

# Properties of a Mutant from *Synechocystis* PCC6803 Resistant to Acetazolamide, an Inhibitor of Carbonic Anhydrase<sup>1</sup>

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## ABSTRACT

A spontaneous mutant of the cyanobacterium *Synechocystis* PCC6803 was isolated for its resistance to acetazolamide, an inhibitor of carbonic anhydrase. The mutant showed a deficiency in oxygen exchange between CO<sub>2</sub> and H<sub>2</sub>O, a lower level of stable internal CO<sub>2</sub> pool and a decreased capacity to adapt its photosynthetic affinity under limited inorganic carbon regime. The initial rate of uptake of inorganic carbon was identical to that of wild-type cells. It is demonstrated that the mutation affects the carbonic anhydrase activity. This could result from either of two impairments: a deficiency in the enzyme activity detectable by mass spectrometric determinations, or a modification of the cellular compartment in which the enzyme is located, preventing its activity.

The involvement of carbonic anhydrase in early events of C<sub>2</sub> assimilation, transport of C<sub>i</sub> and provision of CO<sub>2</sub> to Rubisco, has been suggested in a number of contradictory publications. The affinity of Rubisco from cyanobacteria for its CO<sub>2</sub> substrate is low, around 0.3 μM (2), and makes the accumulation of CO<sub>2</sub> at the vicinity of the enzyme a necessity. Evidence for internal C<sub>i</sub> accumulation has been shown, using different approaches, in several strains of cyanobacteria (3, 6, 9, 11, 14). The existence of a C<sub>i</sub> concentrating system is generally admitted in these species. No carrier responsible for an active transport has yet been identified. The molecular form of C<sub>i</sub> entering the cells, CO<sub>2</sub> and/or HCO<sub>3</sub><sup>-</sup>, is still a much debated question (14). Both molecular species could be involved in the two *Synechococcus* strains UTEX625 (7, 12) and PCC7942 (13) and in *Anabaena variabilis* (1). A carbonic anhydrase activity has been detected in most cyanobacterial strains tested (2). The enzyme is intracellular. It presents a low specific activity as compared with that exhibited by the extracellular enzyme of *Chlamydomonas reinhardtii* (5) or the membranous form of *Chlorella* sp. K (16).

We have demonstrated that the C<sub>i</sub> transport efficiency of

the facultatively phototrophic strain *Synechocystis* PCC6714 is independent of the inorganic carbon regime of the cells (4). In contrast, its carbonic anhydrase activity is very low under high C<sub>i</sub> conditions, and is stimulated by C<sub>i</sub> limitation. The building up of an internal C<sub>i</sub> pool, shown to be under the control of carbonic anhydrase, might be involved in the regulation of the adaptation to varying external C<sub>i</sub> concentrations.

Acetazolamide, an inhibitor of carbonic anhydrase, was lethal to *Synechocystis* PCC6803, as well as the closely related strain *Synechocystis* PCC6714 (4). The behaviors of the two strains were similar when confronted to varying C<sub>i</sub> regimes, as long as C<sub>i</sub> uptake and O<sub>2</sub> exchange between CO<sub>2</sub> and H<sub>2</sub>O were considered. We have isolated spontaneous mutants resistant to this inhibitor from both *Synechocystis* strains. The properties of one of them, AZA<sup>r</sup>-5b, isolated from strain *Synechocystis* PCC6803, are described here, and the questions raised by the existence of this mutant are discussed.

## MATERIALS AND METHODS

### Strain and Growth Conditions

Wild-type *Synechocystis* PCC6803 was obtained from the Pasteur Collection (17). It was grown in Allen's minimal medium modified by omitting the orthosilicate and doubling the NaNO<sub>3</sub> concentration (4). Growths under high (HC, 5% CO<sub>2</sub> in air) or low (LC, air, no carbon added to the medium) C<sub>i</sub> concentrations were as defined (4). Generation times were 5 and 20 h for HC and LC conditions, respectively. Isolation of the acetazolamide resistant mutants (AZA<sup>r</sup>) is described in the "Results" section.

### Inorganic Carbon Uptake and Assimilation

C<sub>i</sub> uptake was measured by the silicone oil filtration technique (11). Incubation times in the presence of 1 mM H<sup>14</sup>CO<sub>3</sub> were 7, 20, and 30 s. C<sub>i</sub> uptake and assimilation into organic compounds were determined as in Bedu *et al.* (4).

### <sup>18</sup>O Exchange between C<sup>18</sup>O<sub>2</sub> and H<sub>2</sub>O

<sup>18</sup>O exchange between C<sup>18</sup>O<sub>2</sub> and H<sub>2</sub>O was measured by mass spectrometry according to Silverman *et al.* (19). Experimental conditions and calculations were as previously described (4). A sample containing 10<sup>8</sup> cells from an exponentially growing culture was suspended in 10 mL Hepes buffer

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<sup>2</sup> Abbreviations: C<sub>i</sub>, inorganic carbon; Rubisco, ribulose biphosphate carboxylase/oxygenase; AZA, acetazolamide; CA, carbonic anhydrase; HC, LC, growth conditions under high (5% in air) and low (0.03%) CO<sub>2</sub> concentrations.

(20 mM, pH 7.5) containing 1 mM  $\text{NaHC}^{18}\text{O}_3$ . Concentrations of  $\text{C}^{18}\text{O}^{18}\text{O}$ ,  $\text{C}^{18}\text{O}^{16}\text{O}$ ,  $\text{C}^{16}\text{O}^{16}\text{O}$  (respective molecular masses 44, 46, and 48) in the external medium were recorded simultaneously. From these measurements, the evolution of the total  $\text{CO}_2$  concentration in the external medium could be calculated.

Depletion of  $^{18}\text{O}$  from the external  $\text{C}^{18}\text{O}^{18}\text{O}$  was taken as a measure of carbonic anhydrase activity (21). This depletion followed first order kinetics, the rate constant of which,  $\theta$ , was calculated according to Silverman *et al.* (19).

### Photosynthetic Affinity

Photosynthetic activity was measured as the rate of  $\text{O}_2$  evolution, and recorded by mass spectrometry. Cells ( $3.10^8/\text{mL}$ ) were incubated at  $25^\circ\text{C}$  in Hepes buffer (20 mM, pH 7.5) under illumination ( $100 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  at the surface of the suspension) until  $\text{O}_2$  evolution due to endogenous  $\text{CO}_2$  had ceased. They were then transferred into the mass spectrometer reaction vessel, under the conditions described in (4). Photosynthesis was started by the injection of  $\text{NaH}^{13}\text{CO}_3$ . Photosynthetic rates were recorded with 0.5 to  $40 \mu\text{M}$  final  $\text{NaHCO}_3$  concentrations. The  $K_{m\text{C}_i}$  for photosynthesis was determined from Lineweaver-Burk plots. Use of  $^{13}\text{C}$  provided a strict reference for the added inorganic carbon, thus avoiding errors due to environmental  $^{12}\text{CO}_2$  or  $\text{H}^{12}\text{CO}_3^-$ .

## RESULTS

### Isolation of Mutants Resistant to Acetazolamide (AZA<sup>r</sup>)

Spontaneous mutants were isolated on solid medium (pH 8.5) containing the standard concentration of  $\text{C}_i$  (12 mM) supplemented with 5 mM AZA. Such mutants were isolated under both photoautotrophic (light, no glucose) and mixotrophic (light, 1% glucose) conditions, at similar frequencies ( $5 \times 10^{-6}$ ). Among the mutants isolated, one of them, AZA<sup>r</sup>-5b, was chosen for further work because of its profile of  $^{18}\text{O}$  exchange capacity (see below). Mutant AZA<sup>r</sup>-5b was initially isolated in the absence of glucose. Its growth capacities were identical to those of the wild-type parental cells, whether under mixotrophic or photoautotrophic conditions, and, in the latter case, in the presence of either LC or HC concentrations.

### $\text{CO}_2$ Fluxes during Illumination

Illumination of wild-type cells preincubated in the dark produced an immediate, rapid decrease of the  $\text{CO}_2$  concentration in the external medium (Fig. 1A, phase c1), followed by a slower, steady one (Fig. 1A, phase c2). As previously established (4), phase c1 corresponds to simultaneous filling up of the internal  $\text{C}_i$  pool and  $\text{C}_i$  assimilation through photosynthetic activity. Phase c2 represents the steady photosynthetic activity. Mutant AZA<sup>r</sup>-5b showed an immediate, rapid uptake of  $\text{CO}_2$  (Fig. 1B, phase c1), of similar magnitude as that of wild-type cells (change of  $\text{CO}_2$  external concentration:  $8 \mu\text{M}$ ). A transitory, but large efflux of  $\text{CO}_2$  ( $3.9 \mu\text{M} \text{CO}_2$ ) then took place, before the cells stabilized to a steady photosynthetic  $\text{C}_i$  assimilation (Fig. 1B, phase c2). The efflux could reflect an

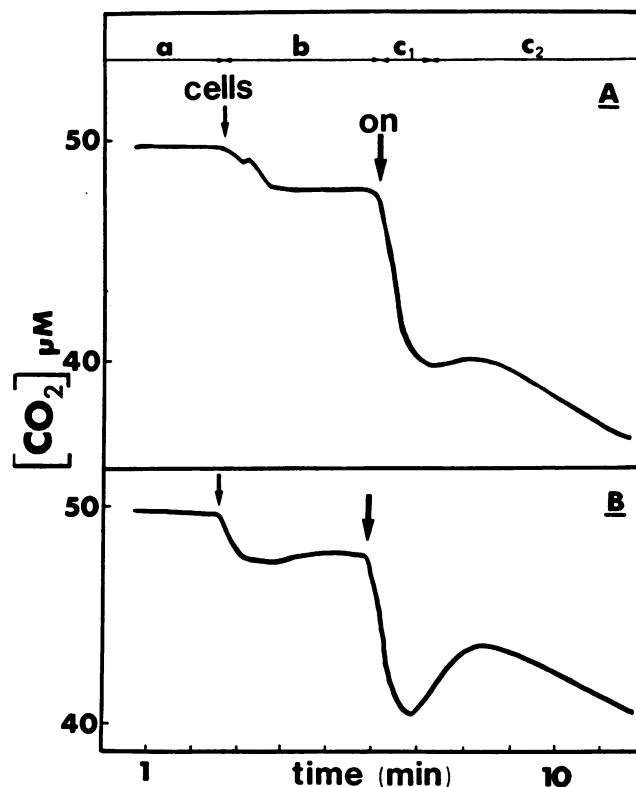
inefficiency of the mutant to maintain an internal  $\text{CO}_2$  pool at the same level as that of the wild type.

### $^{18}\text{O}$ Exchange Capacity of Mutant AZA<sup>r</sup>-5b

The spontaneous interconversion between  $\text{CO}_2$  and  $\text{HCO}_3^-$  can be accelerated by the catalyzing activity of carbonic anhydrase. The rate of  $^{18}\text{O}$  exchange between  $\text{H}_2\text{O}$  and dissolved  $\text{C}^{18}\text{O}^{18}\text{O}$  is representative of this activity (21). The  $^{18}\text{O}$  exchange rate of mutant Aza<sup>r</sup>-5b was measured in cells adapted to LC concentration conditions, which induced maximal CA activity in the wild type. CA activity in the latter cells was increased about threefold upon transfer from HC to LC conditions (data not shown). The mutant showed a rate constant identical to that of the noncatalyzed reaction,  $5 \cdot 10^{-4}$  (Table I). Mutant AZA<sup>r</sup>-5b, which thus appeared devoid of any catalytic exchange activity, was an extreme case among four AZA<sup>r</sup> clones studied. All others had retained an exchange activity, though lower than that of the wild type (data not shown).

### $\text{C}_i$ Uptake Rates

The initial rates of  $\text{C}_i$  uptake, measured by the silicone oil filtration technique, were of the same magnitude ( $10 \mu\text{mol} \text{C}_i \cdot \text{min}^{-1} \cdot \text{mg}^{-1} \text{Chl}$ ) in the wild-type and the mutant (Fig. 2).



**Figure 1.** Evolution of the extracellular concentration of  $\text{C}_i$  upon illumination of wild-type (A) and mutant (B) cells grown under conditions of limited inorganic carbon. Variations of  $\text{CO}_2$  concentrations were determined by mass spectrometry as described in "Materials and Methods." Arrows indicate addition of the cells and illumination.

**Table I.** Rate Constants,  $\theta$ , of  $^{18}\text{O}$  Exchange between  $\text{C}^{18}\text{O}_2$  and  $\text{H}_2\text{O}$  under Steady Photosynthetic Conditions

The rate constants,  $\theta$ , were calculated on low  $\text{C}_i$  grown cells, during steady photosynthetic conditions, corresponding to phase c2 of Figure 1.

Type of Cells	Rate Constants $\theta$ ( $\text{s}^{-1}$ )
None	$5 \cdot 10^{-4}$
Wild-type	$9.3 \cdot 10^{-4}$
AZA <sup>-</sup> 5b	$5 \cdot 10^{-4}$

Changing the  $\text{C}_i$  growth regime did not modify these rates (data not shown). However, the rate slowed down after 7 s of incubation of the mutant cells with  $\text{C}_i$ , while it remained linear during at least 20 s in wild-type cells. This phenomenon, depicting a lower level of saturation of the internal  $\text{C}_i$  pool in the mutant, correlated with the evolution of the pool as followed by mass spectrometry.

### Photosynthetic Activity

Rates of  $\text{CO}_2$  disappearance in the external medium during phase c2 (Fig. 1) were identical in mutant and wild-type cells. This was confirmed when rates of  $\text{C}_i$  incorporation into organic compounds ( $2.5 \mu\text{mol C}_i \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$  Chl) were measured by the silicone oil filtration technique (Fig. 2, up to 7 s of incubation); this rate was afterward increased in the wild-type, whereas it remained at the same low level in the mutant cells. Rubisco activation is dependent on the internal  $\text{CO}_2$  concentration. Since this  $\text{CO}_2$  concentration increased during repletion of the pool in wild-type cells, an increase of the amount of  $\text{C}_i$  assimilation into the organic phase was predictable. The limited size of the pool in the mutant cells might not allow acceleration of Rubisco activation, and thus of  $\text{C}_i$  assimilation.

### Photosynthetic Affinity

The photosynthetic affinities, as defined in "Materials and Methods," were similar in mutant AZA<sup>-</sup>5b and in the wild-type when the cells were grown under HC conditions (Table II). Under  $\text{C}_i$  limitation, conditions which resulted in a 275-fold enhancement of the photosynthetic affinity of the wild-type, the mutant increased its own affinity by only a factor of 27.5

## DISCUSSION

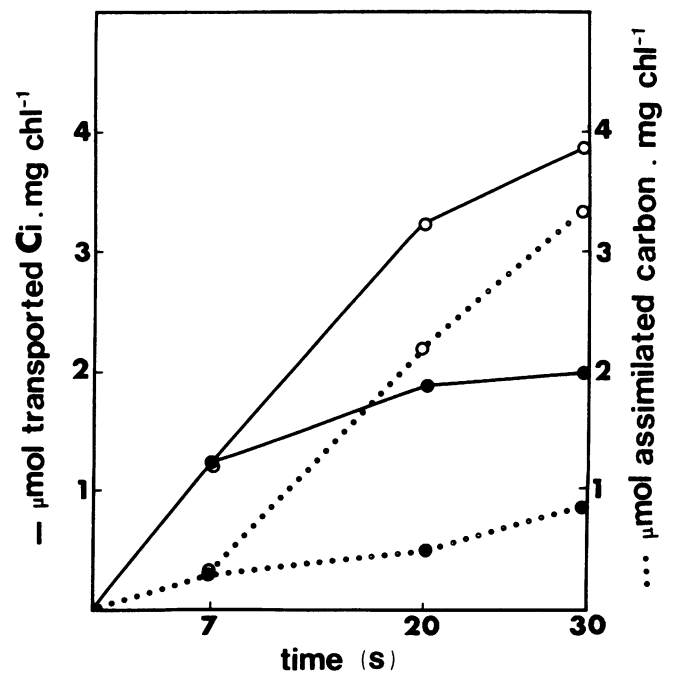
The acquisition of resistance to acetazolamide by spontaneous mutations was observed with a fairly high frequency independently of whether selection was made under photoautotrophic or mixotrophic conditions. The possibility that the resistance was due to a loss of permeation for the inhibitor can be ruled out, at least for mutant AZA<sup>-</sup>5b, on consideration of its metabolic modifications, as compared to wild-type cells.

Among the properties of mutant AZA<sup>-</sup>5b, one is of particular interest: the absence of activity for catalyzed  $\text{O}_2$  exchange between  $\text{CO}_2$  and  $\text{H}_2\text{O}$  measured by mass spectrometry in the external medium. Using this technique, Silverman *et al.* (19)

proposed that the rate of catalyzed exchange is dependent on the individual rates of two successive processes: (a) the entrance of  $\text{CO}_2$  and/or  $\text{HCO}_3^-$  into the cells, by diffusion or/and active transport. This controls availability of its substrate to the intracellular CA; (b) the interconversion between  $\text{CO}_2$  and  $\text{HCO}_3^-$  as catalyzed by the intracellular CA. An impairment, even partial, of  $\text{C}_i$  entry into the cells as the cause for the absence of catalyzed  $\text{O}_2$  exchange activity in mutant AZA<sup>-</sup>5b can be excluded: its  $\text{C}_i$  transport efficiency, whether measured as  $\text{CO}_2$  disappearance in the external medium (Fig. 1B, phase c1) or as  $\text{C}_i$  absorption (Fig. 2), appeared identical to that of wild-type cells.

We have shown that the building and maintenance of the internal  $\text{CO}_2$  pool in strain *Synechocystis* PCC6714 is under the control of the CA (4). Thus, the hypothesis that the mutation in mutant AZA<sup>-</sup>5b corresponds to a deficiency in CA activity tends to be favored.

It is a general rule that photosynthetic microalgae grown under high  $\text{CO}_2$  display a lower photosynthetic affinity for  $\text{C}_i$  than cells grown under low  $\text{CO}_2$  (18). The authors suggested the existence of a correlation between the decrease of apparent photosynthetic  $K_m \text{C}_i$  and the parallel increase of carbonic anhydrase activity when the cells were transferred to limiting  $\text{C}_i$  conditions. Wild-type *Synechocystis* PCC6803 has a similar behavior. The apparent affinity for  $\text{C}_i$  was higher in cells grown under low as compared with high  $\text{C}_i$  conditions ( $K_m \text{C}_i$  0.03 and  $8 \mu\text{M}$ , respectively), while carbonic anhydrase activity decreased threefold under the same conditions. The photosynthetic affinity of the mutant was also increased during



**Figure 2.** Inorganic carbon uptake and assimilation into organic material by wild-type and mutant cells.  $1 \text{ mM H}^{14}\text{CO}_3\text{Na}$  was added to preilluminated cells. As described in "Materials and Methods."  $^{14}\text{C}$  uptake (acid labile, —) and assimilation (acid stable, - - -) were determined in the wild-type (○) and mutant AZA<sup>-</sup>5b (●).

**Table II.** Apparent Photosynthetic Affinity of Wild-Type and AZA<sup>r</sup>-5b Mutant Cells Grown under High (HC) or Low (LC) C<sub>i</sub> Concentrations

Type of Cells	Photosynthetic Affinity	
	HC	LC
	$\mu\text{M}$	
Wild-type	8	0.03
AZA <sup>r</sup> -5b	8	0.30

growth under LC conditions, but to a value ( $K_{m\text{c}}$ , 0.3  $\mu\text{M}$ ) 10 times lower than that of the wild type.

Considering the properties of mutant AZA<sup>r</sup>-5b, it seems reasonable to hypothesize that the mutation responsible for its resistance to acetazolamide lies in a carbonic anhydrase gene. Mutant AZA<sup>r</sup>-5b, however, grew photoautotrophically as efficiently as the wild type, indicating that its reduced C<sub>i</sub> pool and CO<sub>2</sub> assimilation into organic compounds were not limiting. The CO<sub>2</sub> concentration provided to Rubisco must thus be sufficient to promote activation of the enzyme and to maintain a CO<sub>2</sub>:O<sub>2</sub> ratio in favor of the carboxylation activity.

It is difficult, however, to understand how these cells can grow photoautotrophically under low CO<sub>2</sub> conditions without any CA activity. Several localizations of CA have been proposed by different authors, corresponding to different roles of the enzyme: as a constituent of the HCO<sub>3</sub><sup>-</sup> carrier complex it would be localized in or close to the cytoplasmic membrane (8); associated to the thylakoids it would participate in the 'HCO<sub>3</sub><sup>-</sup> effect' on PSII (20); as part of the carboxysomes it would provide its substrate to Rubisco (10, 15). These different locations may not be exclusive of each other. *Synechocystis* PCC6803 could possess several sites of CA activity, corresponding to the same enzyme or to isoenzymes. The O<sub>2</sub> exchange activities as determined in our experimental conditions could reflect only partially the total activity present in the cells. The absence of detection of activity in mutant AZA<sup>r</sup>-5b could then result from either of two impairments: a deficiency in the enzyme whose activity is detectable by mass spectrometric determinations, or a modification of a cellular compartment lodging a carbonic anhydrase. These hypotheses are currently being tested.

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