

The thylakoid carbonic anhydrase associated with photosystem II is the component of inorganic carbon accumulating system in cells of halo- and alkaliphilic cyanobacterium *Rhabdoderma lineare*

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Abstract

The organization of carbonic anhydrase (CA) system in halo- and alkaliphilic cyanobacterium *Rhabdoderma lineare* was studied by Western blot analysis and immunocytochemical electron microscopy. The presence of putative extracellular α -CA of 60 kDa in the glycocalyx, forming a tight sheath around the cell, and of two intracellular β -CA is reported. We show for the first time that the β -CA of 60 kDa is expressed constitutively and associated with polypeptides of photosystem II (β -CA-PS II). Another soluble β -CA of 25 kDa was induced in low-bicarbonate medium. Induction of synthesis of the latter β -CA was accompanied by an increase in the intracellular pool of inorganic carbon, which suggests an important role of this enzyme in the functioning of a CO₂-concentrating mechanism.

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1. Introduction

The zinc-containing metalloenzyme carbonic anhydrase (CA, carbonate hydrolyase, EC 4.2.1.1) is found in most organisms of various organization levels, including animals, plants and microalgae, archaea- and eubacteria [1]. It has been demonstrated that this enzyme is involved in such basic processes as photosynthesis, respiration, transport of inorganic carbon (C_i) and ions, calcification, and regulation of acid–base balance [2].

According to modern nomenclature, all CAs are divided into three basic classes (α , β , and γ) that do not have significant homology in amino acid sequences and presumably evolved independently [1]. It is assumed that the youngest phylogenetic

group of CAs is the α -class, which includes mainly mammalian proteins. The most ancient enzymes are γ - and β -CAs, which are widespread in both Bacteria and Archaea [3].

CAs are characterized by a diversity of structures and physiological functions, and by different locations in cells. In cyanobacteria, this enzyme is found both in outer layers [4,5] and inside the cell [6,7].

Extracellular CAs are either involved in formation of substrates (by maintaining equilibrium between CO₂ and HCO₃⁻) for cytoplasmic transporters of C_i from medium into cell, or play a role as sensors of CO₂ level in the external medium [8]. The CAs that are located in the cell wall may stabilize pH in the pericellular space of the cell; they may also play a role in mineralization of cyanobacteria during photosynthetic assimilation of C_i [5]. The extracellular localization is well documented for the extracellular α -CA (EcaA) from *Anabaena* sp. PCC 7120, *Synechococcus* sp. PCC 7942 [4], and in the relict alkaliphilic cyanobacterium *M. chthonoplastes* [5]. Periplasmic localization was also assumed for EcaB (β -class CA) of *Synechocystis* PCC 6803 [8].

Abbreviations: CA, carbonic anhydrase; CCM, CO₂-concentrating mechanism; C_i, inorganic carbon compounds (CO₂, HCO₃⁻, and CO₃²⁻); PS, photosystem; PAGE, polyacrylamide gel electrophoresis; MW, molecular weight

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The intracellular CAs, which are predominantly located in carboxysomes – the specialized compartments that contain Rubisco, a key enzyme of CO₂ fixation – are involved in production of carbon dioxide from bicarbonate [2,9]. Such localization was suggested for the IcfA β-CA of *Synechococcus* PCC 7942 [6], and for the product of the *ccaA* gene, that was found in *Synechocystis* PCC 6803 [7]. Thus, in cyanobacteria, CAs are the key enzymes in the CO₂-concentrating mechanism (CCM).

Alkaliphilic cyanobacteria from soda lakes are very intriguing species for studies of autotrophic assimilation of C_i and participation of CA in this process [5]. From an evolutionary viewpoint, these cyanobacteria are unique relicts of ancient terrestrial microbiota preserved in extremely severe environments [10]. Thus, the study of the CA system in these organisms is of interest for understanding the evolution of the three classes of CA. The ability of these cyanobacteria to grow in highly alkaline medium (pH 9–10), where inorganic carbon exists only in the carbonate and bicarbonate forms [11], allows researchers to identify available exogenous C_i species and to investigate the role of CA in their utilization.

Earlier we have reported the detection of high activity of CAs in three representatives of cyanobacterial community of soda lakes, including relict halo- and alkaliphilic *Rhabdoderma lineare* [12]. Here we present the data on identification of α- and β-classes of CA in *R. lineare*, their sub-cellular localization, and effects of environmental conditions on the induction of CA synthesis, with an emphasis on the involvement of CA in regulation of intracellular pool of C_i and in the accumulation of C_i in cyanobacteria.

2. Materials and methods

2.1. Strain and growth conditions

We studied an extreme halo- and alkaliphilic plankton cyanobacterium *Rhabdoderma lineare* from the collection of the Institute of Microbiology, Russian Academy of Sciences. The strain was isolated from a hypersaline African lake Magadi [11]. The natural habitat of *R. lineare* is a soda lake with pH near 10 and salinity of 1 M.

R. lineare was grown in sterile batch cultures at 28 °C under continuous illumination with cool white fluorescent lamps at 30 W/m². For cultivation, we used conical flasks with medium S containing (g/l): 1.0 KCl; 16.8 NaHCO₃; 0.5 K₂HPO₄; 2.5 NaNO₃; 1.0 K₂SO₄; 30 NaCl; 0.1 MgSO₄; 0.04 CaCl₂; 0.01 FeSO₄; and 0.08 EDTA, pH 9.5. The medium was supplemented with standard micronutrients (BG-11) [13].

2.2. Isolation of cell fractions

Intact cells were collected by centrifugation and washed once with phosphate buffer (0.06 M Na₂HPO₄, 5 mM cystein, 1 mM EDTA, pH 8.1). The crude homogenate was obtained by cell disruption in a chilled phosphate buffer using a Retch-MMW homogenizer (Germany) with acid-washed glass beads (G4649, Sigma, USA) (equal volumes of buffer and beads). Crude homogenate was separated into soluble proteins and insoluble cell components containing cell membranes, by centrifugation at 100,000×g (1 h, 4 °C). Thylakoid membranes were isolated by centrifugation of membrane fraction in discontinuous gradient of sucrose density [14]. Photosystem I (PS I) and photosystem II (PS II) particles were isolated by the treatment of thylakoid membranes with Triton X-100 according to method of Shim et al. [15]. All preparation steps were carried out at 4 °C and dim light using chilled buffers. The protease inhibitor mix (Sigma, USA) was added to the samples to avoid protein degradation.

2.3. Assay of CA activity

CA activity was assayed electrometrically with an M-901 pH-meter and M-951 plotter (Orion Research, USA) by measuring changes of H⁺ concentration in the reaction of carbon dioxide hydration [16]. The reaction mixture contained intact cells or the crude homogenate (0.5–0.8 mg of protein) in the phosphate buffer. The reaction was carried out at 2 °C and started by a rapid injection of a saturated aqueous solution of CO₂ into an equal volume of reaction mixture (fermentative reaction) or phosphate buffer (non-fermentative reaction, control). The CA activity was calculated as a difference in the initial rate of CO₂ hydration between control and experimental samples and expressed in Wilbur–Anderson units (WAU) per 1 mg of chlorophyll (Chl) or protein [16]. The measurements were carried out in 5–7 replicates.

2.4. Estimation of protein and chlorophyll content

Protein content was estimated in accordance with the method of Lowry et al. [17]. Chlorophyll concentrations were determined spectroscopically after extraction with absolute methanol [18].

2.5. Electrophoresis and immunodetection

Proteins were separated on 12% SDS-PAGE as described by Laemmli [19]. The lanes were loaded with 20 μg protein. The samples were solubilized during 20 min at 55 °C in the sample buffer containing 2% SDS. Immunoblotting was performed as described in the protocol from Bio-Rad Laboratories, and a standard reagent kit was used. The primary antibodies were raised against: (1) Cah-3 protein (α-CA) of *C. reinhardtii* [20] or (2) intracellular β-CA from *Coccomyxa* [21]. Horseradish peroxidase-labeled secondary antibodies (Amersham Life Science) and chemiluminescence solutions (ECL, Amersham) were used to detect an antibody–antigen conjugate.

2.6. Immunoelectronic microscopy

For immunogold labeling experiments, *R. lineare* cells were fixed in 4% paraformaldehyde during 4–10 days at 4 °C. Immunocytochemical reactions were performed after sample washing in 0.1 M phosphate buffer (pH 7.4). The reaction with primary antibodies against Cah-3 protein (α-CA) from *C. reinhardtii* 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 for 24 h. The second step of immunochemical reaction and post-washing were performed under the same conditions using Protein-A-Gold (Sigma). The post-fixation in 1% OsO₄, dehydration in alcohol series, and staining in Epoxy resin were performed as described previously [22]. For the control of immunochemical reaction specificity, the step of sample treatment with primary antibodies was omitted. Thin sections of samples were prepared using an ultramicrotome and then analyzed with a JEM JEOL X-100 transmission microscope (Japan) without any additional contrasting.

2.7. Intracellular pool of inorganic carbon (C_i)

The intracellular pool of C_i was estimated using the method of filtrating centrifugation through the layer of silicon oil [23], adapted for cells of *R. lineare*. This method estimates the pool of C_i taken up by cells from growth medium but not involved in metabolism.

R. lineare cells were separated from the medium by centrifugation at 4000×g (20 min, 25 °C) and washed once with the phosphate buffer. The samples were preincubated under light (30 W/m², 1 h) in the phosphate buffer. After that, suspension aliquots (20 μl) were transferred into eppendorf tubes (200 μl), which contained a bottom layer of 10% KOH in absolute methanol covered by a mixture of silicon oils AR200:DC200 (Fluka, Sweden) at 2:3 (v/v) ratio. The uptake of C_i was initiated by injection of NaH¹⁴CO₃ (specific activity of 18.8 Ci/mol) to a final concentration of 10 μM at 25 °C and irradiance of 30 W/m². After 0.5 min incubation of cells with the labeled bicarbonate, the exposure was stopped by centrifugation (30 s, 14,500×g). The tubes were frozen in liquid nitrogen. Then lower parts of the tubes,

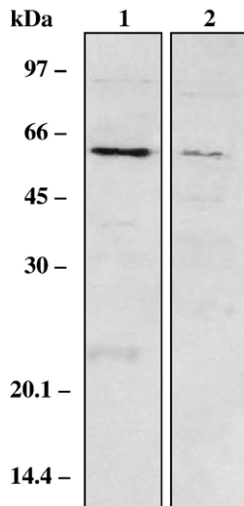


Fig. 1. Identification of α - and β -class CAs in *R. lineare*. 20 μ g proteins of total cell proteins were loaded into each lane of 12% PAG and resolved by electrophoresis. Proteins were transferred onto nitrocellulose membranes and probed with rabbit antibodies raised against intracellular β -CA of *Coccomyxa* (1) and with antibodies against α -CA (Cah-3 protein) from thylakoids of *C. reinhardtii* (2).

which contained cells separated from the medium, were cut off, and their radioactivity was measured on a 1219 Rackbeta counter (LKB, Sweden).

3. Results

3.1. Activity of *R. lineare* CA

The enzymatic activity of CA was detected in both intact cells and crude cell homogenate of *R. lineare*. For the crude homogenate, the average value was about 1.71 WAU/mg protein. In intact cells, the average value was about 0.24 WAU/mg protein. The values of detected CA activity in *R. lineare* cells are comparable with enzyme activities in some other prokaryotic organisms [23,24].

In order to confirm that the acceleration of carbon dioxide hydration in the reaction mixture was mediated by the enzymatic activity of CA, we used the specific inhibitor of CA, ethoxzolamide (6-ethoxybenzothiazole-2-sulfonamide). The constants of CA half-inhibition (I_{50}) by ethoxzolamide were 10^{-7} and 10^{-5} M for extracellular CA of intact cells and for CA in the crude homogenate, respectively. These data indicate the presence of different CAs at the outer surface and inside the cell. These CAs might belong to different classes, since the sensitivity of β -CA to sulfonamides is known to be considerably lower than that of α -CA [3].

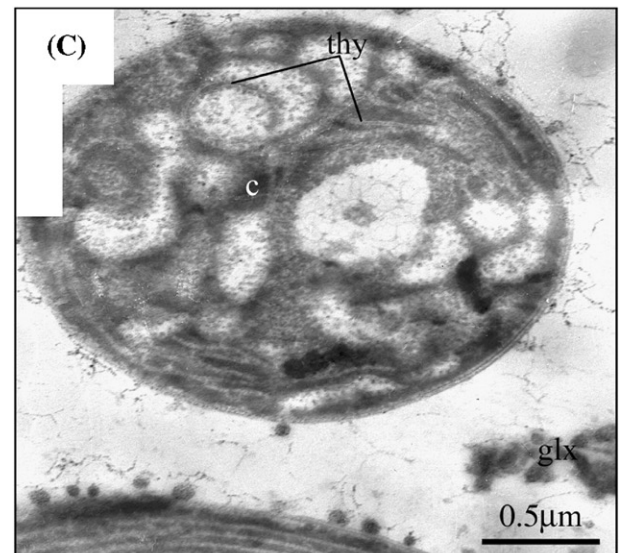
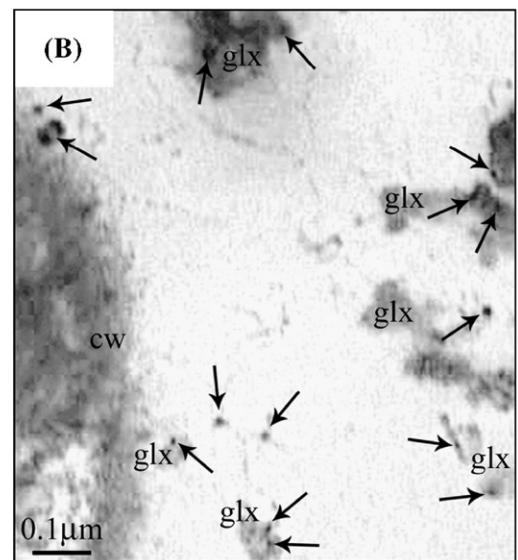
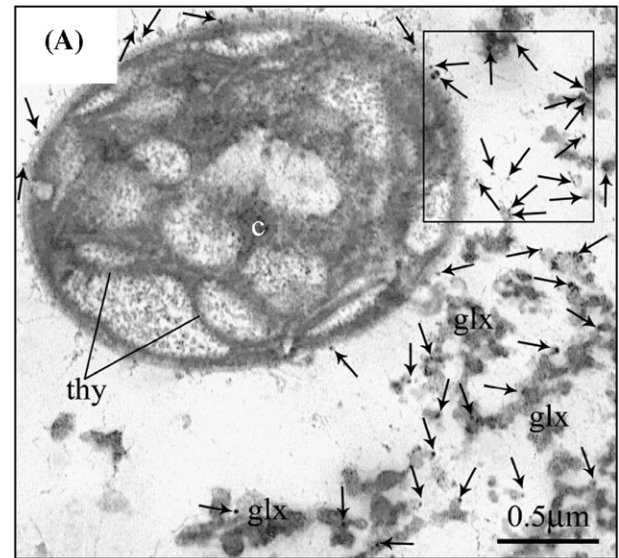


Fig. 2. Immunocytochemical localization of α -CA in the *R. lineare* cells. For immunocytochemical reaction, the antibodies raised against Cah-3 protein (α -CA) from *C. reinhardtii* have been used as primary antibodies and Protein-A-Gold (Sigma) have been used as secondary antibodies. The arrows indicate the localization of α -CA. (A) The cells grown in standard S medium and treated with primary and secondary antibodies; (B) the enlarged fragment of (A); (C) the cells grown in standard S medium and treated only with secondary antibodies (control). Abbreviations: glx—glycocalyx; cw—cell wall; thy—thylakoids; c—carboxysome.

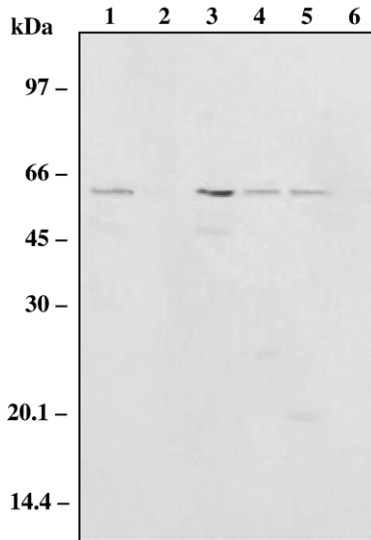


Fig. 3. Western blot analysis of various protein fractions isolated from *R. lineare* cells. The blot was probed with rabbit antibodies raised against intracellular β -CA from *Coccomyxa*. Lanes: (1) crude homogenate, (2) total soluble proteins, (3) total membrane-bound proteins, (4) thylakoid membranes, (5) PS II enriched fraction, and (6) PS I enriched fraction. All lanes contained 20 μ g of protein. The initial SDS-PAGE-12%.

3.2. Identification of *R. lineare* α - and β -class CA

Measurements of the enzymatic activity, in intact cells and crude homogenate of *R. lineare*, suggest the presence of specific CA isoforms associated with the outer cell layers and located inside the cells of this cyanobacterium. Western blotting analysis (Fig. 1) of the crude cell homogenate with antibodies raised against intracellular β -CA from the microalga *Coccomyxa* revealed the presence of a protein of about 60 kDa. A protein of about 60 kDa was also detected with antibodies against α -type CA (Cah-3 protein) of *C. reinhardtii*.

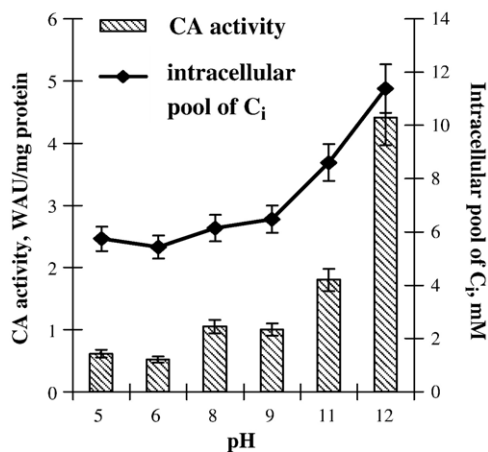


Fig. 4. Total CA activity and intracellular pool of C_i depending on pH of external medium in *R. lineare*. Cyanobacterial cells were grown in standard medium S with pH of 9.5 and then transferred into the phosphate buffer with pH ranged from 5 to 12. CA activity of the crude cell extract and concentration of C_i were measured after incubation of cells in these buffers for 2 h.

The comparison of amino acid sequences of *C. reinhardtii* α -CA (Cah3) and β -CA of *Coccomyxa* revealed a 12.8% similarity between them. Thus the probability of nonspecific cross-hybridization between antibodies and α - and β -CAs seems to be negligibly low.

3.3. Immunocytochemical detection of the α -CA in *R. lineare* cells

The α -CA was localized in *R. lineare* with electron microscopy of thin sections of the cells using antibodies against α -CA (Cah3) of *C. reinhardtii* (Fig. 2). The electron-impermeable spots of colloidal gold have been found predominantly in glycocalyx bounded with the cell envelopes (Fig. 2A). Aggregates of gold particles could also be distinguished in fragments of free glycocalyx, which were easily separated from the cells during fixation (Fig. 2B).

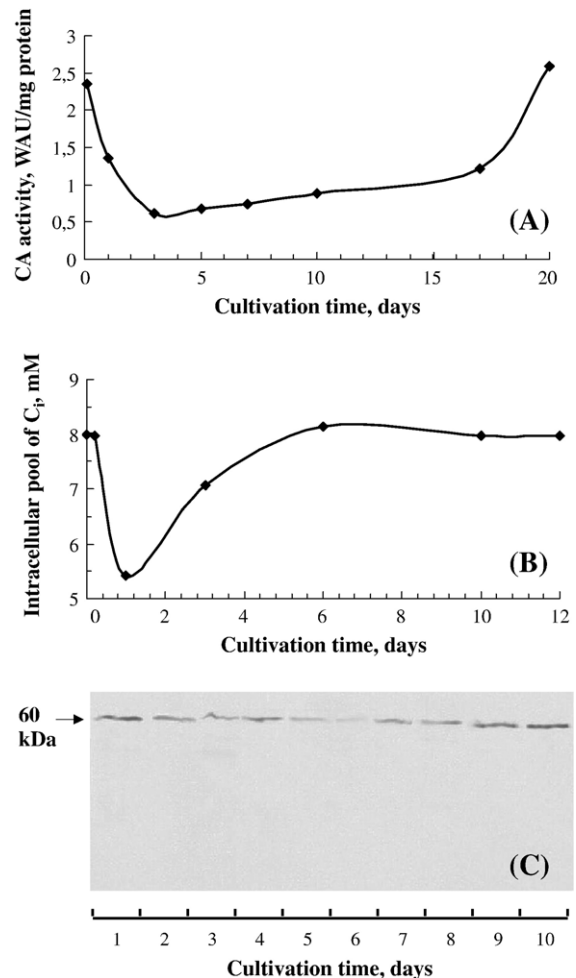


Fig. 5. The effect of illumination on the total activity of CA (A); the intracellular pool of C_i (B) and the amount of putative β -CA-PS II protein in *R. lineare* cells determined by Western blot analysis (C). *R. lineare* cells were grown in standard medium S to the beginning of stationary growth phase and then diluted with fresh S medium providing increased illumination per cell in suspension (point "zero"). The CA activity and intracellular pool of C_i were estimated after 2 h of incubation of the cells in new conditions. The blot was probed with rabbit antibodies raised against intracellular β -CA from *Coccomyxa*. Each lane contained 20 μ g protein of crude cell protein. The initial SDS-PAGE-12%.

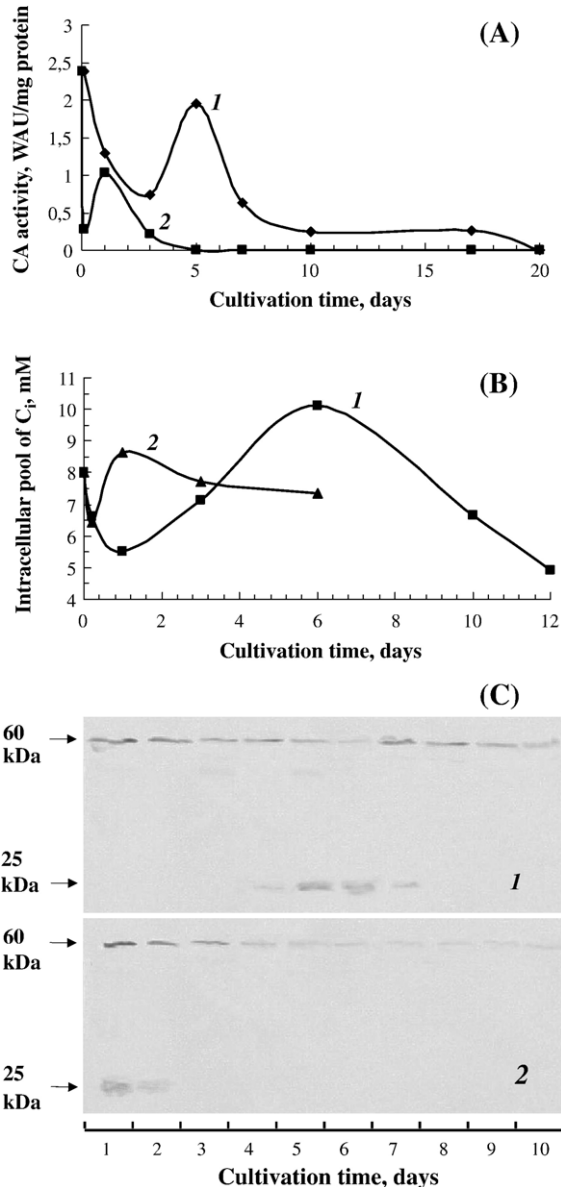


Fig. 6. The effect of bicarbonate concentration in the culture medium on (A) the total activity of CA; (B) the intracellular pool of C_i , and (C) amount of putative β -CA in *R. lineare* cells. *R. lineare* cells were grown in standard medium S with 16.8 g/l NaHCO_3 to the middle of logarithmic phase, and then transferred into medium S where bicarbonate concentration was reduced 2 or 20 times (8.4 and 0.84 g/l of Na HCO_3 respectively). Changes in the amount of β -CA were detected by Western blot analysis as described in legend for Fig. 1. The blots were probed with rabbit antibodies raised against intracellular β -CA from *Coccomyxa*. Each lane contained 20 μg protein of crude cell protein. Arrows indicate the signals from CAs of 60 and 25 kDa. Concentrations of sodium bicarbonate in the growth medium were (g/l): 8.4 (1) and 0.84 (2).

3.4. Cellular localization of *R. lineare* β -class CA

The intracellular distribution of β -CA in *R. lineare* cells was studied by separation of particular cell fractions and subsequent immunoblotting with polyclonal antibodies against β -CA of *Coccomyxa* (Fig. 3). This method revealed the presence of a single protein band in the region of 60 kDa, which gave specific cross-reaction with antibodies in the

crude cell homogenate, as well as in the fractions of total membrane proteins, thylakoid membranes, and subchloroplast particles enriched with PS II. β -CA was not detected among total soluble proteins, nor in the membrane fraction enriched with PS I.

3.5. The dynamic changes in intracellular pool of C_i and CA-system of *R. lineare* depend on environmental conditions

The activity and organization of CA-system in the microalgae and cyanobacterial cell as well as their ability to uptake C_i by initiation of CCM depend on a number of environmental conditions [2,9].

To study the dependence of *R. lineare* CA activity and intracellular C_i pool on external pH, the cyanobacterial cells were cultured under an optimum pH of 9.5 and then transferred to phosphate buffers with different pH (5.0–12.0) with further incubation in the new conditions for 2 h. Other parameters (light, temperature, etc.) remained unchanged. The highest CA activity in *R. lineare* crude homogenate as well as the highest intracellular concentration of C_i were detected for the cells incubated at an alkaline pH (Fig. 4). At the same time, these values decreased together with a decrease of pH of the medium. The intracellular concentration of C_i in *R. lineare* cells grown under the optimal conditions was near 8 mM (Fig. 4), resembling similar values of other cyanobacteria, including some freshwater strains [24].

To study the dependence of total CA-activity and potential β -CA protein synthesis on the light intensity, the *R. lineare* cell suspension was diluted with fresh S medium at the beginning of

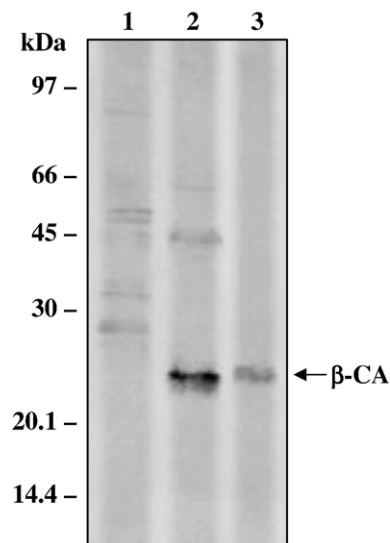


Fig. 7. Western blot analysis of total soluble protein fractions isolated from *R. lineare* cells cultivated in medium S with different concentrations of bicarbonate. 20 μg of total protein was loaded into each well and resolved by electrophoresis. Proteins were transferred onto nitrocellulose membrane, and blots were probed with rabbit antibodies raised against intracellular β -CA from *Coccomyxa*. Concentrations of sodium bicarbonate in the medium were (g/l): 16.8 (1); 8.4 (2), and 0.84 (3). The fractions of soluble proteins were isolated from the cells at the moment when new low-MW β -CA appeared (Fig. 6C).

the stationary growth phase. As a result the light absorbed per cell drastically increased. After adding the inoculate into the fresh medium, the enzyme activity decreased, followed by its recovery to the initial level by the 20th day of cultivation (Fig. 5A), when cell density reached the initial value. There was also a drastic decrease of intracellular pool of C_i which was followed by its recovery to the initial level by the 6th day of cultivation (Fig. 5B).

Western blot analysis with antibodies against β -CA of *Coccomyxa* (Fig. 5C) showed that changes in the enzyme activity were well correlated with changes in the amount of putative β -CA, that is associated (as demonstrated before) with PS II (β -CA-PS II). The amount of β -CA protein clearly declined and then recovered. These fluctuations were accompanied by synchronous changes in CA activity (Fig. 5A, C).

To study the effect of bicarbonate concentration on the intracellular concentration of C_i , CA activity and on the induction of enzyme synthesis, the *R. lineare* cells, grown in standard medium S with 16.8 g/l NaHCO_3 to reach the mid-logarithmic phase, were transferred into medium S, where the bicarbonate concentration was reduced 2 or 20 times (8.4 and 0.84 g/l of NaHCO_3 , respectively). After reduction of the bicarbonate concentration in the growth medium, the CA activity in the crude homogenate decreased approximately up to 3 and 10 times (within 3 days and 2 h, respectively) in the presence of 8.4 g/l and 0.84 g/l NaHCO_3 (Fig. 6A). Then CA activity was recovered on the fifth and first day, respectively, for the medium with 8.4 g/l and 0.84 g/l NaHCO_3 . Thus, the lower was the initial concentration of bicarbonate in the culture medium, the earlier the enzyme activity started to increase. It is noteworthy that even a twofold decrease in the concentration of sodium bicarbonate (from 16.8 to 8.4 g/l) suppressed growth of cyanobacteria, which presumably led to a further decrease in enzyme activity and its complete inhibition on the 20th day (curve 1).

Changes in the intracellular concentration of total C_i displayed similar dynamics (Fig. 6B). Thus, maxima of C_i accumulation coincided with maxima of CA activity. These peaks of CA activity during growth in a low HCO_3^- medium coincided with the appearance of new form of β -CA of about 25 kDa (Fig. 6C). This form of β -CA was not detected in cells that had been grown in a standard S medium (Fig. 5C).

Western blot analysis was used to identify the subcellular localization of this new β -CA of 25 kDa (Fig. 7). The fractions of total membrane and soluble proteins were isolated from the cells that have been grown in low NaHCO_3 medium (8.4 and 0.84 g/l) during 5 and 1 day, respectively, when the maximum of CA activity and appearance of new β -CA of 25 kDa were observed (Fig. 6C). These fractions were tested with primary antibodies raised against *Coccomyxa* β -CA. These antibodies cross-reacted with only one specific protein in the region of 25 kDa among the polypeptides of the soluble protein fraction, isolated from cells grown at low concentrations of bicarbonate (Fig. 7). At the same time, no cross-reaction was detected among the polypeptides of membrane protein fractions (data not shown).

4. Discussion

The dependence of total CA activity on pH of the external medium in the cells of alkaliphilic cyanobacterium *R. lineare* reveals the possible involvement of this enzyme in accumulation of C_i , which is induced by alkaline pH (Fig. 4). When cells were transferred into an alkaline environment, the increase in accumulation of C_i was apparently conditioned by changes in the ratio of $\text{HCO}_3^-/\text{CO}_3^{2-}$ substrates in accordance with the Henderson–Hasselbalch equation. It has been suggested that cells sense limitation in a suitable exogenous form of C_i rather than a reduction of total C_i concentration in the environment [2]. If so, it would be reasonable to assume that induction of C_i -accumulation in *R. lineare* cells might be caused either by the excess of carbonate, or by low content of bicarbonate at high alkaline pH.

Reduction of the concentration of bicarbonate in culture medium resulted in maximum accumulation of intracellular C_i , which coincided with the maximum activity of CA (Fig. 6). This fact implies the involvement of CAs in C_i -concentration in bicarbonate limiting conditions.

We have detected three potential forms of CA in *R. lineare* cells. Western blot analysis and immunoelectronic microscopy revealed the constitutive extracellular CA of 60 kDa, which is located in glycocalix of *R. lineare* (Figs. 1, 2), and possibly belongs to the family of α -class. In addition, there were at least two intracellular enzymes of 60 and 25 kDa, which belong to β -class of CA (Figs. 1, 7). Constitutive 60 kDa β -CA was localized in thylakoid membranes. The soluble β -CA of 25 kDa was inducible and appeared only at low bicarbonate conditions (Fig. 6).

Investigation of the phylogenetic relationship between the amino acid sequences of different CAs suggested that the most ancient classes of CA are β - and γ -CA [3]. The existence of α -class of CA in relict organisms such as *R. lineare*, as well as in other cyanobacteria [4,5], might indicate that α -CAs are as ancient as β - and γ -CAs.

The potential extracellular CA, which is located in the outer layers of *R. lineare*, is possibly involved in the uptake of exogenous bicarbonate and pH stabilization in the pericellular space, as it was earlier demonstrated for other extracellular CAs [9,25,26]. Besides this, extracellular CA can play a role as a sensor for the level of CO_2 in the external medium, as suggested for the EcaA of *Synechococcus* PCC 7942 [4,8]. The α -CA located in glycocalix of *R. lineare* may also prevent leakage of CO_2 from cells by converting it back to HCO_3^- and by providing the substrates for cytoplasmic carriers of C_i similarly to the extracellular CA of the alkaliphilic cyanobacterium *Microcoleus chthonoplastes* [5].

The constitutive β -CA of *R. lineare* that was expressed at both optimal and bicarbonate limiting condition represents an intracellular enzyme associated with polypeptides of PS II (Fig. 3). It is noteworthy that in plants and microalgae, such PS II-associated CAs belong to the α -class [27,28]. Up to now, the thylakoid CAs, found in some microalgae and higher plants, have not been detected in thylakoid membranes of cyanobacteria.

The physiological role of such thylakoid CAs is not sufficiently clear. It was shown that the operation of CA-PS II is important for CCM in microalgae [28,29]. Supposedly, these enzymes use light-dependent proton uptake to catalyze formation of CO₂ from HCO₃⁻ in the thylakoid lumen [30]. Nevertheless, the functional role of thylakoid CAs does not seem to be limited to bicarbonate conversion, since it was demonstrated that CAs are involved in regulation of water oxidation in microalgae and higher plants [27,31,32].

The β-CA-PS II of *R. lineare* may be involved in carbon dioxide generation from bicarbonate in the lumen of thylakoids, similarly to Cah3 of *C. reinhardtii*. Alternatively, this CA may maintain the equilibrium between CO₂ and HCO₃⁻ at the outer site of thylakoid membrane depending on cytoplasmic pH. As we have shown previously, the cytoplasmic pH of *R. lineare* remains near 7.5 [12].

Excess of light caused a 2-fold decrease in the intracellular pool of C_i in *R. lineare* with subsequent recovery to the initial level within 6 days of cultivation (Fig. 5B), while the amount of β-CA-PS II (60 kDa) in cyanobacterial cell remained at its minimum level (Fig. 5C). Thus, it is likely that C_i level is not associated with activity of β-CA-PS II, and it might be restored due to the function of C_i transporting systems [26].

It is known that the intracellular pool of C_i in cyanobacterial cells is represented by HCO₃⁻ in accordance with its pH. In carboxysomes, it is converted to CO₂ by the intracellular CA. In its turn, CO₂ is fixed by Rubisco [2]. This cooperation of CA and Rubisco in carboxysomes is very important, because the increase in intracellular concentration of CO₂ may lead to leakage of CO₂ from the cell. However, some cyanobacterial genomes lack DNA sequences similar to standard carboxysomal CA such as *icfA* and *ccaA* [26]. This fact assumes the existence of some other HCO₃⁻/CO₂ conversion mechanism. In *R. lineare* cells, such a mechanism might operate through the location thylakoid CAs of β-class. The results of immunoelectron microscopy reveal that thylakoid membranes of *R. lineare* are situated near the carboxysomes (Fig. 2).

Reduction of bicarbonate in the culture medium caused a rapid drop in the intracellular pool of C_i with its transient increase (Fig. 6B). The latter event was accompanied by the synthesis of a new form of the soluble β-CA of 25 kDa (Fig. 6C; Fig. 7). It should be noted that under low exogenous concentration of bicarbonate, the ability to uptake C_i remains high, while the total measured activity of CA seems to be negligible (Fig. 6). Strong correlation between the dynamics of the enzyme activity, enzyme amount and the intracellular pool of C_i within initial several days of bicarbonate limitation suggests the participation of this CA in C_i-concentration of *R. lineare*, although the exact localization of this form of CA remains to be identified. We suggest that the later steps of the C_i uptake might be maintained by the induced specific C_i transporters [26].

In any case, the 25 kDa β-CA of *R. lineare* seems to act as a stress-induced protein, which is induced due to exogenous bicarbonate limitation and a subsequent increase in the intracellular pool of C_i.

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References

- [1] D. Hewett-Emmett, R.E. Tashian, Functional diversity, conservation and convergence in the evolution of α-, β- and γ-carbonic anhydrase gene families, *Mol. Phylogenet. Evol.* 5 (1996) 50–77.
- [2] A. Kaplan, L. Reinhold, CO₂ concentrating mechanism in photosynthetic microorganisms, *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 50 (1999) 539–570.
- [3] K.S. Smith, J.G. Ferry, Prokaryotic carbonic anhydrases, *FEMS Microbiol. Rev.* 24 (2000) 335–366.
- [4] E. Soltes-Rak, M.E. Mulligan, J.R. Coleman, Identification and characterization of gene encoding a vertebrate-type carbonic anhydrase in cyanobacteria, *J. Bacteriol.* 179 (1997) 769–774.
- [5] E.V. Kupriyanova, A.G. Markelova, N.V. Lebedeva, L.M. Gerasimenko, G.A. Zavarzin, N.A. Pronina, Carbonic anhydrase of the alkaliphilic cyanobacteria *Microcoleus chthonoplastes*, *Microbiology (Moscow)* 73 (2004) 307–311.
- [6] H.E. Fukuzawa, Y. Suzuki, Y. Komukai, S.A. Miyachi, Gene homologous to chloroplast carbonic anhydrase (*icfA*) is essential to photosynthetic carbon fixation in the cyanobacterium *Synechococcus* PCC 7942, *Proc. Natl. Acad. Sci. U. S. A.* 89 (1992) 4437–4441.
- [7] A.K. So, G.S. Espie, Cloning, characterization and expression of carbonic anhydrase from the cyanobacterium *Synechocystis* PCC 6803, *Plant Mol. Biol.* 37 (1998) 205–215.
- [8] A.K.C. So, H.G.C. Van Spall, J.R. Coleman, G.S. Espie, 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 (1998) 1153–1160.
- [9] G.D. Price, D. Sultemeyer, V. Klughammer, M. Ludwig, M.R. Badger, The functioning of the CO₂ concentrating mechanism in several cyanobacterial strains: a review of general physiological characteristics, genes, proteins, and recent advances, *Can. J. Bot.* 76 (1998) 973–1002.
- [10] L.M. Gerasimenko, G.A. Zavarzin, Relict cyanobacterial communities, *Problems of the Evolution of Primary Components of Biosphere*, (1993), pp. 221–252, Nauka, Moscow.
- [11] A.V. Dubinin, L.M. Gerasimenko, G.A. Zavarzin, Ecological physiology and species variety of cyanobacteria from Magadi lake, *Microbiology (Moscow)* 64 (1995) 845–849.
- [12] E.V. Kupriyanova, N.V. Lebedeva, M.V. Dudoladova, L.M. Gerasimenko, S.G. Alekseeva, N.A. Pronina, G.A. Zavarzin, Carbonic anhydrase activity of alkaliphilic cyanobacteria from soda lakes, *Russ. J. Plant Physiol.* 50 (2003) 598–606.
- [13] R.Y. Stanier, R. Kunisawa, M. Mandel, G. Cohen-Bazire, Purification and properties of unicellular blue-green algae (order *Chroococcales*), *Bacteriol. Rev.* 35 (1971) 171–205.
- [14] K.D. Allen, L.A. Stachelin, Polypeptide composition assembly and phosphorylation patterns of photosystem II antenna system of *Chlamidomonas reinhardtii*, *Planta* 194 (1994) 42–54.
- [15] H. Shim, J. Cao, G. Debrunner, P.G. Debrunner, Purification of high active oxygen-evolving photosystem II from *Chlamydomonas reinhardtii*, *Photosynth. Res.* 26 (1990) 223–228.

- [16] K.M. Wilbur, N.G. Anderson, Electrometric and colorimetric determination of carbonic anhydrase, *J. Biol. Chem.* 176 (1948) 147–154.
- [17] D. Lowry, N. Rosebrough, A.L. Farr, R.T. Randall, Protein measurement with the Folin phenol reagent, *J. Biol. Chem.* 193 (1951) 265–275.
- [18] R.J. Porra, W.A. Thompson, P.E. Kriedemann, Determination of accurate extinction coefficients and simultaneous equation for assaying chlorophyll a and b with four different solvents: verification of the concentration of chlorophyll by atomic absorption spectroscopy, *Biochim. Biophys. Acta* 975 (1998) 384–394.
- [19] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of the bacteriophage T₄, *Nature* 227 (1970) 680–685.
- [20] J. Karlsson, A.K. Clarke, Z.Y. Chen, S.Y. Huggins, Y.I. Park, H.D. Husic, J.V. Moroney, G. Samuelsson, A novel α -type carbonic anhydrase associated with the thylakoid membrane in *Chlamydomonas reinhardtii* is required for growth at ambient CO₂, *EMBO J.* 17 (1998) 1208–1216.
- [21] T. Hiltunen, N. Bjorkbacka, C. Forsman, A.K. Clarke, G. Samuelsson, 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 (1998) 1341–1349.
- [22] A.G. Markelova, M.G. Vladimirova, V.E. Semenenko, Ultrastructural localization of Rubisco from algal cells, *Russ. J. Plant Physiol.* 37 (1990) 907–911.
- [23] A. Kaplan, M.R. Badger, J.A. Berry, Photosynthesis and the intracellular inorganic carbon pool in the blue-green alga *Anabaena variabilis*: response to external CO₂ concentration, *Planta* 149 (1980) 219–226.
- [24] K. Aizawa, S. Miyachi, Carbonic anhydrase and CO₂-concentrating mechanism in microalgae and cyanobacteria, *Fed. Eur. Microbiol. Soc. Microbiol. Rev.* 39 (1986) 215–233.
- [25] J.V. Moroney, S.G. Bartlett, G. Samuelsson, Carbonic anhydrase in plants and algae, *Plant Cell Environ.* 24 (2001) 141–153.
- [26] M.R. Badger, G.D. Price, CO₂ concentrating mechanisms in cyanobacteria: molecular components, their diversity and evolution, *J. Exp. Bot.* 54 (2003) 609–622.
- [27] N.A. Pronina, S.I. Allakhverdiev, E.V. Kupriyanova, G.L. Klyachko-Gurvich, V.V. Klimov, Carbonic anhydrase in subchloroplast particles of pea plants, *Russ. J. Plant Physiol.* 49 (2002) 341–349.
- [28] Y.I. Park, J. Karlsson, I. Rojdestvenski, N. Pronina, V. Klimov, G. Oquist, G. Samuelsson, Role of a novel photosystem II-associated carbonic anhydrase in photosynthetic carbon assimilation in *Chlamydomonas reinhardtii*, *FEBS Lett.* 444 (1999) 102–105.
- [29] M. Mitra, S.M. Lato, R.A. Ynalvez, Y. Xiao, J.V. Moroney, Identification of a new chloroplast carbonic anhydrase in *Chlamydomonas reinhardtii*, *Plant Physiol.* 135 (2004) 173–182.
- [30] N.A. Pronina, V.V. Borodin, CO₂-stress and CO₂-concentration mechanism: investigation by means of photosystem-deficient and carbonic anhydrase-deficient mutants of *Chlamydomonas reinhardtii*, *Photosynthetica* 28 (1993) 515–522.
- [31] Y.K. Lu, A.J. Stemler, Extrinsic photosystem II carbonic anhydrase in maize mesophyll chloroplasts, *Plant Physiol.* 128 (2002) 643–649.
- [32] A. Villarejo, T. Shutova, O. Moskvina, M. Forssen, V.V. Klimov, G. Samuelsson, A photosystem II-associated carbonic anhydrase regulates the efficiency of photosynthetic oxygen evolution, *EMBO J.* 21 (2002) 1930–1938.