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EXPERIMENTAL ARTICLES

Carbonic Anhydrase of the Alkaliphilic Cyanobacterium *Microcoleus chthonoplastes*

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Abstract—The activity of carbonic anhydrase (CA) was studied in different cell fractions of the alkaliphilic cyanobacterium *Microcoleus chthonoplastes.* The activity of this enzyme was found in the soluble and membrane protein fractions, as well as in intact cells and in a thick glycocalyx layer enclosing the cyanobacterium cells. The localization of CA in glycocalyx of *M. chthonoplastes* was shown by western blot analysis and by immunoelectron microscopy studies with antibodies to the thylakoid CA from *Chlamydomonas reinhardtii* (Cah3). At least one of the CA forms occurring in *M. chthonoplastes* CA was shown to be an α-type enzyme. A possible mechanism of the involvement of the glycocalyx CA in calcification of cyanobacteria is discussed.

Key words: alkaliphilic cyanobacteria of soda lakes, *Microcoleus chthonoplastes*, carbonic anhydrases, glycocalyx, calcification.

The foundations of the modern-type biosphere were laid about 2 billion years ago, when the Earth was dominated by bacteria [1]. It is at that time that a structural change in the geosphere–biosphere system occurred that opened the way to its evolvement to the present-day state. The major phases in the evolution of the biosphere over the last 3.8 billion years of the Earth's history have been outlined by geologists and paleontologists on the basis of the available geological sediment record. The prokaryotic biosphere phase is evidenced by the occurrence in sedimentary rock deposits of stromatolites, which are shell-type limestone deposits of fossil cyanobacterial communities. The early stage of the Earth's evolution was characterized by a paramount geochemical process—the global sink of carbon dioxide—consisting in binding of carbon dioxide to carbonates and above all calcium. A major part in this process was played by ancient cyanobacteria involved in calcification, i.e., binding of inorganic calcium to carbonates followed by their mineralization.

It was suggested [1, 2] that the enzyme carbonic anhydrase (CA, carbonate hydrolyase, EC 4.2.1.1) could be involved in calcification in cyanobacteria and eukaryotic algae by catalyzing the reversible reaction of carbon dioxide hydration according to the equation $CO_2 + H_2O \longleftrightarrow HCO_3^- + H^+$. One well-studied function of CA in photosynthetic microorganisms is its participation in the CO_2–concentrating mechanism [4–7], allowing the photosynthetic efficiency to be increased by saturating ribulose bisphosphate carboxylase (RuBPC) with carbon dioxide occurring at low concentrations in the environment. Meanwhile, it has often been claimed recently the biological significance of CA could be much wider and more diverse. CA is believed to be implicated in regulating the supply and removal of the substrates CO_2 and HCO_3^-, utilized in many carboxylation and decarboxylation processes, and in the ion transport associated with bicarbonate transport. In addition, CA was shown to contribute to the formation of the calcareous skeleton in corals [8] and to deposition of calcium scales in coccolithophorides [9].

Analysis of genomic sequences in a wide range of prokaryotes [10–12] showed that carbonic anhydrases are very ancient enzymes and existed even before the division of Archaea and Bacteria. According to the contemporary taxonomy, all CAs fall into three major classes (α, β, and γ), the amino acid sequences of which are not homologous and which are supposed to have evolved independently [10]. The enzymes of the γ and β classes are widespread in Bacteria and Archaea [10–12]. The vertebrates are known to have only the α class of CA, represented in man by ten isoforms [10]. Cyanobacterial cells were found to have all three CA classes [12]. It can be hypothesized that these enzymes of ancient cyanobacteria had an important role to play in the global sink of carbon dioxide coupled with stromatolite formation in the Precambrian. The organiza-

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Activity distribution of carbonic anhydrase (CA) in cell fractions of *Microcoleus chthonoplastes*

Fraction	CA activity, arb. units/mg chlorophyll	CA activity, arb. units/mg protein in fraction
Homogenate	737 ± 53	26 ± 5
Soluble protein	676 ± 47	48 ± 3
Membrane protein	56 ± 8	4 ± 1
Intact cells	132 ± 6	5 ± 0.2
Glycocalyx	–	66 ± 12

tion of the CA system in representatives of relict communities has not been studied until now. This issue, however, has important implications for understanding not only the geochemical process that unfolded on the Earth in the long past but also the evolution of the three CA classes.

The goal of this work was to study the CA activities and localization in one member of such communities—the alkaliphilic cyanobacterium *Microcoleus chthonoplastes*. The focus of our attention was on finding an extracellular form of the enzyme that can participate in calcification.

MATERIALS AND METHODS

The object of study was the alkaliphilic cyanobacterium *Microcoleus chthonoplastes* from the collection of the Institute of Microbiology, Russian Academy of Sciences. The culture was isolated from the Khilganta soda lake, Chita oblast, Russia [13].

The culture was grown under sterile conditions on the alkaline medium S [13] containing 17 g/l carbonates, pH 9.5–9.8, at 20°C, radiated by luminous tubes at 30 W/m^2.

Cells were disintegrated in a phosphate buffer composed of Na_2HPO_4, 0.06 M; cysteine, 5 mM; and EDTA, 1 mM; pH 8.1; in the cold in a Retch-MMW homogenizer (Germany) containing glass beads in a 1 : 1 ratio to the buffer volume. The obtained homogenate was fractionated by centrifuging at 18000 *g* for 40 min [14] into soluble proteins and insoluble cell components consisting of cell membranes.

Glycocalyx was isolated by passing homogenate through a capron filter. The material trapped on the filter was washed repeatedly in the phosphate buffer to remove membranes and soluble protein. The completeness of glycocalyx separation from the cell material was tested by measuring the chlorophyll content of the isolated fraction.

The CA activity was determined by the electrometric method using an M-901 pH meter (Orion, USA) to measure the concentration change of H^+ in the reaction of carbon dioxide hydration [14]. The reaction mixture (2 ml) contained intact cells washed off the culture medium, the entire homogenate, or its fractions (0.1–0.5 mg protein) in the phosphate buffer. The reaction was carried out at 2°C and started with a fast introduction of a saturated solution of CO_2 into an equal volume of the reaction mixture. The CA activity was determined in relative units as proposed by Wilbur and Anderson per one milligram of chlorophyll or protein from the observed change in the initial reaction rate. The nonenzymatic reaction was used as a control. Reaction rate measurements were replicated three to five times.

Electrophoretic separation of polypeptides was carried out under denaturing conditions in 12% polyacrylamide gel [3].

The western blot analysis was performed according to the standard Bio-Rad Laboratories protocol. Antibodies to the CA encoding the CA α-type (Cah3) in *C. reinhardtii* [6] were kindly provided by Prof. G. Samuelsson (Umea University, Sweden).

The protein content was determined by the Lowry method. Chlorophyll was determined spectrophotometrically in methanol extracts [3, 14].

The electron microscopic immunochemical analysis of carbonic anhydrase localization in cells of *M. chthonoplastes* followed the procedure described in [15]. In the immunocytochemical reaction, carried out upon material fixing with 4% formalin, the same antibodies to α-CA (Cah3) from *C. reinhardtii* as in western blotting and Protein-A-Gold (Sigma) were used. Ultrathin sections were examined in a JEOL X-100 microscope (Japan) without any additional contrasting.

RESULTS

High CA activity was observed in the entire cell-free homogenate of *M. chthonoplastes* (table), comparable in strength to enzymatic activities in eukaryotic cells of microalgae [7, 11, 12, 16]. The CA activity calculated per unit total chlorophyll differed with the cell fraction and was higher in the soluble protein fraction than in the membrane fraction. Calculated per unit protein of the isolated fraction, the activity of membrane-bound CA in cells of *M. chthonoplastes* was as low as 8% of the CA activity in the soluble fraction. The enzyme activity was also detected in intact undisrupted cells, indicating the presence of extracellular CA having access to external substrate. A particularly high activity of the enzyme (per unit protein) was found in glycocalyx (gCA) of *M. chthonoplastes*, which is composed mostly of lipopolysaccharides but also contains about 4.5% of the total cell protein.

By western blotting the entire cell homogenate and the glycocalyx preparation isolated from *M. chthonoplastes* with polyclonal antibodies to α-CA (Cah3) from *C. reinhardtii*, the occurrence of a single protein strip in

both samples was established (Fig. 1). The CA signal corresponds to a molecular mass of about 20 kDa.

The location of carbonic anhydrase in the glycocalyx is also confirmed by immunoelectron microscopy of cell sections and glycocalyx preparations from *M. chthonoplastes* using the same antibodies to α-CA (Cah3) from *C. reinhardtii* (Fig. 2). In the presented micrographs, electron-dense colloid gold markers are clearly visible only in the polysaccharide capsule enclosing the cell (Fig. 2a). Multiple particles of colloid gold, indicating the presence of CA, can also be seen in the isolated glycocalyx preparations (Fig. 2b), which, as evidenced by the micrograph, contain no residues of thylakoid membranes. The specificity of the immunochemical reactions was confirmed by controls (no serum specific to α-CA), in which gold particles were never observed.

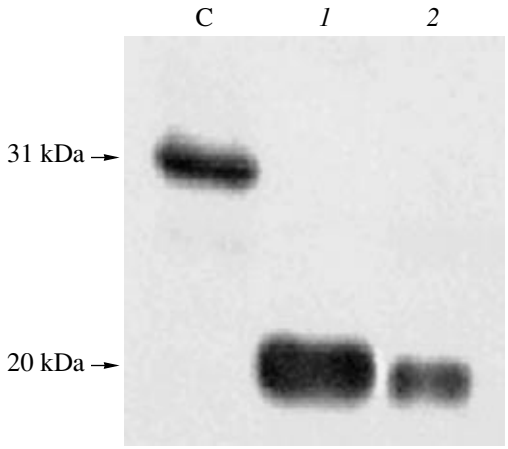

Fig. 1. Western blot assay of protein fractions from *M. chthonoplastes*: (*1*) total cell protein and (*2*) glycocalyx. (C) *C. reinhardtii* homogenate serving as control. Each lane contained 20 μg protein. The immunohybridization reaction was carried out with antibodies to the thylakoid α-CA (Cah3) from *C. reinhardtii.*

DISCUSSION

Alkaliphilic cyanobacterial communities could be relicts of ancient mainland biota persisting under extreme soda lake conditions [17]. These organisms are believed to have played an important part in the development of the biosphere in early stages of the Earth's evolution [1]. By binding large amounts of CO_2 in carbonates, above all, calcium, and releasing oxygen in photosynthesis, they radically changed the relative abundances of gas components of the atmosphere and made it oxygenic. This gave rise to a major change in the entire geosphere–biosphere system and allowed new forms of life other than prokaryotes to emerge. The participation of ancient cyanobacteria in carbonate sedimentation is attested to by the occurrence in sedimentary deposits of fossil microorganisms strongly resembling present-day cyanobacteria and by the calcification capacity of relict organisms represented in part by alkaliphilic communities of soda lakes [1, 18].

CA was previously implicated in the construction of the calcareous skeleton in the eukaryotic unicellular alga *Emiliania huxleyi* [9]. The calcareous coccolith is first formed in intracellular cisterns of *E. huxleyi* and then emerges to the cell surface to form its external skeleton. In corals, CA also operates in the intracellular medium [8]. In cyanobacteria, calcium and magnesium

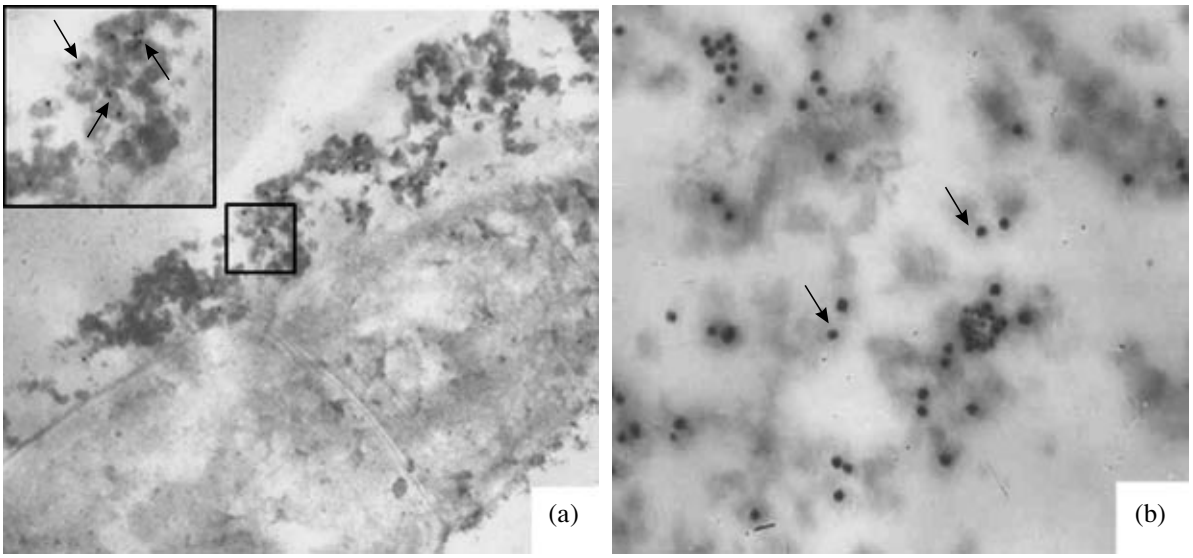

Fig. 2. Electron immunochemical assay of carbonic anhydrase localization in cells of *M. chthonoplastes*: (a) a cell section of *M. chthonoplastes* and (b) an isolated glycocalyx preparation. (The immunocytochemical reaction was carried out using antibodies to the thylakoid α-CA (Cah3) from *C. reinhardtii* and Protein-A-Gold (Sigma).)

carbonates are produced outside the cell by the mechanism of ciscalcification [2, 19], which, therefore, implies the action of an extracellular CA. In benthic cyanobacteria, to which *M. chthonoplastes* belongs, this takes place in a glycocalyx enveloping the entire population of cyanobacteria.

In this work, the localization of CA in glycocalyx of the typical mat-former *M. chthonoplastes* was first shown. The occurrence of extracellular CA in this cyanobacterium is evidenced by the activity of CA found in intact cells and in the glycocalyx preparations isolated from *M. chthonoplastes* (table). By using western blot tests (Fig. 1) with antibodies to α-CA, a homology between gCa and Cah3 was shown, suggesting a structural similarity between the gCa in *M. chthonoplastes* and the thylakoid CA in *C. reinhardtii.* The localization of carbonic anhydrase in glycocalyx is fully supported by the results of electron microscopic immunochemical localization analysis performed with the same antibodies to Cah3 from *C. reinhardtii* (Fig. 2). In the light of the symbiotic theory, the established structural similarity between the thylakoid Cah3 in *C. reinhardtii* and gCA in *M. chthonoplastes* looks even more interesting.

The presence of an extracellular α-type CA (EcaA) was previously shown in only two cyanobacteria—*Anabaena* sp. PCC 7120 and *Synechococcus* sp. PCC 7942 [20]. Using the cytoimmunological method, the extracellular CA was shown to be localized in cells of these cyanobacteria in the periplasmic space. At the same time, the authors of the cited work, having isolated the CA gene (*ecaA*), failed to detect the activities of the enzyme either in entire cells or in any of the protein fractions of these cyanobacteria. They explained this fact by a low enzyme activity in cyanobacterial cells and by the low sensitivity of the electrometric method employed to measure the CA activity.

Based on the data obtained, the following model of the gCA role in calcification in *M. chthonoplastes* and the relation of this process with photosynthesis was proposed. Given that the soda lake pH is normally 9–10, most inorganic carbon dissolved in water occurs in the form of bicarbonate ions. It is this form of carbon that predominantly makes its way into the cell to be eventually taken up in photosynthesis. Part of the bicarbonate flowing into the cell is converted spontaneously or by the action of intracellular CA to carbon dioxide, which can escape the cell by the pH gradient. The CO_2 draining from the cell acidifies the enclosing medium, as a result of which this cyanobacterium becomes coated in a layer having a lower pH than the surrounding medium. The CA enzyme located in the glycocalyx (gCA) participates in establishing a new balance between CO_2 and HCO_3^- and facilitates the production of one or several substrates for bicarbonate carriers. The Ca^{2+} ions present in the near-cell space can combine with bicarbonate ions to produce $CaCO_3$ minerals. The occurrence of CA in glycocalyx of *M. chthonoplastes* established in this work and the activity peaks of extracellular CA at pH around 7 and 10, which we showed elsewhere [3], are evidence in favor of this hypothesis.

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REFERENCES

1. Zavarzin, G.A., Rise of the Biosphere, *Vestn. Ross. Akad. Nauk*, 2001, vol. 71, no. 11, pp. 988–1001.
2. Zavarzin, G.A., Microbial Geochemical Calcium Cycle, *Mikrobiologiya*, 2002, vol. 71, pp. 5–22.
3. Kupriyanova, E.V., Lebedeva, N.V., Dudoladova, M.V., Gerasimenko, L.M., Alekseeva, S.G., Pronina, N.A., and Zavarzin, G.A., Activity of Carbonic Anhydrases of Alkaliphilic Cyanobacteria of Soda Lakes, *Fiziol. Rast.*, 2003, vol. 50, no. 1, pp. 14–22.
4. Pronina, N.A. and Borodin, V.V., CO_2-Stress and CO_2-Concentration Mechanism: Investigation by Means of Photosystem-Deficient and Carbonic Anhydrase-Deficient Mutants of *Chlamydomonas reinhardtii, Photosynthetica*, 1993, vol. 28, pp. 515–522.
5. Kaplan, A. and Reinhold, L., CO_2 Concentrating Mechanism in Photosynthetic Microorganisms, *Annu. Rev. Plant Mol. Biol.*, 1999, vol. 50, pp. 539–570.
6. Park, Y., Karlsson, J., Rojdestvenski, I., Pronina, N., Klimov, V., Oquist, G., and Samuelsson, G., Role of a Novel Photosystem II–Associated Carbonic Anhydrase in Photosynthetic Carbon Assimilation in *Chlamydomonas reinhardtii, FEBS Lett.*, 1999, vol. 444, pp. 102–105.
7. Badger, M., CO_2 Acquisition, Concentration and Fixation in Cyanobacteria and Algae, *Photosynthesis: Physiology and Metabolism*, Leegood, R.C., Sharkey, T.D., and Caemmerer, S., Eds., Dordrecht: Kluwer Academic, 2000, pp. 369–397.
8. Lucas, J.M. and Knapp, L.K., A Physiological Evaluation of Carbon Sources for Calcification in the Octocoral *Leptogordia virgulata* (Lamark), *J. Exp. Biol.*, 1997, vol. 200, pp. 2653–2662.
9. Numer, N.A., Guan, Q., and Merrett, M.J., Extra- and Intra-Cellular Carbonic Anhydrase in Relation to Culture Age in a High-Calcifying Strain of *Emiliania huxleyi* Lohmann, *New Phytol.*, 1994, vol. 126, pp. 601–607.
10. Hewett-Emmett, D. and Tashian, R.E., Functional Diversity, Conservation and Convergence in the Evolution of α-, β-, and γ-Carbonic Anhydrase Gene Families, *Mol. Phylogenet. Evol.*, 1996, vol. 5, pp. 50–77.
11. Smith, K.S., Jakubzick, C., Whittam, T.S., and Ferry, J.G., Carbonic Anhydrase Is an Ancient Enzyme Widespread in Prokaryotes, *Proc. Natl. Acad. Sci. USA*, 1999, vol. 96, pp. 15184–15189.

12. Smith, K.S. and Ferry, J.G., Prokaryotic Carbonic Anhydrases, *FEMS Microbiol. Rev.*, 2000, vol. 24, pp. 335–366.

13. Gerasimenko, L.M., Dubinin, A.V., and Zavarzin, G.A., Alkaliphilic Cyanobacteria of Tuva Soda Lakes and Their Ecophysiology, *Mikrobiologiya*, 1996, vol. 65, pp. 844–849.

14. Pronina, N.A. and Semenenko, V.E., Localization of Membrane-Bound and Soluble Forms of Carbonic Anhydrase in Chlorella Cells, *Fiziol. Rast.* (Moscow), 1984, vol. 31, pp. 241–251.

15. Markelova, A.G., Vladimirova, M.G., and Semenenko, V.E., Ultrastructural Localization of RuBPC in Algal Cells, *Fiziol. Rast.* (Moscow), 1990, vol. 37, pp. 907–911.

16. Graham, D. and Smillie, R.M., Carbonate Dehydratase in Marine Organisms of the Great Barrier Reef, *Aust. J. Plant Physiol.*, 1976, vol. 3, pp. 113–119.

17. Zavarzin, G.A., Epicontinental Soda Lakes as Possible Biotopes for Terrestrial Biota Formation, *Mikrobiologiya*, 1993, vol. 62, pp. 789–800.

18. Rozanov, A.Yu., Fossil Bacteria and a New Approach to the Processes of Sedimentogenesis, *Sorosovskii Obrazovatel'nyi Zh.*, 1999, no. 10, pp. 63–67.

19. *Microbial Sediments*, Riding, R.E. and Awramik, S.M., Eds., Berlin: Springer, 2000.

20. Soltes-Rak, E., Mulligan, M.E., and Coleman, J.R., Identification and Characterization of Gene Encoding a Vertebrate-Type Carbonic Anhydrase in Cyanobacteria, *J. Bacteriol.*, 1997, vol. 179, pp. 769–774.