Extracellular carbonic anhydrases of the stromatolite-forming cyanobacterium *Microcoleus chthonoplastes*

Elena Kupriyanova,¹ Arsenio Villarejo,^{2,3} Alexandra Markelova,¹ Lyudmila Gerasimenko,⁴ Georgy Zavarzin,⁴ Göran Samuelsson,² Dmitry A. Los¹ and Natalia Pronina¹

Correspondence
Natalia Pronina
pronina@ippras.ru

¹Institute of Plant Physiology, Russian Academy of Sciences, Botanicheskaya Street 35, Moscow, 127276 Russia

²Umeå Plant Science Centre, Department of Plant Physiology, University of Umeå, S-901 87 Umeå, Sweden

³Department of Biology, Universidad Autonoma de Madrid, 28049 Madrid

⁴Institute of Microbiology, Russian Academy of Sciences, Prospect 60-Letiya Oktyabrya 7/2, Moscow, 117312 Russia

Active extracellular carbonic anhydrases (CAs) were found in the alkaliphilic stromatolite-forming cyanobacterium *Microcoleus chthonoplastes*. Enzyme activity was detected in intact cells and in the cell envelope fraction. Western blot analysis of polypeptides from the cell envelope suggested the presence of at least two polypeptides cross-reacting with antibodies against both α and β classes of CA. Immunocytochemical analysis revealed putative α -CA localized in the glycocalyx. This α -CA has a molecular mass of about 34 kDa and a pI of 3.5. External CAs showed two peaks of activity at around pH 10 and 7.5. The possible involvement of extracellular CAs of *M. chthonoplastes* in photosynthetic assimilation of inorganic carbon and its relationship to CaCO_3 deposition during mineralization of cyanobacterial cells are discussed.

Received 30 October 2006

Revised 6 January 2007

Accepted 11 January 2007

INTRODUCTION

The main features of the ‘modern’ biosphere were formed about 2 billion years ago, when prokaryotes dominated on Earth. The participation of cyanobacterial communities in deposition of atmospheric CO_2 at early stages of the Earth’s history have been confirmed by their presence in stromatolites. The latter represent layered limestone deposits that consist of lithified cyanobacterial communities, which are somewhat similar to modern benthic mats in terms of their morphology and ultrastructure (Sergeev *et al.*, 2002). At the present point in geological time, stromatolite formation is rather insignificant, and the mechanisms of their formation are poorly known. It is still unclear whether mineralization occurs due to some metabolic processes in cyanobacteria, or whether the cyanobacteria just serve as a matrix for binding and structuring of carbonates in the natural process of calcium precipitation.

Carbonic anhydrase (CA, EC 4.2.1.1) is a zinc-containing enzyme catalysing a reversible hydration of carbon dioxide: $\text{CO}_2 + \text{H}_2\text{O} \leftrightarrow \text{H}^+ + \text{HCO}_3^-$. CA operates with forms of

inorganic carbon (C_i), including bicarbonate, which participate in calcium precipitation in nature. CAs have been found in all groups of living organisms. According to the accepted classification, CAs are divided into three main classes, α , β and γ , which have no significant primary sequence identity and, supposedly, are evolutionarily independent (Smith & Ferry, 2000). The existence of additional δ and ϵ classes of CAs have also been reported (Tripp *et al.*, 2001; So *et al.*, 2004).

Some eukaryotic algae are able to precipitate intracellular calcium carbonate due to the activity of intracellular CAs (Quiroga & Gonzalez, 1993). In contrast, cyanobacteria can deposit calcium only outside the cells, and such precipitation is strictly controlled by pH (Zavarzin, 2002). It is likely that extracellular CAs of cyanobacterial cells might stabilize the pericellular pH and participate in cell mineralization. However, the cyanobacterial CAs of benthic communities have not been investigated so far.

It is well known that cyanobacterial communities are rather conserved and have not changed significantly during the past two billion years, in terms of their physiology and morphology. Thus, today’s microbial communities can be used as a model system for the study of ancient

Abbreviations: CA, carbonic anhydrase; C_i , inorganic carbon; WAU, Wilbur–Anderson units.

mineralization. This is especially true for so-called 'relict' communities that are considered to be the analogues of ancient ecosystems. At present, such communities exist in extreme environments where no traces of higher organisms have been detected (Sergeev *et al.*, 2002).

We studied CAs of the benthic cyanobacterium *Microcoleus chthonoplastes*. It dominates in alkaliphilic and halophilic cyanobacterial mats worldwide (Gerasimenko *et al.*, 2003). The formation of calcium minerals on the outer mucous cover (glycocalyx) of this cyanobacterium has been reported both in natural samples in the mats (Gerasimenko *et al.*, 2003) and also in laboratory culture (Zavarzin *et al.*, 2003). We have identified extracellular CAs of *M. chthonoplastes* with access to outer C_i substrates and indicated their possible participation in extracellular calcium carbonate precipitation.

METHODS

Strain and growth conditions. *Microcoleus chthonoplastes* was obtained from the Culture Collection of the Institute of Microbiology, Russian Academy of Sciences, Moscow, Russia, originally isolated from Khilganta soda lake (Buryatiya, Russia; 50° 25' N, 106° 53' E, altitude 606 m) (Gerasimenko *et al.*, 2003). The organism was grown in medium S (Castenholz & Waterbury, 1989) (g l⁻¹: KCl, 1.0; NaHCO₃, 16.8; K₂HPO₄, 0.5; NaNO₃, 2.5; K₂SO₄, 1.0; NaCl, 30; MgSO₄, 0.1; CaCl₂, 0.04; FeSO₄, 0.01; EDTA, 0.08, pH 9.8) and micronutrients (Rippka *et al.*, 1979) in batch cultures at 31 °C under continuous irradiance of 50 µmol m⁻² s⁻¹ from cool white fluorescent lamps. All experiments were carried out with biomass after 4–5 days cultivation, corresponding to the mid-exponential phase of growth.

Isolation of cell envelopes and their fractions. Cells were broken in cooled buffer (30 mM HEPES-KOH, pH 8.2) using a French press (5 × 10⁷ Pa). Unbroken cells were sedimented by centrifugation at 700 g for 10 min at 4 °C. Cell envelopes of *M. chthonoplastes* were isolated by centrifugation of the cell homogenate at 12 000 g according to Weckesser & Jürgens (1988). To remove chlorophyll and soluble protein contaminations, the cell envelope fraction was washed four to five times with 30 mM HEPES-KOH (pH 8.2).

The isolation of cell walls and other components of the envelope was performed as described by Weckesser & Jürgens (1988). The cell envelope fraction was loaded on a discontinuous sucrose gradient (60, 55, 50, 45 and 40 % sucrose in 30 mM HEPES-KOH buffer, pH 8.2) and centrifuged at 20 000 r.p.m. in a Beckman SW 41 rotor for 4 h at 4 °C. As a result, three main fractions were obtained: (1) two lower fractions of cell walls (at the bottom of the tube and at the 55/60 % sucrose interface); (2) a fraction of some cell envelope fragments at the 50/55 % sucrose interface (supposedly glycocalyx); and (3) a lighter fraction of plasma membranes located above 40 % sucrose. All these fractions were collected, and the two cell-wall fractions were combined. Traces of sucrose were removed by washing in 30 mM HEPES-KOH buffer with centrifugation at 176 000 g for 1 h at 4 °C. Cell walls were further purified as described by Weckesser & Jürgens (1988). During all isolation steps, protease inhibitor mix (Sigma) was added to avoid protein degradation.

Assay of CA activity. CA activity was measured electrometrically (Wilbur & Anderson, 1948) by monitoring the rate of pH change during carbon dioxide hydration using a fast-response 'blue glass' microelectrode (MI-710; Microelectrodes) and an 18-bit A/D converter (IOtech). Measurements were performed with intact cells, cell

homogenate and cell envelopes in 30 mM HEPES-KOH buffer, pH 8.2. The reaction was carried out at 2 °C and started by a rapid injection of saturated CO₂ solution into an equal volume of the sample (fermentative reaction) or buffer (non-fermentative reaction, control). The reaction kinetics were recorded for 100 s. CA activity was calculated as the difference in the initial rate of CO₂ hydration between control and samples, and expressed in Wilbur–Anderson units (WAU) per 1 mg chlorophyll (Chl) or protein. One WAU is defined as $10 \times (t_0 - t) t^{-1}$, where t_0 and t are the times required for the pH to change by ΔpH in the control and the samples, respectively. The measurements were carried out in three to five replicates.

To study the dependence of *M. chthonoplastes* extracellular CA activity on external pH, the cyanobacterium was cultured at the optimum pH of 9.8 and then transferred to S medium at different pH values (6.5, 7.5, 8.0, 9.0 and 9.8) and grown under similar culture conditions (light and temperature were not changed). CA activity in intact cells was measured after 1.5 h incubation. CA activity was expressed in relative units compared to the activity at the optimal pH of 9.8.

Estimation of chlorophyll and protein content. Protein content was estimated in accordance with Bio-Rad Laboratories protocols using commercial kits with standard solutions (Bio-Rad DC Protein Assay Kit). Chlorophyll content was determined spectroscopically after extraction with absolute methanol (Porra *et al.*, 1989).

Electrophoresis and immunodetection. Proteins were separated by 10 or 12 % SDS-PAGE, following Laemmli (1970), or by two-dimensional (2D) electrophoresis. A mix of protein standards (Bio-Rad) was used as molecular mass markers.

For electrophoresis, a standard protocol and Bio-Rad buffers and solutions were used. The lanes were loaded with 15 µg protein for silver-stained gels, and 20 or 45 µg protein for Coomassie-stained gels and immunoblot analysis, respectively. The samples were solubilized for 5 min at 95 °C in the sample buffer.

2D electrophoresis was performed according to the manufacturer's instructions (Amersham). The protein content was 100 µg for immunodetection or 300 µg for silver-stained gels. Each sample was mixed with a rehydration solution containing 8 M urea, 2 % CHAPS, 20 mM DTT, 0.5 % IPG buffer (pH 3–10; Amersham) and 0.001 % bromophenol blue. IPG dry strips (Immobiline DryStrip gels, 7 cm, with a linear pH range coverage of 3–10; Amersham) were allowed to rehydrate in the presence of samples in the IPGphor Isoelectric Focusing System (Amersham), followed by isoelectric focusing of proteins at 20 °C. The following voltage/time profile was used: rehydration for 12 h; increasing voltage by the 'step-and-hold' procedure from 500 to 1000 V over 1 h; final phase of 8000 V for 12 h. After the first dimension run, the individual strip was equilibrated for 15 min in SDS buffer (50 mM Tris/HCl, pH 6.8; 6 M urea; 30 %, v/v, glycerol; 2 % SDS) supplemented with 1 % (w/v) DTT, and then for another 15 min in SDS buffer supplemented with 2.5 % (w/v) iodoacetamide. Thereafter, the strips were put on the top of the second dimension gel (12 %) and covered with 0.5 % agarose in SDS buffer. Each gel was run at 10 mA at the beginning and then at 15 mA per gel after all the proteins were transferred from the strip into the gel.

Immunoblotting was performed as described in the Bio-Rad Laboratories protocol. The primary antibodies were raised against: (1) *Chlamydomonas reinhardtii* α-CA (Cah-3) (Karlsson *et al.*, 1998) (affinity-purified); (2) *C. reinhardtii* mitochondrial β-CA (Eriksson *et al.*, 1996); (3) spinach chloroplast β-CA (Fawcett *et al.*, 1990); (4) *Coccomyxa* sp. intracellular β-CA (Hiltonen *et al.*, 1998); and (5) *C. reinhardtii* D1 protein (PsbA) (Nishiyama *et al.*, 2001). Horseradish

Fig. 1. pH-dependence of extracellular *M. chthonoplastes* CA activity. Cyanobacterial cells were grown at the optimum pH of 9.8 and then transferred into S medium at different pH values (6.5, 7.5, 8.0, 9.0 and 9.8). CA activity of intact cells was measured after incubation in these media for 1.5 h. Since during the incubation the non-pH 9.8 culture media became more alkaline due to the buffering properties of medium S, we have used the mean pH values in the figure. CA activity is expressed in relative units compared to the activity at the optimal pH of 9.8.

peroxidase-labelled secondary antibodies (Amersham Life Science) and chemiluminescence solutions (ECL, Amersham) were used to detect an antibody-antigen conjugate.

Immunoelectron microscopy. For immunogold labelling experiments, *M. chthonoplastes* cells or the isolated fraction of glycocalyx were fixed in 4% paraformaldehyde for 4–10 days at 4 °C. Immunocytochemical reactions were performed after washing the sample in 0.1 M phosphate buffer (pH 7.4). The reaction with primary antibodies against *C. reinhardtii* α -CA (Cah-3) was carried out for 1 h at 24 °C and then for 23 h at 4 °C. Thereafter, the samples were washed three times with phosphate buffer over a 24 h period. The second step of the immunochemical reaction and post-washing were performed under similar conditions using Protein-A-Gold (Sigma). The samples were post-fixed in 1% OsO₄, dehydrated in an alcohol series and embedded in Epoxy resin (Sigma). As a control for immunochemical reaction specificity, the sample treatment step with primary antibodies was omitted. Thin sections of samples were prepared using an ultramicrotome and then analysed with a JEM JEOL X-100 transmission electron microscope (Japan) without any additional contrast.

RESULTS

Activity of *M. chthonoplastes* extracellular CA

CA activity was detected both in the intact cells [0.238 ± 0.01 WAU (mg protein)⁻¹, 24 ± 4 WAU (mg chlorophyll)⁻¹] and in the cell envelope fraction [0.318 ± 0.01 WAU (mg protein)⁻¹] of *M. chthonoplastes* (mean values from three independent experiments). Thus, in spite of some possible CA inactivation during the isolation of cell envelopes, the results showed enrichment of CA activity in the cell envelope

fraction compared to intact cells. CA activity was not detected in the cell homogenate. This could be due to (1) low enzyme activity as a result of inhibition by compounds in the homogenate, or (2) low CA content relative to the total protein content of the fraction.

The highest activity of *M. chthonoplastes* extracellular CA was detected in intact cells incubated at an alkaline pH (~10) which is optimal for the growth of this alkaliphilic cyanobacterium. Enzyme activity was also recorded at a near neutral pH (~7.5) (Fig. 1). There was no detectable CA activity in intact cells incubated at pH 6.7 and 8.2.

Identification of *M. chthonoplastes* extracellular α -CA

To identify the protein responsible for this enzyme activity, Western blotting of the fractions with antibodies raised against *C. reinhardtii* α -CA (Cah-3) was carried out. These antibodies cross-reacted with only one specific protein of approximately 34 kDa (Fig. 2b). The protein cross-reacting with the Cah3 antibodies was clearly enriched in the cell

Fig. 2. Identification of *M. chthonoplastes* extracellular α -CA. (a) SDS-PAGE (10%) analysis of the polypeptide composition of *M. chthonoplastes* cell homogenate and cell envelope fraction. The gel was stained with Coomassie brilliant blue R-250. (b) Immunodetection of α -CA in cell envelopes of *M. chthonoplastes*. The blot was probed with antibodies raised against *C. reinhardtii* α -CA (Cah-3). (c) Immunoblot with antibodies against the D1 (PsbA) protein (test for cross-contamination of *M. chthonoplastes* cell envelopes with thylakoid membranes). Lanes: 1, cell homogenate; 2, isolated cell envelopes. The lanes contained 20 and 45 μ g protein for the Coomassie-stained gel and for immunoblotting, respectively. The arrow indicates the position of the Cah-3-like protein in the gel.

envelope fraction when compared to the cell homogenate (Fig. 2b). This enrichment corresponded with the higher enzyme activity seen in the cell envelope fraction (see above).

As Cah-3 is known to be associated with polypeptides of photosystem (PS) II of *C. reinhardtii* thylakoid membranes (Park *et al.*, 1999; Villarejo *et al.*, 2002), it was necessary to be sure that the signal observed in the cell envelope fraction of *M. chthonoplastes* did not result from cross-contamination of this fraction with thylakoid membranes from the cyanobacterium. Antibodies against D1 (PsbA), a major protein of PS II, was used as a specific marker for thylakoid membranes (Nishiyama *et al.*, 2001). Fig. 2(c) shows that cell envelopes did not cross-react with these antibodies, which implies the absence of thylakoid membrane contamination in this fraction.

Immunocytochemical detection of α -CA

Localization of α -CA in *M. chthonoplastes* cells was also investigated by immunogold electron microscopy using antibodies against *C. reinhardtii* α -CA (Cah-3) (Fig. 3). The electron-impermeable spots of colloidal gold are clearly visible in the glycocalyx (Fig. 3b). This structure and analogous specific signals are also typical of the fragments located at the interface between 50 and 55 % sucrose during cell envelope fractionation (Fig. 3d). Thus, one can conclude that this fraction consists of glycocalyx.

It is known that the cell envelopes of cyanobacteria have a quite complex structure and are composed of the outer membrane with glycocalyx, a peptidoglycan layer and a plasma membrane (Gantt, 1994). Our attempts to detect CA activity in different fractions obtained after further fractionation of the cell envelopes from *M. chthonoplastes* failed. Apparently, in spite of the presence of protease inhibitors, enzyme activity decreased drastically during fractionation. However, the presence of a potential α -CA in these fractions was tested by Western blot analysis with antibodies against *C. reinhardtii* α -CA (Cah-3). Fig. 4 shows that the signal was specifically enriched in a fraction characterized as glycocalyx, but there was no signal in the cell wall or plasma membrane fractions. It thus appears that the specific signal observed in the cell envelopes (Fig. 2b) was due to the presence of a Cah-3-like enzyme in the *M. chthonoplastes* glycocalyx.

Analysis of *M. chthonoplastes* glycocalyx protein composition by 2D electrophoresis

2D electrophoresis followed by Western blotting with antibodies against *C. reinhardtii* α -CA (Cah-3) enabled us to estimate the protein composition of the *M. chthonoplastes* glycocalyx fraction as well as the isoelectric point of extracellular α -CA (Fig. 5). The analysis of the silver-stained 2D gel showed the presence of approximately 15–20 various polypeptides in the *M. chthonoplastes* glycocalyx. Practically all the proteins in this fraction were acidic, with pI values

Fig. 3. Immunocytochemical localization of α -CA in *M. chthonoplastes* cells and isolated glycocalyx (The glycocalyx is the fraction isolated from the interface between 50 and 55 % sucrose during the cell envelope fractionation.). (a, b) Thin sections of *M. chthonoplastes* cell (control and experimental sample, respectively). Gold particles localized in the glycocalyx are shown in the enlarged inset in the top left of panel (b). (c, d) Isolated glycocalyx from *M. chthonoplastes* (control and experimental sample, respectively). For the immunocytochemical reaction, antibodies against *C. reinhardtii* α -CA (Cah-3) and Protein-A-Gold (Sigma) were used. The arrows indicate the localization of α -CA. Bars, 1 μ m.

Fig. 4. Immunodetection of α -CA in *M. chthonoplastes* cell envelope fractions following SDS-PAGE (12%). The blot was probed with antibodies against *C. reinhardtii* α -CA (Cah-3). Lanes: 1, cell homogenate; 2, cell envelopes; 3, cell walls; 4, glycocalyx; 5, plasma membranes. All lanes contained 45 μ g protein.

ranging from 3 to 5, as has been reported in other cyanobacteria (Huang *et al.*, 2004). However, the most abundant protein of this fraction was basic, with an estimated pI value of 9.5 and a approximate molecular mass of 18 kDa.

Immunoblotting showed that the Cah-3-like protein, which was recognized by the antibodies, was located in a region of low pI (about 3.5). The molecular mass of this protein was about 34 kDa, i.e. similar to that of Cah-3-like protein observed in 1D electrophoresis analysis.

Identification of *M. chthonoplastes* extracellular β -CAs

For identification of β -CAs among *M. chthonoplastes* cell envelope proteins, primary antibodies against β -CAs from

various organisms were used, i.e. spinach chloroplasts and *C. reinhardtii* mitochondria, as well as antibodies against intracellular β -CA from the photobiont microalga *Coccomyxa* sp. This decision to use several types of antibody was determined by the multiplicity of the β -CA isoforms.

The cross-reaction between the *M. chthonoplastes* cell envelope polypeptides and the antibodies against spinach and *C. reinhardtii* β -CAs revealed the presence of several specific signals (Fig. 6). These signals were much stronger than those of the cell homogenate. The molecular masses of polypeptides detected by different antibodies were not identical. No cross-reaction was detected by Western blotting when the antibodies against the intracellular CA of *Coccomyxa* sp. was probed (not shown).

DISCUSSION

CA activity detected in intact cells as well as in the cell envelopes of *M. chthonoplastes* indicates that the cyanobacterium possesses extracellular enzyme(s), which can use external C_i as a substrate. Western blot analysis has suggested a multiplicity of potential extracellular CAs related to either the α - or β -classes.

Until now, the presence of external α -CA (possibly periplasmic) had been shown in only two species of cyanobacteria, *Anabaena* sp. PCC 7120 and *Synechococcus* sp. PCC 7942. However, enzyme activity of the identified α -CA (EcaA) was not detected either in whole-cell lysates or in cell fractions of these cyanobacteria (Soltes-Rak *et al.*, 1997). It was postulated that the cause of this failure could be due to the low sensitivity of the electrometrical method used for CA activity measurement. Extracellular localization in the periplasmic space was also assumed for EcaB (β -CA) of *Synechocystis* PCC 6803 (So *et al.*, 1998).

The levels of *M. chthonoplastes* extracellular CA activity detected are comparable with enzyme activities of some other prokaryotic CAs, for example the intracellular enzyme activity of *Anabaena variabilis* (Yagawa *et al.*, 1984), as well as with the activities of CAs from microalgal chloroplasts

Fig. 5. 2D electrophoresis (a) and Western blot analysis (b) of the glycocalyx fraction from *M. chthonoplastes*. The silver-stained gel and gel for immunodetection were loaded with 300 and 100 μ g protein, respectively. The blot was probed with antibodies directed against *C. reinhardtii* α -CA (Cah-3). 1, Localization of Cah-3-like protein in the gel; 2, localization of the major protein in the glycocalyx fraction.

Fig. 6. Identification of *M. chthonoplastes* extracellular β -CAs using immunoblot analysis with antibodies raised against various β -CAs following SDS-PAGE (12%). (a) Antibodies against mitochondrial β -CA from *C. reinhardtii*. (b) Antibodies against β -CA from spinach chloroplasts. Lanes: 1, cell homogenate; 2, isolated cell envelopes. Lanes were loaded with 45 μ g protein.

(Katzman *et al.*, 1994). In addition to a potential α -class enzyme, at least two putative β -CAs were found in the cell envelopes of *M. chthonoplastes*: one of them is similar to chloroplastic β -CAs and the other to mitochondrial β -CAs (Fig. 6). Numerous other specific signals probably resulted

from incomplete denaturation of native β -CAs consisting of several subunits (Smith & Ferry, 2000). The exact topology of these extracellular β -CAs within cell envelopes of *M. chthonoplastes* is still not clear.

Studies of the CAs of alkaliphilic cyanobacteria from soda lakes are important with respect to evolutionary theory, because these organisms are supposed to be relicts of ancient microbiota (Sergeev *et al.*, 2002). Genome analysis of many prokaryotic organisms has revealed the prevalence of β - and γ -CAs, which have been proposed to be the most ancient classes of CAs (Smith & Ferry, 2000). It has been assumed that α -CAs evolved from a common ancestral gene about 0.5–0.6 billion years ago (Smith & Ferry, 2000). This is consistent with the evidence that only a few prokaryotic genomes encode α -class enzymes. However, α -CAs have been found in some bacteria (Nafi *et al.*, 1990; Chirica *et al.*, 1997) and cyanobacteria (Soltes-Rak *et al.*, 1997; Dudoladova *et al.*, 2004) where they have been characterized as extracellular enzymes, similar to the α -CA of *M. chthonoplastes*. Thus, the presence of this class of CAs in relict organisms, as well as in other bacteria, indicates that α -CAs are as ancient as β - and γ -CAs.

The participation of CAs in the autotrophic assimilation of C_i by cyanobacteria is well known. It is supposed that the function of extracellular forms of the enzyme (α -EcaA and β -EcaB) is associated with C_i transport from the medium into the cell by CO_2 and/or HCO_3^- substrate formation for carbon transporters located in the plasma membrane. For intracellular β -CAs (IcfA and CcaA), which are located in the carboxysome, a role in CO_2 generation is

Fig. 7. A hypothetical scheme showing the role of *M. chthonoplastes* extracellular CAs in C_i assimilation and its connection with calcium carbonate deposition outside the cell. The size of the type is proportional to the C_i concentration in the medium and cell compartments. The localization of the carboxisomal CAs (IcfA and CcaA) is as described by Smith & Ferry (2000). An outer alkaline pH of about 10 corresponds to the soda lake environment whereas an outer pH of about 7.5 corresponds to conditions where $CaCO_3$ deposition in cyanobacterial mats usually occurs (seashore conditions where oceanic water is mixed with continental river waters.) *Alkalinization of the pericellular layer as a result of C_i assimilation is only relevant when the outer medium pH approaches neutral.

presumed in the place of the fixing of CO₂ by Rubisco (Smith & Ferry, 2000).

A general scheme of extracellular CA participation in C_i assimilation of *M. chthonoplastes* is presented in Fig. 7. In soda lakes, where the pH is highly alkaline (near 10), all dissolved C_i is present as carbonate and bicarbonate ions. In cyanobacterial cells, the existence of several HCO₃[−] transport systems has been demonstrated (Badger & Price, 2003). The majority of bicarbonate ions absorbed by the cell enter the carboxysome where they are converted to CO₂ by intracellular β-CAs, which is then fixed by Rubisco. Some of the absorbed HCO₃[−] can also be converted into CO₂ before being fixed by photosynthesis in accordance with the Henderson–Hasselbalch equation because of the lower intracellular pH compared to that of the outer medium. It has been shown previously that the cytoplasmic pH of alkaliphilic cyanobacteria is neutral (Kupriyanova *et al.*, 2003). According to the concentration gradient, there must be some leakage of the CO₂ produced in the intracellular space out of the cell. However, α- and β-CAs, which are located in the cell envelope of cyanobacteria, might prevent this leakage of CO₂ by converting it to HCO₃[−]. This is then transported through the membrane back into the cell by carbon transporters. This is in accordance with high enzyme activity of extracellular CA observed under alkaline pH conditions (Fig. 1) typical of the natural habitat of *M. chthonoplastes* in soda lakes.

The second peak of extracellular enzyme activity at pH near 7.5 (Fig. 1) reflects the ability of *M. chthonoplastes* to live in the seashore environment with a pH close to this value. The mineralization of cyanobacteria usually occurs under such conditions because seawater has a large reserve of Ca²⁺ ions (Zavarzin *et al.*, 2003). Stromatolite formation does not occur in soda lakes because calcium ions almost disappear from the medium at high pH, having been precipitated as CaCO₃.

The mechanisms of bicarbonate uptake discovered in cyanobacterial cells are associated solely with the alkalization of the pericellular space. Bicarbonate assimilation could be accompanied by hydroxyl excretion into the outer medium (McConnaughey, 1994) and/or by sodium symport (Badger & Price, 2003). The latter mechanism leads to alkalization of cytoplasm followed by activation of Na⁺-ATPase that assists Na⁺ exchange for H⁺ from the outer medium (Balnokin *et al.*, 2004). These mechanisms are shown schematically in Fig. 7.

When the pH of the pericellular layers rises to 9.0, calcium carbonate granules are produced in the cyanobacterial glycocalyx if free Ca²⁺ is available in the medium. The glycocalyx facilitates mineralization because it helps in the formation of a physicochemical/chemical microgradient by decreasing the rate of diffusion flow, and it binds divalent cations, including Ca²⁺, from the external medium due to the presence of polysaccharide carboxyl groups (Arp *et al.*, 2001). Thus, photosynthetic assimilation of C_i by *M.*

chthonoplastes cells appears to be accompanied by their mineralization under pH conditions found in seawater. CAs possibly stabilize pH in the pericellular space and maintain the substrate (HCO₃[−]) concentration essential for both photosynthesis and CaCO₃ deposition.

ACKNOWLEDGEMENTS

We are grateful to Dr M. Eriksson (Umeå University, Sweden) who kindly provided antibodies against mitochondrial CA from *C. reinhardtii*. We thank Dr N. L. Klyachko (Institute of Plant Physiology, Russia) for helpful discussions and her advice at the stage of preparation of the manuscript. The work was supported by grants from European Molecular Biology Organization (EMBO Short-term Fellowship ASTF 12-02) and from the Ministry of Science and Education (MK-3393.2005.4) to E.K., grants from the Russian Foundation for Basic Research (04-04-49407 and 05-04-50883) to N.P. and D.A.L., grants from the 'Molecular and Cell Biology Program' and 'Evolution of Biosphere Program' of the Russian Academy of Sciences to D. A. L and G. Z., and a grant from the Swedish Foundation for International Cooperation in Research and Higher Education (STINT) to G. S.

REFERENCES

- Arp, G., Reimer, A. & Reitner, J. (2001). Photosynthesis-induced bio-film calcification and calcium concentrations in phanerozoic oceans. *Science* **292**, 1701–1704.
- Badger, M. R. & Price, G. D. (2003). CO₂ concentrating mechanisms in cyanobacteria: molecular components, their diversity and evolution. *J Exp Bot* **54**, 609–622.
- Balnokin, Y. V., Popova, L. G., Pagis, L. Y. & Andreev, I. M. (2004). The Na⁺-translocating ATPase in the plasma membrane of the marine microalga *Tetraselmis viridis* catalyzes Na⁺/H⁺ exchange. *Planta* **219**, 332–337.
- Castenholz, R. W. & Waterbury, J. B. (1989). Group I. Cyanobacteria. In *Bergey's Manual of Systematic Bacteriology*, vol. 3, pp. 1710–1727. Edited by J. T. Staley, M. P. Bryant, N. Pfennig & J. G. Holt. Baltimore, MD: Williams & Wilkins.
- Chirica, L. C., Elleby, B., Jonsson, B. H. & Lindskog, S. (1997). The complete sequence, expression in *Escherichia coli*, purification and some properties of carbonic anhydrase from *Neisseria gonorrhoeae*. *Eur J Biochem* **244**, 755–760.
- Dudoladova, M. V., Markelova, A. G., Lebedeva, N. V. & Pronina, N. A. (2004). Compartmentation of α- and β-carbonic anhydrases in the cells of halo- and alkaliphilic cyanobacterium *Rhabdoderma lineare*. *Russ J Plant Physiol* **51**, 806–814.
- Eriksson, M., Karlsson, J., Ramazanov, Z., Gardestrom, P. & Samuelsson, G. (1996). Discovery of an algal mitochondrial carbonic anhydrase: molecular cloning and characterisation of a low-CO₂-induced polypeptide in *Chlamydomonas reinhardtii*. *Proc Natl Acad Sci U S A* **93**, 12031–12034.
- Fawcett, T. W., Browse, J. A., Volokita, M. & Bartlett, S. G. (1990). Spinach carbonic anhydrase primary structure deduced from the sequence of a cDNA clone. *J Biol Chem* **265**, 5414–5417.
- Gantt, E. (1994). Supermolecular membrane organization. In *The Molecular Biology of Cyanobacteria*, pp. 119–138. Edited by D. A. Bryant. Dordrecht: Kluwer.

- Gerasimenko, L. M., Mityushina, L. L. & Namsaraev, B. B. (2003). *Microcoleus* mats from alkaliphilic and halophilic communities. *Mikrobiologiya* 72, 71–79.
- Hiltonen, T., Bjorkbacka, H., Forsman, C., Clarke, A. K. & Samuelsson, G. (1998). Intracellular β -carbonic anhydrase of the unicellular green alga *Coccomyxa*. Cloning of the cDNA and characterization of the functional enzyme overexpressed in *Escherichia coli*. *Plant Physiol* 117, 1341–1349.
- Huang, F., Hedman, E., Funk, C., Kieselbach, T., Schroder, W. P. & Norling, B. (2004). Isolation of outer membrane of *Synechocystis* sp. PCC 6803 and its proteomic characterization. *Mol Cell Proteomics* 3, 586–595.
- Karlsson, J., Clarke, A. K., Chen, Z. Y., Huggins, S. Y., Park, Y.-I., Husic, H. D., Moroney, J. V. & Samuelsson, G. (1998). A novel alpha-type carbonic anhydrase associated with the thylakoid membrane in *Chlamydomonas reinhardtii* is required at ambient CO₂. *EMBO J* 17, 1208–1216.
- Katzman, G. L., Carlson, S. J., Marcus, Y., Moroney, J. V. & Togasaki, R. K. (1994). Carbonic anhydrase activity in isolated chloroplasts of wild-type and high-CO₂-dependent mutants of *Chlamydomonas reinhardtii* as studied by a new assay. *Plant Physiol* 105, 1197–1202.
- Kupriyanova, E. V., Lebedeva, N. V., Dudoladova, M. V., Gerasimenko, L. M., Alekseeva, S. G., Pronina, N. A. & Zavarzin, G. A. (2003). Carbonic anhydrase activity of alkaliphilic cyanobacteria from soda lakes. *Russ J Plant Physiol* 50, 532–539.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680–685.
- McConnaughey, T. A. (1994). Calcification, photosynthesis and global carbon cycles. *Bull Inst Oceanogr* 13, 137–161.
- Nafi, B. M., Miles, R. J., Butler, L. O., Carter, N. D., Kelly, C. & Jeffery, S. (1990). Expression of carbonic anhydrase in neisseriae and other heterotrophic bacteria. *J Med Microbiol* 32, 1–7.
- Nishiyama, Y., Yamamoto, H., Allakhverdiev, S. I., Inaba, M., Yokota, A. & Murata, N. (2001). Oxidative stress inhibits the repair of photodamage to the photosynthetic machinery. *EMBO J* 20, 5587–5594.
- Park, Y.-I., Karlsson, J., Rojdestvenski, I., Pronina, N., Klimov, V., Oquist, G. & Samuelsson, G. (1999). Role of a novel photosystem II-associated carbonic anhydrase in photosynthetic carbon assimilation in *Chlamydomonas reinhardtii*. *FEBS Lett* 444, 102–105.
- Porra, R. J., Thompson, W. A. & Kriedemann, P. E. (1989). Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls a and b extracted with four different solvents: verification of the concentrations of chlorophyll standards by absorption spectroscopy. *Biochim Biophys Acta* 975, 384–394.
- Quiroga, O. & Gonzalez, E. L. (1993). Carbonic anhydrase in the chloroplast of a coccolithophorid (Prymnesiophyceae). *J Phycol* 29, 321–324.
- Rippka, R., DeReuelles, J., Waterbury, J. B., Herdman, M. & Stanier, R. Y. (1979). Generic assignments, strain histories and properties of pure cultures of cyanobacteria. *J Gen Microbiol* 111, 1–61.
- Sergeev, V. N., Gerasimenko, L. M. & Zavarzin, G. A. (2002). The proterozoic history and present state of cyanobacteria. *Mikrobiologiya* 71, 623–637.
- Smith, K. S. & Ferry, J. G. (2000). Prokaryotic carbonic anhydrases. *FEMS Microbiol Rev* 24, 335–366.
- So, A. K., Van Spall, H. G. C., Coleman, J. R. & Espie, O. S. (1998). Catalytic exchange of ¹⁸O from ¹³C¹⁸O-labelled CO₂ by wild type cells and *ecaA*, *ecaB*, and *ccaA* mutants of the cyanobacteria *Synechococcus* PCC 7942 and *Synechocystis* PCC 6803. *Can J Bot* 76, 1153–1160.
- So, A. K., Espie, G. S., Williams, E. B., Shively, J. M., Heinhorst, S. & Cannon, G. C. (2004). A novel evolutionary lineage of carbonic anhydrase (epsilon class) is a component of the carboxysome shell. *J Bacteriol* 186, 623–630.
- Soltes-Rak, E., Mulligan, M. E. & Coleman, J. R. (1997). Identification and characterization of gene encoding a vertebrate-type carbonic anhydrase in cyanobacteria. *J Bacteriol* 179, 769–774.
- Tripp, B. C., Smith, K. & Ferry, J. G. (2001). Carbonic anhydrase: New insights for an ancient enzyme. *J Biol Chem* 276, 48615–48618.
- Villarejo, A., Shutova, T., Moskvina, O., Forssen, M., Klimov, V. V. & Samuelsson, G. (2002). A photosystem II-associated carbonic anhydrase regulates the efficiency of photosynthetic oxygen evolution. *EMBO J* 21, 1930–1938.
- Weckesser, J. & Jürgens, U. J. (1988). Cell walls and external layers. *Methods Enzymol* 167, 173–188.
- Wilbur, K. M. & Anderson, N. G. (1948). Electrometric and colorimetric determination of carbonic anhydrase. *J Biol Chem* 176, 147–154.
- Yagawa, Y., Shiraiwa, Y. & Miyachi, S. (1984). Carbonic anhydrase from the blue-green alga (cyanobacterium) *Anabaena variabilis*. *Plant Cell Physiol* 25, 775–783.
- Zavarzin, G. A. (2002). Microbial geochemical calcium cycle. *Mikrobiologiya* 71, 1–17.
- Zavarzin, G. A., Orleanskii, V. K., Gerasimenko, L. M., Pushko, S. N. & Ushatinskaya, G. T. (2003). Laboratory simulations of cyanobacterial mats of the alkaline geochemical barrier. *Mikrobiologiya* 72, 80–85.

Edited by: K. Forchhammer