activities.

Under conditions (1 mM AZA) giving 100% inhibition of a commercial enzyme (Sigma beef heart CA C-7500), total inhibition of the CA activity present in extracts prepared from LC or HC cells was observed. A 2 h incubation of whole cells in 5 mM AZA reduced the rates of catalysed ¹⁸O exchanges to levels close to that of the noncatalysed reaction (Table III). Though less efficient, the inhibition was measurable in HC cells.

In parallel with this effect on CA, AZA showed an inhibition of the assimilation of Ci into organic compounds, determined as the incorporation into the acid soluble fraction of the ¹⁴Ci taken up by the cells (Fig. 3, curves B). The efficiency of this inhibition, however, was lower than that on CA activity, since 10% incorporation still took place after 24 h of treatment.

The building of the internal Ci pool was also prevented by AZA (Table I). The extent of this inhibition (about 50%),

Table II. Carbonic Anhydrase Activity in LC and HC Cells

CA activity was measured on protein extracts obtained as described in "Materials and Methods". Cells were grown under LC and HC conditions, respectively. Total Chl content was 1 mg/10⁷ cells for both LC and HC cells. Experiments I and II correspond to two separate preparations performed with two different batches of both LC and HC cells.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Specific Activity of LC Cells</th>
<th>Specific Activity of HC Cells</th>
<th>Induction Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>units/mg protein⁻¹ of the extract</td>
<td>units/mg Chl⁻¹</td>
<td>units/mg protein⁻¹ of the extract</td>
</tr>
<tr>
<td>I</td>
<td>2.5</td>
<td>0.2</td>
<td>0.35</td>
</tr>
<tr>
<td>II</td>
<td>0.9</td>
<td>0.075</td>
<td>0.085</td>
</tr>
</tbody>
</table>
Figure 2. Kinetics of $^{18}$O exchanges between CO$_2$ and H$_2$O by LC (A) and HC (B) cells. The rate of disappearance of $^{18}$O from external CO$_2$ was calculated as indicated in the text, from data obtained as in Figure 1B. The arrows indicate addition of the cells and illumination. Treatment with AZA (5 mM) was for 2 h prior to the measures. (A), No AZA; (C), 5 mM AZA.

Table III. Rate Constants, $\theta$, of $^{18}$O Exchanges under Steady Photosynthetic Conditions, for LC and HC Cells

The kinetics of $^{18}$O exchanges were determined as in Figure 2. The rate constants, $\theta$, were calculated from the slopes of the curves, during phase d. LC and HC cells were treated with AZA for 2 h prior to the measure, the cells being grown under LC and HC conditions, respectively.

<table>
<thead>
<tr>
<th>Type of Cells</th>
<th>Rate Constants $\theta$ (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>None</td>
<td>3.10$^{-4}$</td>
</tr>
<tr>
<td>LC</td>
<td>4.7.10$^{-3}$</td>
</tr>
<tr>
<td>HC</td>
<td>1.10$^{-3}$</td>
</tr>
</tbody>
</table>

however, was smaller than that of CA, measured on the same samples (Table III).

**DISCUSSION**

As shown for other organisms (4, 7–9), CA activity was enhanced by Ci depletion. The amount of increase in CA activity obtained in vivo was in agreement with the in vitro determinations (Tables II and III). Maximal induced activities were similar to those known for other cyanobacteria (6, 7), but largely inferior to that of the inducible periplasmic enzyme of *Chlamydomonas reinhardtii* (3, 9, 22). The internal constitutive CA described in this alga (9), on the other hand, shows a very low activity.

The first step in Ci metabolism, its entry into the cell, as defined by the initial uptake rate, was not modified by variations of the external Ci concentration during growth. The absence of sensitivity to AZA of the uptake of Ci excluded an implication of CA at this level. By contrast, its role in the provision of CO$_2$ to Rubisco was evidenced by the inhibition by AZA on Ci incorporation into organic compounds.

The dependency of the buildup of a Ci pool on the external Ci concentration and its inhibition by AZA revealed the implication of CA in this process. This original observation suggests that a proportion of the Ci pool is maintained as CO$_2$, generated by CA and used as substrate source by Rubisco.

The apparent discrepancy between the kinetics of inhibition by AZA on CA activity on one hand (90% in 2 h for LC cells, Table III) and on Ci incorporation into organic compounds (30% in 2 h, Fig. 3) on the other, could reflect the presence of this CO$_2$ pool.

The existence of *Synechococcus R2* of a similar capacity to modulate Ci accumulation in response to availability of external Ci (11) could be explained by the same scheme.

Carbonic anhydrase might, as proposed by Stemler (16), be involved in the regulation of electron transfer through PSII. Such a hypothesis would imply that the apparent sensitivity to AZA of Ci accumulation observed here be a secondary effect, due to, for instance, a depletion of available energy or a modification of the internal pH equilibrium.

**LITERATURE CITED**

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