DOI: 10.1007/s11099-017-0685-4 PHOTOSYNTHETICA 55 (1): 3-19, 2017

REVIEW

Carbonic anhydrase – a universal enzyme of the carbon-based life

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Abstract

Carbonic anhydrase (CA) is a metalloenzyme that performs interconversion between CO_2 and the bicarbonate ion (HCO_3^-). CAs appear among all taxonomic groups of three domains of life. Wide spreading of CAs in nature is explained by the fact that carbon, which is the major constituent of the enzyme's substrates, is a key element of life on the Earth. Despite the diversity of CAs, they all carry out the same reaction of CO_2/HCO_3^- interconversion. Thus, CA obviously represents a universal enzyme of the carbon-based life. Within the classification of CAs, here we proposed the existence of an extensive family of CA-related proteins (γCA-RPs) – the inactive forms of γ-CAs, which are widespread among the Archaea, Bacteria, and, to a lesser extent, in Eukarya. This review focuses on the history of CAs discovery and integrates the most recent data on their classification, catalytic mechanisms, and physiological roles at various organisms.

Additional key words: active site; carbon metabolism; convergent evolution; distribution; functional role; inhibitor; activator; inorganic carbon concentration.

Introduction

"*A diversity of transformations in organic matter is determined by the fact that the basis of its structure is Carbon – the element of outstanding properties*" (Nichiporovich 1955).

Carbon is a key element of life on the Earth, being the basis of all bioorganic compounds. A chain of organic interconversion begins at the moment when the inorganic carbon (C_i) is fixed by autotrophs – the primary producers of organic matter in the biosphere – and continues in heterotrophic organisms. Decomposition of the organics closes this chain and releases carbon in order to enter into a new cycle of fixation and interconversion.

Dissolved in an aqueous solution, C_i may exist in various forms (carbonic acid, carbon dioxide, bicarbonate, and carbonate ions) in equilibrium (Eq. 1):

$$H_2CO_3 \leftrightarrows CO_2 + H_2O \leftrightarrows H^+ + HCO_3^- \leftrightarrows 2H^+ + CO_3^{2-} \quad (1)$$

The ratio of C_i form concentrations depends on the pH of the solution according to Henderson-Hasselbalch equation (Eq. 2).

$$pH = 6.3 + \log([HCO_3^-]/[CO_2]) \quad (2)$$

At pH 6.3, there are equal amounts of CO_2 and bicarbonate. Below this value, the equilibrium shifts toward the formation of carbon dioxide, whereas HCO_3^- dominates at alkaline pH. At pH above 10.3, C_i is mainly represented by the carbonate ion (Rabinowitch 1945).

Along with organic molecules, living cells always contain C_i, which serves either as a substrate for biochemical reactions, or as their end-products. Since the pH of physiological solutions is maintained at neutral values, the main forms of C_i in a living cell are presented by carbon dioxide and bicarbonate ions. Effective interconversion between these two forms of C_i is a guarantee to the successful flow of biochemical processes.

In living cells, acceleration of interconversion between CO_2 and HCO_3^- is ensured by the metalloenzyme carbonic anhydrase (CA, carbonate dehydratase, carbonate hydrolyase, EC 4.2.1.1), which catalyzes the following reversible reaction (Eq. 3):

$$CO_2 + H_2O \leftrightarrows H^+ + HCO_3^- \quad (3)$$

Interconversion between CO_2 and HCO_3^- may also occur nonenzymatically, although this process is relatively slow, according to biochemical standards: a value of rate constant for the noncatalyzed CO_2 hydration is ~ 0.037 s^{-1} at 25°C and an ionic strength of 0.2 (Khalifah 1971).

Received 30 September 2016, *accepted* 28 November 2016.
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Abbreviations: CA – carbonic anhydrase; CAI – carbonic anhydrase inhibitor; CA-RP – carbonic anhydrase-related protein; CCM – CO_2-concentrating mechanism; C_i – inorganic carbon compounds (CO_2 + HCO_3^-); hCA – human carbonic anhydrase; PSR – proton shuttle residue.
Acknowledgements: This work was supported by the grant from Russian Science Foundation No. 14-24-00020.

Rate constant of the reverse reaction (HCO_3^- dehydration) is about 20 [s^{-1}] at 25°C (Kern 1960). This may significantly limit the rate of biochemical reactions. CA functions as a protein catalyst providing multiple acceleration of CO_2/HCO_3^- interconversions. It is noteworthy that the CA is one of the fastest enzymes, which turnover number (k_{cat}) reaches the value of 10^6 [s^{-1}] at pH 9 and 25°C (Lindskog 1997).

CAs are found in all groups of living organisms. Despite the diversity of CAs, which differ in their amino acid sequences and in the architecture of the active center, they catalyze the same reaction. Obviously, this is a good example of convergent evolution. CA, being absolutely necessary for optimal functioning of any living organism, was created several times as a different proteins that evolved to perform identical function (Hewett-Emmett and Tashian 1996, Liljas and Laurberg 2000). Thus, CA is a universal enzyme of the carbon-based life. This is undoubtedly due to the key role of CA in the carbon cycle, which includes synthesis of various biomolecules from C_i, their turnover, and decomposition back to the C_i-containing substances.

In animals, CAs are involved into a wide range of physiological reactions including global catabolic respiration, where they participate in the removal of large CO_2 amounts from tissues to the external environment.

CAs of photosynthetic cells has an exceptional significance at ecological and biospheric levels. The participation of CA in photosynthesis of phytoplankton and land plants, which generate the entire atmospheric oxygen (Field *et al*. 1998, Coleman 2000), determines its role in the maintenance of the "Fifth Ocean" of the Earth. Another aspect of the ecological importance of aquatic photosynthetic organisms may consist in mediated regulation of ocean acidification by withdrawal of massive amounts of CO_2 from the global carbon cycle by its fixation into organic compounds, followed by disposal of the latter in the form of sediments.

The whole-biosphere value of CA is determined by its crucial role in the formation of modern atmosphere. It is thought that this event happened about 2 billion years ago due to the vital function of ancient microbiota (Zavarzin 1997, Blank and Sánchez-Baracaldo 2010). A drain of huge amounts of CO_2 (as a result of mineralization of cyanobacterial communities and formation of stromatolites) with simultaneous release of photosynthetic oxygen led to a radical change in the composition of the atmosphere. Both processes, mineralization and photosynthesis, have been mediated by CAs (Zavarzin 2002, Jansson and Northen 2010, Kupriyanova and Pronina 2011). This hypothesis has been recently supported by finding of active extracellular CAs in photosynthetic relict cyanobacteria that survive in extreme habitats (Kupriyanova *et al*. 2007, 2013a).

This review focuses on recent advances in CA studies: their classification, functioning, and physiological roles at all organismic levels – from Archaea to mammals.

The history of CA studies and the distribution of CAs in the organic world

The existence of CAs was first predicted theoretically. Since the nonenzymatic reaction of interconversion between CO_2 and bicarbonate proceeds rather slowly, it has been suggested that living organisms should have some catalytic factor for the effective functioning. The survey of such factor was concentrated on animal red blood cells, where it could accelerate the transformation of bicarbonate to CO_2 during gas exchange in lungs. In 1933, CA has been independently discovered by two research groups, who determined its activity and performed crude purification of the enzyme (Meldrum and Rounghton 1933, Stadie and O'Brien 1933). Further protein purification and biochemical characterization revealed that this animal enzyme carries Zn^{2+} in its active site (Keilin and Mann 1944).

The enzymatic activity of CAs from different organisms may differ significantly. In part, it depends on the stability of the enzyme and on isolation procedure. Thus, during the early studies of CAs in photosynthetic objects, the absence of its specific enzymatic activity was frequently reported. Later, however, the reasons of such negative results have been uncovered. First reason was inappropriate cultivation conditions, when CA was expressed at a relatively low level, whereby the enzyme activity was below the level of assay sensitivity. Another problem was the inadequate protein isolation buffer that inactivated the enzyme. Some CAs have low resistance to oxidizing agents, whereas others, in contrast, lose their functional activity in the presence of reduced agents. For example, before the discovery of the carboxysomal CA in cyanobacteria, its specific enzymatic activity in total cell extracts could not be detected despite the fact that the assimilation rate of C_i and photosynthetic affinity to C_i was inhibited by the specific inhibitor of CAs, ethoxyzolamide (Kaplan *et al*. 1980). Later it was found that dithiothreitol (DTT), a common antioxidant in enzyme isolation buffers, inactivates the carboxysomal CA (Price *et al*. 1992). Another reason for the failure in detection of CA activity could be the enzyme inhibition by intracellular compounds of total cell homogenate.

The existence of CAs in plants was initially questioned because of the aforementioned lack of the enzymatic activity in the early attempts to detect it. First plant CA was isolated from chloroplasts of *Trifolium pratense* and *Onoclea sensibilis* (Neish 1939). In support to the theory of endosymbiotic origin of Eukaryota, a gene encoding a chloroplast-like CA was later discovered in cyanobacteria (Fukuzawa *et al*. 1992). In 1963, the first report on a

bacterial CA appeared (Veitch and Blankenship 1963). In 1989, a specific enzyme activity of CA was found in archaea, *Methanosarcina barkeri* (Karrasch *et al*. 1989). A bit later, it was purified and characterized from a related organism, *Methanosarcina thermophila* (Alber and Ferry 1994). In 2005, a gene encoding fungal CA was cloned from *Saccharomyces cerevisiae* (Amoroso *et al*. 2005).

Currently, there is no known organism, which lives without CAs. The enzyme was found in representatives of all taxonomic groups of three life domains: Archaea, Bacteria, and Eukarya (Supuran 2016a). CAs are present in many (in some organisms – in all) organs, tissues, and cellular compartments. In addition, one organism or cell may have simultaneously several different CAs that perform different functions. CAs are widespread among various groups of organisms due to the variety of enzymatic reactions, in which CO_2 or bicarbonate are used as substrates or products. In addition, two other participants of the reversible CO_2 hydration, H^+ and H_2O, are also important for the maintenance of cellular homeostasis.

Barely a bit more than 80 years passed since first CA was discovered in 1933. Initially, all of the studies were mainly focused on biochemical properties and catalytic activity of animal enzymes. More detailed studies on CA structure and catalytic mechanisms appeared only after the development of X-ray crystallography and the methods of bioorganic chemistry and molecular cloning. Indeed, the complete primary amino acid sequences of two enzymes from human erythrocytes (hCA I and hCA II) were identified only in the early 1970s (Andersson *et al*. 1972, Henderson *et al*. 1973, Lin and Deutsch 1973, Lin and Deutsch 1974). Almost simultaneously, the crystal structure for hCA II has been determined (Liljas *et al*. 1972). However, catalytic mechanism of hCA II has been proposed only in 1988 based on the crystallographic data (Silverman and Lindskog 1988), 55 years after the discovery of the enzyme.

Despite the fact that higher plant chloroplast CAs were found just six years after the discovery of animal CAs, studies of structure and function of plant proteins are still far behind. However, over the past decades, a significant progress has been made in the study of microalgal and cyanobacterial CAs, whose participation in the process of photosynthesis is well documented (Giordano *et al.* 2005, Price *et al*. 2008, Kupriyanova *et al*. 2013b, Long *et al*. 2016). In addition, two new classes of CA were discovered in diatoms (Roberts *et al*. 1997, Lane *et al*. 2005). As for the CAs of bacteria and archaea, their structures and functions are currently available for some individual proteins (Kumar and Ferry 2014). Among those, the unique prokaryotic CAs have been discovered that can operate in anaerobic conditions (Alber and Ferry 1994, Zimmerman *et al*. 2010).

CA classes, their distribution and phylogeny

As mentioned above, CA was first isolated from mammalian red blood cells, and early assays of the enzymatic properties were carried out on animal proteins. After the discovery and subsequent investigations of plant CAs, it turned out that some of them are quite different from animal enzymes in their biochemical and catalytic properties. Compared to animal enzymes, the native chloroplastic CA of higher plants had higher molecular mass and lower sensitivity to the primary sulfonamides – classical inhibitors of CAs (Everson 1970, Atkins *et al*. 1972).

Once the partial amino acid sequence of spinach chloroplast CA was determined in 1984, it appeared that it has no significant homology with any of known mammalian enzymes (Hewett-Emmett *et al*. 1984). Following this, several cDNA sequences encoding chloroplastic CAs were cloned from other plants. Again, it appeared that these enzymes cannot be assigned to a group of vertebrate CAs (Hewett-Emmett and Tashian 1996). Thus, it became clear that, despite the common catalytic function, individual CAs may be structurally unrelated.

It should be noted that first amino acid sequences of CAs were determined by N-terminal amino acid sequencing according to Edman degradation method (Edman and Begg 1967), which has a number of limitations. After the invention of DNA sequencing method, which allows quick and precise determination of nucleotide sequences (Sanger *et al.* 1977), the information on sequences of CA genes and their corresponding proteins rapidly accumulated.

The current databases, such as the database of NCBI (http://www.ncbi.nlm.nih.gov), make possible the analysis of relationships between all known CAs, as well as the reconstruction of the evolutionary pathways of the enzyme. At the same time, next generation sequencing technologies allow determination of nucleotides sequences at a whole-genome scale. This develops a fairly rapid rate of continuous accumulation of new information and provides a wealth of material for molecular systematics. Comparisons of newly determined nucleotide or amino acid sequences with already known CAs of organisms with completely or partially characterized genomes, allow searching and identification of all types of hypothetical CAs.

Currently, all known CAs are divided into six classes or families, referred to the Greek alphabet - α, β, γ, δ, ζ, and η (Supuran 2016a). This classification takes into account both the amino acid sequence of a protein, and its three-dimensional structure, organization of the active site, and catalytic properties. It is believed that α-, β-, and γ-CAs are convergently evolved, whereas δ-, ζ-, and η-CAs seem to be highly divergent variants of the β- or α-CAs, though constituting rather diverse genetic families of their own (Hewett-Emmett and Tashian 1996, Liljas and Laurberg 2000, Supuran 2016a). Another look at the CAs

molecular evolution, however, considers the origin α-, β- and γ-classes of CAs from a common ancestral gene (Smith *et al*. 1999).

Previously, an additional class of CA, ε-class, was described, which was represented by the only protein, CsoS3, which is a part of the carboxysomal shells of alpha-cyanobacteria and chemoautotrophic bacteria (So *et al*. 2004). Two years later, on the basis of crystallographic data, the same group of researchers attributed this CsoS3 to β-CAs, and renamed this protein as CsoSCA (Sawaya *et al*. 2006).

Many organisms express genes for CAs of several classes. Simultaneously, the same organism may have a different number of genes for the representatives of each individual CA class. But even within the same class, CAs may be represented by a number of (sometimes multiple) isoenzymes with different molecular features, oligomeric arrangement, cellular localization, distribution in organs and tissues, expression levels, kinetic properties, and responses to different classes of inhibitors (Alterio *et al*. 2012a).

α-CAs: Isolated from animal red blood cells, α-class representatives are historically the first described CAs. Most α-CAs are monomers with a molecular mass of about 30 kDa, although some human and bacterial enzymes were reported as homodimers (Supuran 2016a), and two periplasmic α-CAs (Cah1 and Cah2) from the green alga *Chlamydomonas reinhardtii* – as heterotetramers (Kamo *et al*. 1990). The folding of all α-CAs is highly similar possessing mainly antiparallel β-sheets as the dominating secondary structure (Fig. 1*A*).

α-CAs are considered as an evolutionarily youngest group, because they were found in mammals, including humans, and their distribution in prokaryotes is rather restricted (Smith and Ferry 2000). According to phylogenetic analysis, α-CAs have been evolved from a common ancestral gene ~ 0.5–0.6 billion years ago (Smith *et al*. 1999). Presumably, the parents of all α-CAs were extracellular enzymes associated with a cell membrane or localized outside a cytoplasmic membrane; whereas intracellular α-CAs appeared later. At least, all prokaryotic α-CAs seem to evolve from an ancestral extracellular form of the enzyme (Smith and Ferry 2000).

Currently the α-class covers all 16 mammalian CA isoforms with different organ/tissue distribution and subcellular location, 15 of which are present in humans (Alterio *et al*. 2012a, Supuran 2016a). α-CAs are also characteristic for higher plants and eukaryotic algae, some types of fungi and Protozoa, some bacteria and cyanobacteria (Elleuche and Pöggeler 2010, Supuran 2016a). In the Archaea, however, α-CAs have not been detected (Kumar and Ferry 2014).

β-CAs: The founders of β-class CAs are plant enzymes discovered after α-CA of mammals (Hewett-Emmett and Tashian 1996). β-CAs are much more diverse in their quaternary structure, representing oligomers – dimers, tetramers, and octamers with molecular masses ranging from 45 to 200 kDa with a dimer as the catalytically active unit (Rowlett 2014). The monomeric subunits, with molecular mass ranging from 25 to 30 kDa (Supuran 2004), are characterized by a unique α/β-fold that associates to form dimers. In some cases [for example, in red alga, *Porphyridium purpureum* (Fig. 1*B*), or in bacterium, *Halothiobacillus neapolitanus*] a fundamental structural unit appears to be a dimer equivalent: their monomers are represented by a single amino acid sequence with two identical repeats connected with a short polypeptide linker. The latter represent so-called pseudo-dimers (Mitsuhashi *et al.* 2000, Sawaya *et al.* 2006).

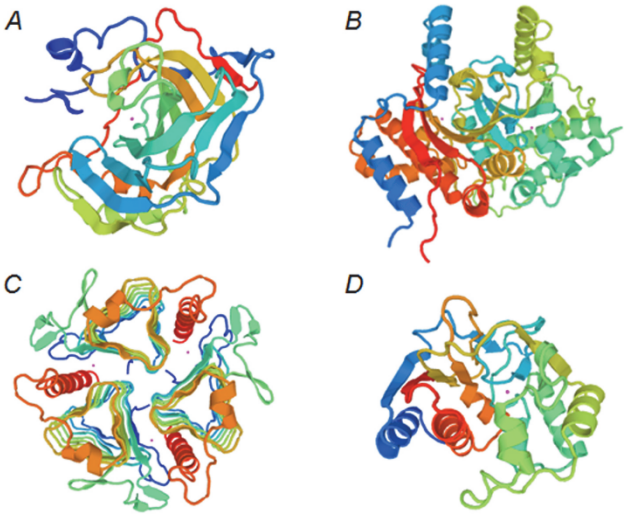

Fig. 1. The spatial structure of full-length representatives of different classes of CAs (α, β, γ, ζ) or their specific domains, for which the crystallographic data is currently available. (*A*) human α-CA II (SMTL 1g6v.1.A), a native monomer; (*B*) β-CA of red algae *Porphyridium purpureum* (SMTL 1ddz.1.A), homo-dimer of a native tetramer formed by a single amino acid sequence comprising two identical repeats joined by a polypeptide linker (*see* details in the text); (*C*) γ-CA Cam of the anaerobic archaea *Methanosarcina thermophila* (SMTL 1qq0.1), a native trimer. (*D*) R3 domain of ζ-CA CDCA of the diatom *Thalassiosira weissflogii* (SMTL 3uk8.1.A). Metal ions in the active centers of enzymes are shown as spheres. PDB files were obtained from the database of templates at https://swissmodel.expasy.org.

All β-CAs are divided into two types depending on the architecture of the active site (for details, please, refer to subsection "Active site and catalytic mechanism of CAs. Inhibitors and activators"). Nevertheless, the folding of the monomer units of both types of β-CAs is quite similar. All structural details of β-class CAs are recently reviewed by Rowlett (2014).

For quite a long time it was thought that the β-class of CAs consists exclusively of the enzymes found in the chloroplasts of higher plants, and these enzymes have been called as "plant CAs". However, since the 1990s, with

accumulation of new data, it became clear that this class of enzymes is fairly widespread. Unlike α-CAs, β-CAs have been found in all three domains of life – eukaryotes, bacteria, and archaea. Many organisms possess this class of enzymes, including microalgae, cyanobacteria, bacteria, fungi, archaea, higher C_3 and C_4 plants, and invertebrates (Rowlett 2014).

Phylogenetic analysis conducted by Smith and Ferry (2000) for 76 β-CAs of organisms, which belong to different domains, revealed that only monocotyledons and dicotyledons plants form monophyletic groups of β-CAs. The rest of β-CAs form four separate subgroups (clades). Two of these clades are formed by the enzymes of Eukarya and Bacteria. The other two clades are solely prokaryotic: one includes the enzymes from Gram-negative Bacteria only, and the other consists primarily of sequences from Archaea and Gram-positive species of Bacteria. The analysis indicates that there is a strong difference in the primary structure of β-CAs allocated to different clades, which correlates with the differences in their quaternary structure. This divergence complicates the phylogenetic evaluation of the appearance time of β-CAs. Nevertheless, the presence of these enzymes in the thermophilic Archaea – most ancient branch of the universal tree of life – suggests the ancient origin of β-CAs (Smith and Ferry 2000).

γ-CAs: In 1994, Cam protein with CA activity was isolated from methanogenic anaerobic archaea, *M. thermophila*. Amino acid sequence of Cam did not show any significant homology to known members of β- or α-classes of CAs (Alber and Ferry 1994). Thus, Cam protein has become the archetype of the third, γ-class, of CAs (Hewett-Emmett and Tashian 1996). The active form of the Cam is a homotrimer (Fig. 1*C*) with monomers of ~ 23 kDa that carry a distinctive left-handed parallel β-helix fold (Ferry 2010).

Cam homologues have been found in all three domains of life, although they were mostly widespread among Bacteria and Archaea (Ferry 2010, Kumar and Ferry 2014). In prokaryotes (archaea, bacteria, and cyanobacteria), γ-CAs have different subcellular localizations and perform different functions (Ferry 2010, Kumar and Ferry 2014). In Eukarya, γ-CAs are mainly located in mitochondria (Klodmann *et al*. 2010, Moroney *et al*. 2011).

At present, some additional γ-CAs have been characterized: CamH proteins from *M. thermophila* and from another anaerobic archaeon, *Pyrococcus horikoshii* (Jeyakanthan *et al*. 2008, Zimmerman *et al*. 2010), as well as two carboxysomal proteins, CcmM, from cyanobacteria *Thermosynechococcus elongatus* BP-1 and *Anabaena* sp. strain PCC 7120 (Peña *et al.* 2010, de Araujo *et al.* 2014). Strictly speaking, CcmM is a bifunctional protein with both structural and catalytic functions. It is a part of a multiprotein bicarbonate dehydration complex of β-cyanobacteria; and only the N-terminal domain of CcmM represents the γ-CA (Cot *et al.* 2008).

It is believed that the γ-CAs separated from the common ancestral branch ~ 3.0–4.5 billion years ago. Thus, this class of CAs, being the most ancient, existed before the divergence of domains Archaea and Bacteria ~ 2.2 billion years ago (Smith *et al*. 1999). The ancient origin of γ-class enzymes is confirmed by the presence of their active forms in anaerobic archaea. Phylogenetic analysis shows that all γ-CAs are divided into two subclasses, which are represented by Cam and CamH. CamH lacks a part of the amino acid sequence that is essential for the catalytic activity of Cam, although CamH is also an active CA. Thus, both proteins differ in the organization of the active site, and have a different mechanism of catalysis (Ferry 2010).

δ-CAs: In 1997, new CA of marine diatom *Thalassiosira weissflogii* was reported (Roberts *et al*. 1997). The enzyme of ~ 34 kDa was named as TWCA1. The structure and geometry of its active site was similar to that of α- and γ-CAs (Cox *et al*. 2000). However, the amino acid sequence of TWCA1 had no significant similarity to the representatives of three known CA classes, and, therefore, it was allocated as a new class, δ-CA (Tripp *et al*. 2001).

Later, other δ-CAs, homologous to TWCA1, have been found in some representatives of marine phytoplankton (Supuran 2016a). These δ-CAs are currently quite poorly studied. Indeed, the crystallographic data for δ-CAs are missing, and the exact structure of these proteins remains unknown. Although the first representative of δ-CAs was discovered almost 20 years ago (Roberts *et al*. 1997), the data on its physico-chemical, biochemical, and catalytic properties, as well as inhibitor assays, became available only recently (Del Prete *et al*. 2014a,c; Vullo *et al*. 2014).

ζ-CAs: The representative of ζ-CAs, also called cadmium-CAs, was also first isolated from the marine diatom, *T. weissflogii* (Lane and Morel 2000). Sea water is basically depleted by Zn^{2+} ions. Therefore, under strict Zn^{2+} limitation, the activity of Zn-required δ-CA TWCA1 sharply drops, and TWCA1 is replaced by another CA that employs Cd^{2+} ions in its active site. Such purified protein of 43 kDa was designated as CDCA (Lane and Morel 2000). After another 5 years, the amino acid sequence of CDCA and its exact molecular mass (69 kDa) have been determined, contradicting to an earlier estimate (Lane *et al*. 2005). CDCA had no significant homology to proteins of other known classes of CAs, and thus, was attributed to a new ζ-class of CAs. Later, genes encoding CDCA homologs have been found in several other diatoms (Park *et al*. 2007).

CDCA is a monomer, the amino acid sequence of which is composed of three repeats (R1-R3) exhibiting 85% identity to each other, while the coding DNA sequences demonstrate much smaller percentage of homology (Lane *et al*. 2005). Each of R1-R3 repeats comprises one active site (Fig. 1*D*), which structurally mimics that of β-CA type I (*see* below) (Xu *et al*. 2008, Alterio *et al*. 2012b).

ηCAs: This class of CAs was discovered recently (Del Prete *et al*. 2014b). A gene for this CA has been cloned ten years earlier from the protozoan parasite, *Plasmodium falciparum*, which causes human malaria. The corresponding protein was designated PFCA, and it was initially assigned to α-CA class (Reungprapavut *et al*. 2004). However, a closer look on the protein, as well as on other homologous enzymes from the other *Plasmodium* strains, revealed significant differences between PFCA and α-CAs.

In addition to differences in the inhibitor-analysis data, it turned out that the protozoan enzymes have certain unique conserved features in their amino acid sequences. These features theoretically implied the organization of the active site structurally different from that of α-CAs (Del Prete *et al*. 2014b). Computational approach confirmed this hypothesis and constructed a completely different pattern of metal ion coordination in the active site of the protozoan enzymes if compared to α-CAs (De Simone *et al*. 2015). Simultaneously, phylogenetic analysis indicated that the enzymes of *Plasmodium* spp. form a separate branch of CAs. On the basis of the aforementioned properties, the enzymes of PFCA group were assigned to the new class of η-CAs (Del Prete *et al*. 2014b).

At the moment, η-CAs are represented only by enzymes of the genus *Plasmodium*. Exact data on the structure of these proteins are not yet available because none of η-CAs have been crystallized. In general, η-CAs are represented by the polypeptides of about 30 kDa (similarly to α-CAs) that have significant specific activity (Del Prete *et al*. 2014b, Supuran 2016a).

Carbonic anhydrase-related proteins

CA-RPs: Carbonic anhydrase-related proteins (CA-RPs) are represented by the proteins homologous to α-CAs, but lacking the specific activity due to deletion or substitution of one to three His ligands that are necessary for coordination of Zn^{2+} ion in the active site of the enzyme (Aspatwar *et al*. 2014). CA-RPs appear as separate proteins or as domains of other proteins in animals and viruses. The phylogenetic analysis shows that this group of proteins is highly conserved across the species.

The function of CA-RPs in cells is not entirely clarified. It was shown that these proteins may be associated with motor coordination or certain types of cancers in animals. They may also coordinate the functions of other proteins through protein-protein interaction (reviewed by Aspatwar *et al*. 2014).

CA-RPs in DNA-containing viruses are of special interest. Modern classification of living organisms defines three main domains – Archaea, Bacteria, and Eukarya (Woese *et al*. 1990), and ignores Viruses considering them as noncellular infectious agents. Meanwhile, it was suggested that viruses represent a fourth domain of life (Nasir *et al*. 2012). Moreover, this form of life is preceded or coexisted with the last universal common ancestor (LUCA) of all living organisms on the Earth. The theory of Virus World suggests that the ancestral forms of viruses existed before the appearance of cellular life (Koonin and Dolja 2014).

According to current views, viruses acquired CA-RP genes, presumably evolved from animal CA XIII, by horizontal transfer (Aspatwar *et al*. 2014). This event took place not earlier than ~ 0.5–0.6 billion years ago when α-CAs evolved from a common ancestor (Smith *et al*. 1999). However, considering that phylogenetic analysis establishes evolutionary relationships only of those objects that have a common ancestor, such evaluation of the evolutionary appearance of different CA classes may be invalid.

The alternative theory of sovereign origin of different CA classes (at least, α, β, and γ), implies that these proteins appeared independently and ultimately evolved to a similar function (Hewett-Emmett and Tashian 1996, Liljas and Laurberg 2000, Supuran 2016a).

The presence of CA-RPs in viruses, which presumably represent the most ancient domain of life, raises the question about the origin of CAs evolution. Since CA-RPs do not reveal CA specific activity, their physiological roles remain unobvious. It is possible to assume, however, that CA-RPs could serve as a "starting point" of CAs evolution. The presence of α-CAs in cyanobacteria, whose ancestors already inhabited the Earth ~ 3.5 billion years ago (Soltes-Rak *et al*. 1997, Smith and Ferry 2000, Konhauser 2009), may also indicate much earlier origin of α-CAs than it is now predicted by phylogenetic analysis.

γCA-RPs: γ-CAs include a number of CamH homologs that are widespread among Archaea, Bacteria, and Eukarya (Fig. 2*A*). In databases, these proteins are usually annotated as hypothetical proteins or as ferripyochelin-binding proteins, and, rarely, as γ-CAs. These CamH-like proteins usually possess three His ligands required for Zn^{2+} coordination in the active site of the enzyme, as well as all other conserved catalytic residues, characteristic for γ-CAs, except those that correspond to Glu^{53} and Asn^{173} in CamH of *M. thermophila* (Fig. 2*B*). Thus, CamH-like proteins *a priori* cannot be active as CAs. Within their group, these proteins are up to 60% or even more identical.

It is noteworthy that CamH-like protein of *M. thermophila* (the source of two characterized γ-CAs, Cam, and CamH), has another amino acid substitution at the conservative asparagine corresponding to Asn^{65} of CamH.

CamH-like proteins are widespread among the Archaea and Bacteria including cyanobacteria. Within the Eukarya, CamH-like proteins are present only among photoautotrophs – eukaryotic microalgae and higher plants

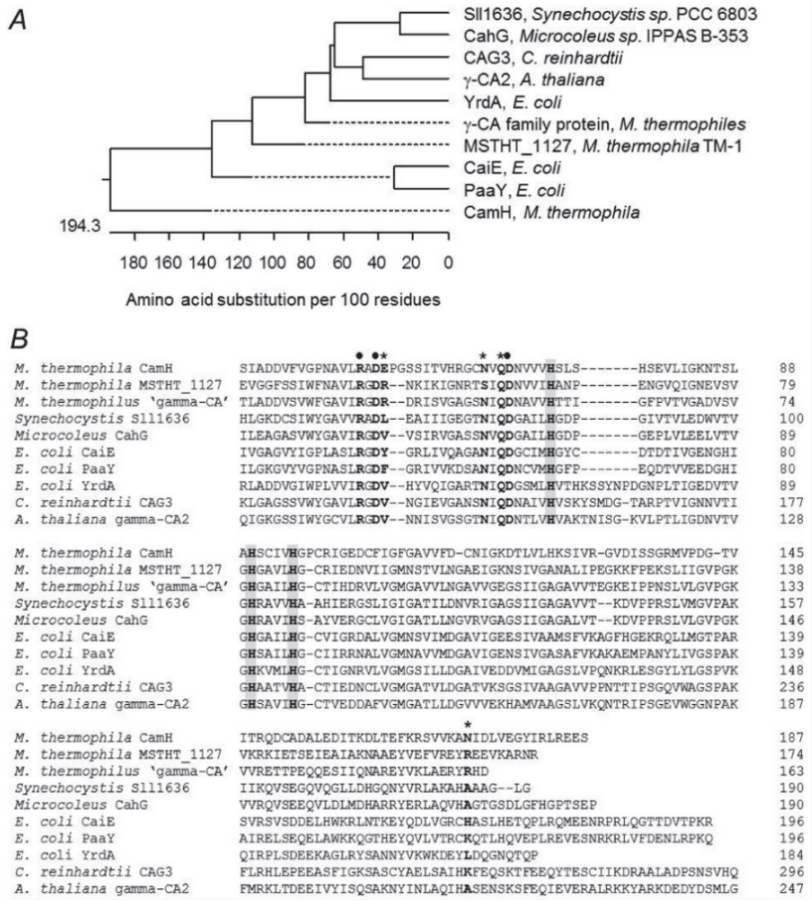

Fig. 2. Distribution of catalytically inactive γ-CAs (γCA-RPs), which are homologous to the active γ-CA CamH from the archaea *Methanosarcina thermophila*, among taxonomic groups of three domains of life. (*A*) Relationship of γCA-RPs based on phylogenetic analysis. (*B*) Partial alignment of amino acid sequences of γCA-RPs with γ-CA CamH of *M. thermophila* TM-1 (GenBank ACQ57353). The following γCA-RPs were used for the analysis: γ-CA family protein MSTHT_1127 of archaea *M. thermophila* TM-1 (NCBI WP_048166988); γ-CA family protein of archaea *Methanoculleus thermophiles* (NCBI WP_066958771); ferripyochelin-binding protein Sll1636 of freshwater cyanobacterium *Synechocystis* sp. PCC 6803 (NCBI WP_010871699, CyanoBase Sll1636); CahG (MBR2563) of alkaliphilic cyanobacterium *Microcoleus* sp. IPPAS B-353 (NCBI PRJNA203668; Kupriyanova *et al*. 2016); CaiE of *Escherichia coli* (NCBI WP_032314210); PaaY of *E. coli* (NCBI WP_001123466); YrdA of *E. coli* (GenBank ABE09161); CAG3 of green alga *Chlamydomonas reinhardtii* (NCBI XP_001696746); γ-CA2 of *Arabidopsis thaliana* (NCBI NP_175159). Positions of amino acids that are critical for the activity of CamH and that have replacements in γCA-RPs, are marked in bold according to Ferry (2010) and Zimmerman *et al*. (2010). Additionally, amino acids, which are ligands of metal ions in the active site, are shown in gray; amino acids involved in catalysis, are marked with an asterisk (*); amino acids necessary for the formation of the correct structure of the active site are marked with a point (•). Amino acid sequences of proteins were obtained from NCBI databases (http://www.ncbi.nlm.nih.gov) and *CyanoBase* of Kazusa DNA Research Institute (http://genome.kazusa.or.jp/cyanobase).The alignments of amino acid sequences as well as phylogenetic tree were performed with *Clustal W* algorithm of *Lasergene* module *MegAlign v. 10.1.2* software (*DNASTAR Inc.*).

(Fig. 2*A,B*). These proteins were not reported in animals, fungi, true yeasts, and protists.

At present, there is no hypothesis about the origin and evolution of CamH-like proteins. An extensive family of these proteins, in fact, is represented by CA-RPs, in terms of classification proposed for inactive forms of α-CAs (Aspatwar *et al*. 2014). Here, we propose to designate the family of noncatalytic CamH-like γ-CAs as γCA-RPs. Accordingly, the inactive forms of α-CA should be designated as αCA-RPs.

Well-studied γCA-RPs are the proteins of *Escherichia coli* – PaaY, YrdA, and CaiE. CaiE likely contributes to generation or regeneration of a coenzyme for the enzymes involved in the carnitine biosynthesis pathway (Merlin *et al*. 2003). PaaY has a thioesterase activity and participates in phenylacetic acid catabolism, exercising regulatory functions (Fernández *et al*. 2014a). Crystallographic data has been recently obtained for YrdA (Park *et al*. 2012), but physiological role of this protein is still unknown. The data on location and biological roles for other γ-CA-RPs are still missing.

Active site and catalytic mechanism of CAs: Inhibitors and activators

All CAs are metal-containing enzymes carrying one metal ion per one protein subunit and, thus, per active center. The metal ion in the active site of the enzyme is present in the form of doubly charged cation (Me^{2+}). To maintain the catalytic activity, all six classes of CAs may use Zn^{2+} as a metal ion. In ζ-CAs, however, Zn^{2+} is interchangeable with Cd^{2+} (Xu *et al*. 2008). γ-CAs may use Fe^{2+} under anaerobic conditions (Zimmerman *et al*. 2010) and retain the activity with Co^{2+} (Iverson *et al.* 2000). Co^{2+} may also substitute Zn^{2+} in many α-CAs without a significant loss of the catalytic activity (Supuran 2016a).

Crystallographic studies show that the active site of CAs is usually a cavity of tetrahedral geometry formed by three amino acid residues and one deprotonated water molecule (hydroxide ion) that operate as coordinating ligands for Me^{2+} (Fig. 3). In turn, the latter is bound to amino acids surrounding the active site by hydrogen bonds (Supuran 2016a).

Those ligands are strictly conserved within each class of CAs, as well as amino acids that are either involved in catalysis or formation of the correct structure of the active site (center). In α-, γ-, and δ-CAs, three ligands, which coordinate a Me^{2+} ion in the active center, are represented by three His residues, and the fourth ligand is a hydroxide ion. At least one exception is γ-CA Cam of *M. thermophila*, which uses an additional hydroxide ion to coordinate Me^{2+} (Iverson *et al*. 2000).

All β-CAs are divided into two types according to

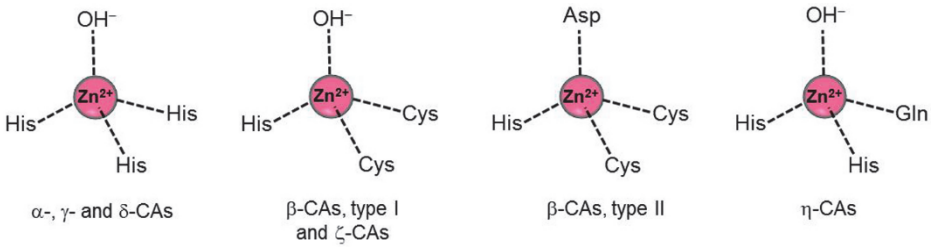

Fig. 3. A schematic representation of the active sites of different classes of CAs. The ligands are shown that coordinate a metal ion (Zn^{2+}) in the active site of enzymes.

nature of the fourth ligand in the active center and of neighboring amino acid residues (Rowlett 2014, Supuran 2016a). In β-CAs of type I (enzymes with so-called "open active site"), Me^{2+} is coordinated by one His, two Cys residues, and one hydroxide ion. β-CAs of type II (enzymes with "closed active site") employ Asp instead of hydroxide ion. These enzymes are active only at pH above 8.3 (Suarez Covarrubias *et al*. 2005). This particular environment favors a transition from "closed active site" into "open site", where a water molecule (hydroxide ion) occupies the position of the fourth ligand, which is necessary for catalysis (*see* below). Since a dimer formation is necessary for the catalysis in all β-CAs (Rowlett 2014), the individual catalytically active subunit has two active centers, each carrying Me^{2+}.

The structure of the active site of ζ-CAs is similar to that of type I in β-CAs (Xu *et al*. 2008).

In η-CAs, Me^{2+} is coordinated by two His and one Gln residues in addition to the hydroxide ion (De Simone *et al*. 2015).

The catalytic mechanism of CAs was first described for human α-CA II (Silverman and Lindskog 1988). The open catalysis mechanism was named as `Zn-hydroxide', as the catalytically active group of hCA II was the Zn-bound water ionized to a hydroxide ion. Metal ion by itself is not nucleophilic enough to act as a catalyst. Later it was found that all CA classes employ metal-hydroxide mechanism, using divalent metal hydroxide derivatives as catalytically active species (Supuran 2016a).

The catalysis is carried out in two stages (Boone *et al*. 2014). First stage involves the conversion of CO_2 into HCO_3^-. Metal hydroxide derivative in the active site of the enzyme (E) is a strong nucleophile, which attacks a CO_2 molecule. This step leads to the formation of bicarbonate ion associated with Me^{2+} (Eq. 4). Then, bicarbonate ion is replaced with water molecule and released into solution. This generates an inactive acid form of the enzyme, with water coordinated to Me^{2+}.

$$\text{E-Me}^{2+}\text{-OH}^- + \text{CO}_2 \leftrightarrows \text{E-Me}^{2+}\text{-HCO}_3^- \xleftrightarrow{\text{H}_2\text{O}} \text{E-Me}^{2+}\text{-H}_2\text{O} + \text{HCO}_3^- \quad (4)$$

Second stage regenerates the active basic form of the enzyme by ionization of a water molecule associated with metal and removal of a proton from the active site to external buffer (Eq. 5).

$$\text{E-Me}^{2+}\text{-H}_2\text{O} \leftrightarrows \text{E-Me}^{2+}\text{-OH}^- + \text{H}^+ \quad (5)$$

In some cases, this step is promoted by individual amino acid residues located in the vicinity of the active center of the enzyme – the proton shuttle residues (PSRs) – and acting as intermediates (Tripp *et al*. 2001). Removal of a proton from the active site and the recovery of metal hydroxide species of the enzyme is the rate-limiting step of the catalytic process. This is actually the basis for the action of CA activators (*see* below).

Enzymes that employ PSRs at the second phase of the catalysis regenerate typically their active sites more efficiently and it results in the enhanced enzymatic activity (Supuran 2016a). In highly active hCA II, PSRs are complemented by a cluster of His residues, which protrudes from the rim of the active site to the surface of the enzyme. This cluster facilitates even more effective proton removal. This is why hCA II is the most active among all currently known enzymes – its rate of catalysis, in fact, is limited by the rate of ions diffusion (Supuran 2004).

A characteristic feature of all CAs, at least, those proteins that have been crystallized, is the 'bipolar' architecture of the active center: one half is lined with hydrophobic residues, while another, opposing part, is lined with hydrophilic amino acid residues (Domsic *et al*. 2008). This dual nature can be explained by the fact that the enzyme substrates, CO_2 and HCO_3^-, have different chemical properties.

At the beginning of the reaction, CO_2 molecule binds to a specific part of the hydrophobic region of the CA active site (hydrophobic pocket); this facilitates a subsequent nucleophilic attack (Domsic *et al*. 2008). The hydrophilic part provides regeneration of the active site, contributing to elimination of a proton from the zinc-bound water and its disposal into external environment with the help of PSRs (Silverman and McKenna 2007).

The inhibitors of CAs (CAIs) are substances that do not allow the enzyme to carry out its catalytic function, partially or completely. Currently, quite a large number of CAIs are known, which operate in one of four different mechanisms (Supuran 2016a,b):

(*1*) Formation of a coordination bond with Me^{2+} ion by the replacement of the metal-associated water/hydroxide ion or by the attachment as a fifth ligand;

(*2*) Attachment to the metal-coordinated water/hydroxide ion;

(*3*) Blockage of the entrance to the active center of CA by the penetration of the inhibitor part into its cavity;

(*4*) Attachment out of the active site cavity, which also leads to the blockage of the entrance.

In addition, there are a number of CAIs, whose mechanism of action is yet unknown. The most studied class of CAIs are sulfonamides, zinc binders, which operate according to the mechanism 1. They are widely used in research and in clinical practice. The above mentioned mechanisms of inhibition are common to all CAs. However, representatives of different CA classes may vary in the degree of sensitivity to a particular type of inhibitors. Moreover, the effectiveness of the same inhibitor can vary for different CAs within one class.

The activators of CAs are substances that accelerate the passage of the second, rate-limiting step of catalysis (Eq. 5). Such compounds bind to the enzyme near the entrance to its active site, forming a complex that facilitates proton-transfer processes between the active site and the reaction medium (Supuran 2004, 2016a). Accordingly, the recovery of metal hydroxide derivative in the active site of the enzyme is much faster, and the turnover rate of the enzyme increases.

Functional roles of CAs

Functional roles of the CA are determined by the catalysis of reversible hydration of carbon dioxide. There is a number of biological processes, where the enzyme is involved. CAs are present wherever the acceleration of CO_2/HCO_3^- interconversion or rapid changes in the concentration of the participants of the Eq. 1 are required. The role of the enzyme may consist (Henry 1996, Smith and Ferry 2000, Frost and McKenna 2014) in:

(*1*) CO_2/HCO_3^- delivery for transport across cellular membranes;

(*2*) Delivery of CO_2/HCO_3^- substrates for enzymes;

(*3*) Removal of CO_2/HCO_3^- – the products of other enzymatic reactions;

(*4*) Changes in H^+ concentration at the places of CA localization;

(*5*) Changes in C_i forms in certain cellular compartments or tissues.

First four of the five listed biological roles unlikely need any comment. The need to change the C_i forms is associated with differences in their permeability through the cell membrane. This role of CA is clearly represented in animal respiration. Bicarbonate ion and CO_2 are quite different in their physico-chemical properties. While HCO_3^- is a charged molecule insoluble in cell membranes, neutral CO_2 is highly soluble in lipids. CAs of red blood cells convert CO_2 formed in tissues into bicarbonate, which is transported to the lungs. In the alveoli, the enzyme carries out a reverse conversion of HCO_3^- into CO_2, thus contributing to gas exchange. Another example of CAs role in changing of C_i forms is the function of CO_2-uptake systems of cyanobacteria, which are equipped with CA-like proteins, ChpX/Y, that presumably convert the absorbed CO_2 into HCO_3^-, thereby "locking" the intracellular carbon to ensure the efficient photosynthesis (Price 2011).

In addition to the main reaction of reversible hydration of CO_2, which defines the role of CA in nature, some CAs are able to catalyze a number of side reactions (Supuran 2016a). These are reversible hydration reactions of small molecules that are structurally similar to the CO_2 – carbonyl sulfide (COS), carbon disulfide (CS_2), cyanamide, and some aldehydes. CA may also exhibit esterase activity, carrying out a reversible hydrolysis of esters of carboxylic or sulfonic acids, as well as phosphate esters and thioesters. There is some evidence that CA can hydrolyse carbobenzoxy chloride ($PhCH_2OCOCl$) and sulfonyl-chlorides ($R\text{-}SO_2Cl$). However, the physiological significance of these alternative reactions is still not clear.

Physiological roles of CAs in living organisms

Physiological roles of the CA refer to enzyme participation in certain vital processes of living organisms, by performing the above mentioned functional roles. Despite of the universal function of all CAs, which is CO_2/HCO_3^- interconversion, the importance of the enzyme at the organismic level is determined by metabolic features of the individual organism and by characteristics of its C_i exchange system. In general, depending on its subcellular localization, CA is involved in many physiological processes: respiration, photosynthesis, pH maintenance, ion transport/homeostasis, biosynthetic reactions, calcification and osteogenesis, *etc*.

Along with the physiological functions, CAs play physio-pathological roles: excessive CAs activity contributes to a number of human diseases, such as glaucoma, atherosclerosis, appearance of tumors, obesity, and some others (Supuran and Scozzafava 2007). A lack of CAs activity leads to violations in learning and memory, *e.g*. Alzheimer's disease or aging (Alterio *et al*. 2012a). For this reason, along with the fundamental studies of CAs (catalytic mechanisms, structural features, *etc*.), some applied areas are quickly developing, such as studies of activation or inhibition with the subsequent drug design for clinical medicine (McKenna and Supuran 2014). The latter is also important for the treatment of invasive infections caused by numerous pathogens: *Helicobacter pylori*, *Mycobacterium tuberculosis*, *Plasmodium falciparum*, *Candida albicans*, vital activity of which is not possible without functional activity of their intracellular CAs. More information about participation of CAs in pathophysiological processes is available in specialized comprehensive reviews (Supuran 2004, Supuran and Scozzafava 2007, McKenna and Supuran 2014). Here, we further focus on the physiological role of this enzyme.

Organisms from the Archaea domain: Archaea contain just two (β- and γ-) of the six known classes of CAs. The intracellular function of these CAs is not precisely known. Cab protein of methanogenic anaerobe, *Methanobacterium thermoautotrophicum*, is currently the only characterized β-CA from archaea (Smith and Ferry 1999). Both methanogenic pathways known to date use CO_2 either as a substrate (CO_2 reduction pathway) or as a product (aceticlastic pathway) (Kumar and Ferry 2014). It is assumed that the Cab plays a role in CO_2 reduction pathway, where it can participate in CO_2 uptake with its simultaneous conversion into HCO_3^- for the retention of C_i within the cell (Fig. 4). Alternatively, Cab may participate in the reverse conversion of HCO_3^- into CO_2 in the cytoplasm for further CO_2 reduction to methane (Kumar and Ferry 2014).

For two γ-CAs (Cam and CamH proteins) of *M. thermophila* (Alber and Ferry 1994, Zimmerman *et al*. 2010), the role in aceticlastic methanogenesis was assumed, a pathway of acetate conversion to methane and carbon dioxide. It was suggested that Cam and CamH can work on different sides of the cell membrane to facilitate transport of acetate into the cell and CO_2 out of the cell *via* an acetate/bicarbonate anion exchanger (Fig. 4) (Ferry 2010, Kumar and Ferry 2014).

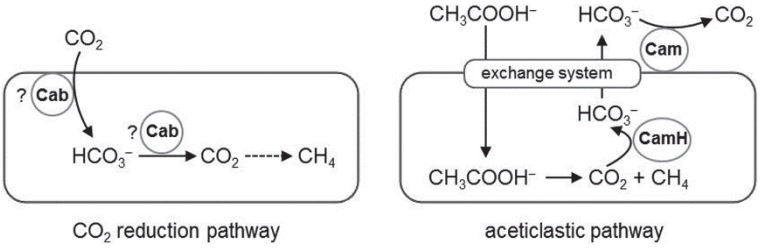

Fig. 4. A proposed scheme of CAs participation in two methanogenic pathways of anaerobic archaea. Cab – β-CA of *M. thermoautotrophicum*, Cam and CamH – γ-CAs of *M. thermophila*.

Organisms from the Bacteria domain: Bacteria carry three CA classes – α, β, and γ. The Bacteria domain includes all prokaryotes that are not included in the Archaea. The structure of this domain, reflecting the modern taxonomy of bacteria, is extremely diverse. These include organisms that differ in structure, nutrition, metabolism features, *etc*. Organization of CA systems of these organisms (*i.e.* a set of all CAs as well as their cell location) and their physiological relevance are determined by the above mentioned features. Groups of organisms, which are similar in trophic organization and C_i metabolism, show typically similar principles of their CA systems.

In most cases, the role of CAs in prokaryotes is limited to C_i transport and its supply for enzymatic reactions, or to pH maintenance. Here, we provide some examples of the function of CAs in prokaryotic cells. More information on bacterial CAs is available in reviews of Smith and Ferry (2000) and Kumar and Ferry (2014).

Physiological role of CA is well studied in *E. coli*. This facultative anaerobe is capable to use cyanate as a nitrogen source. However, since NCO^- ion is toxic, it is first converted to ammonium by cyanase (Kozliak *et al.* 1994). Cyanase is encoded by the *cynS* gene, which is part of a cyanate-inducible operon, *cyn*. This operon consists of *cynS* for cyanase, *synX* for unknown protein, and *cynT* for β-CA. The biological function of CynT in the cytosol is to maintain a certain concentration of HCO_3^- required for the degradation of cyanate. This is achieved by conversion of CO_2, which is evolved due to the activity of cyanase, into

bicarbonate (Eqs. 6-7):

$$NCO^- + 3H^+ + HCO_3^- \xrightarrow{CynS} NH_4^+ + 2CO_2 \quad (6)$$

$$H^+ + HCO_3^- \xleftarrow{CynT} H_2O + CO_2 \quad (7)$$

In photosynthetic prokaryotes (cyanobacteria), CAs participate in the CO_2-concentrating mechanism, CCM (Price *et al.* 2008, Kupriyanova *et al.* 2013b, Long *et al.* 2016). CCM operates in order to maintain a high concentration of CO_2 molecules near Rubisco. This is achieved with the assistance of C_i-uptake systems (bicarbonate transporters and CO_2-uptake systems, NDH-$1_{3/4}$), which generate the intracellular pool of bicarbonate followed by its rapid conversion by CA into CO_2 near the active site of Rubisco (Fig. 5). Such a directed increase in the intracellular C_i concentration is an adaptation to low atmospheric CO_2 concentrations, which facilitates photosynthetic C_i assimilation. CCM scheme requires the presence of only one CA, located in carboxysomes, the polyhedral bodies, in which CA is co-localized with Rubisco. In β-cyanobacteria, carboxysomal CAs are represented by β-CA, CcaA, and γ-CA, CcmM. In α-cyanobacteria, carboxysomal β-CA is represented by CsoSCA. In addition to carboxysomal CA, cyanobacteria may also contain external enzymes of α- and β-classes (EcaA and EcaB proteins, respectively) (Smith and Ferry 2000). However, their role in the CCM or in other physiological cell processes remains unclear.

In cyanobacteria, two proteins of the CO_2 uptake systems, ChpX and ChpY, presumably work as CAs (Fig. 5), although their specific CA-activities are not yet confirmed. They are suspected in unidirectional conversion of consumed CO_2 into bicarbonate, thereby preventing a leakage of C_i back to the environment (Price 2011). They can also intercept CO_2 leakage from the carboxysomes by the same mechanism. At present, ChpX/Y are considered as CA-like proteins. They are unclassified and do not belong to any of the CA classes. If their specific CA-activity is confirmed, they may become the progenitors of a new class of CAs.

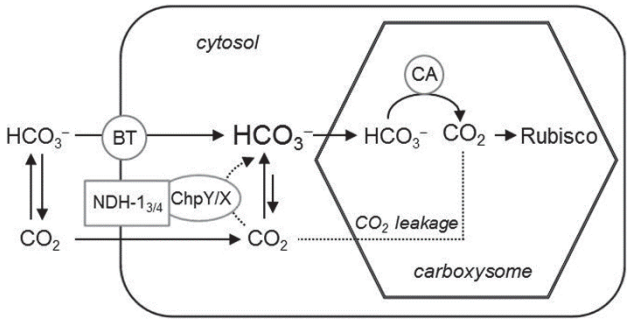

Fig. 5. The circuit operation of the CCM in cyanobacteria with participation of CAs. NDH-$1_{3/4}$ – CO_2 uptake systems, ChpY/X – CA-like proteins of NDH-$1_{3/4}$, respectively, BT – bicarbonate transporters.

Nonphotosynthetic chemoautotrophic bacteria, *e.g.* *Halothiobacillus neapolitanus*, apparently, are also capable of intracellular accumulation of C_i for improving of photosynthesis efficiency (Kimber 2014). Such bacteria possess a α-type carboxysome with β-CA, CsoSCA (Sawaya *et al.* 2006). Like α-cyanobacteria, this carboxysomal CA serves to supply a substrate (CO_2) for Rubisco.

In addition to intracellular CAs, which participate in regulation of homeostasis, extracellular cyanobacterial CAs should be also mentioned, as indirect depositors of carbonates during cells mineralization (Jansson and Northen 2010, Kupriyanova and Pronina 2011). This process, which reached enormous intensity in the Precambrian, ultimately led to the formation of a modern atmosphere and promoted CAs to the level of the biosphere importance (Zavarzin 2002).

Organisms from the Eukarya domain: The Eukarya domain includes organisms belonging to the kingdoms of Animalia, Plantae, and Fungi, as well as organisms belonging to a large group, formerly called protists. More information on the modern system of classification of eukaryotic organisms can be found in the review of Parfrey *et al*. (2006). Eukarya organisms are more diverse in their structural organization than members of Bacteria: along with humans, Eukarya includes unicellular green algae (Chlorophyta) and amoeboid protists. All variety of eukaryotic CA systems cannot be covered within this review, and it is limited only to some specific examples. Eukarya carries five of the six known classes of CAs: α, β, δ, ζ, and η. Enzymes of γ-class are not found among eukaryotes. The biological roles of CAs depend on their subcellular and suborganismal localization and they support a variety of physiological processes.

In animals, there are 16 α-CA isoforms. Depending on the subcellular localization, one may distinguish cytosolic, mitochondrial, and membrane-bound enzymes, as well as a secreted CA forms (Alterio *et al.* 2012a, Frost 2014). The most studied enzymes are cytosolic CAs located in erythrocytes (CA I and CA II), which are involved in the transfer of large amounts of CO_2 generated in the tissues outside the organism. Membrane-bound enzymes are located in various tissues and participate in transport of CO_2 and HCO_3^- across membranes. Tumor-related membrane-bound CAs contribute to pathological cell proliferation and tumorigenesis. Mitochondrial CAs are involved in gluconeogenesis, lipogenesis, and ureagenesis. The physiological role of secreted CA isoforms is assumed to pH maintenance, but it is not sufficiently clear. More information about animal CAs and their functions can be found in the review of Frost (2014).

Fungal cells have the CAs of α- and β-classes (Elleuche and Pöggeler 2010). These enzymes perform

different physiological functions. In *Saccharomyces cerevisiae*, β-CA Nce103 supplies bicarbonate for carboxylation reactions under low CO_2, whereas in *Sordaria macrospora*, two β-CAs (CAS1 and CAS2) are involved in fruiting body development, while the functions of other two enzymes (β-CAS3 and α-CAS4) remain unknown.

Numerous species of protozoan parasite, *Plasmodium*, are the only organisms that carry η-CAs (Del Prete *et al*. 2014b). The physiological role of these enzymes is not completely clear, but there is some evidence that they may be involved in the *de novo* purine/pyrimidine biosynthetic pathways (Supuran 2016a). Since *Plasmodium* is the pathogen, which causes human malaria, an intensive survey for new inhibitors of η-CAs is now conducted for drug treatment of this disease.

In eukaryotic photoautotrophs, the main role of CAs is in the regulation of photosynthesis and related biosynthetic reactions. The architecture and operation of their CA-systems differ depending on the type of C_i metabolism (Fig. 6). Most photoautotrophs require intensive C_i accumulation from the environment due to low concentration of CO_2 in the atmosphere and low affinity of Rubisco to CO_2 – two main factors that limit their photosynthetic efficiency (Raven and Beardall 2014). These organisms increase CO_2 concentration in the active centers near Rubisco *via* carbon concentration. Such mechanisms operate in CAM and C_4-plants, as well in aquatic photosynthetic organisms – microalgae and cyanobacteria possessing C_3-type of carbon fixation.

In C_4 plants, C_i is concentrated through cooperative photosynthesis, where atmospheric CO_2 fixation occurs in one type of cells, and then concentrated CO_2 is delivered to the Calvin-Benson cycle (C_3 pathway) located in another type of cells. In such metabolic scheme, a cytosolic β-CA located in mesophyll cells is the most active. There, the enzyme converts the consumed CO_2 into HCO_3^- that is used by phospho*enol*pyruvate carboxylase to form oxaloacetate (Ludwig 2016) (Fig. 6*A*). In CAM plants, with uncoupled concentration and fixation of CO_2 in time, similar function is expected for cytosolic CA (Tiwari *et al*. 2005) (Fig. 6*B*).

The schemes of CCM for cyanobacteria and microalgae are depicted in Figs. 5 and 6*C*, respectively. Unlike

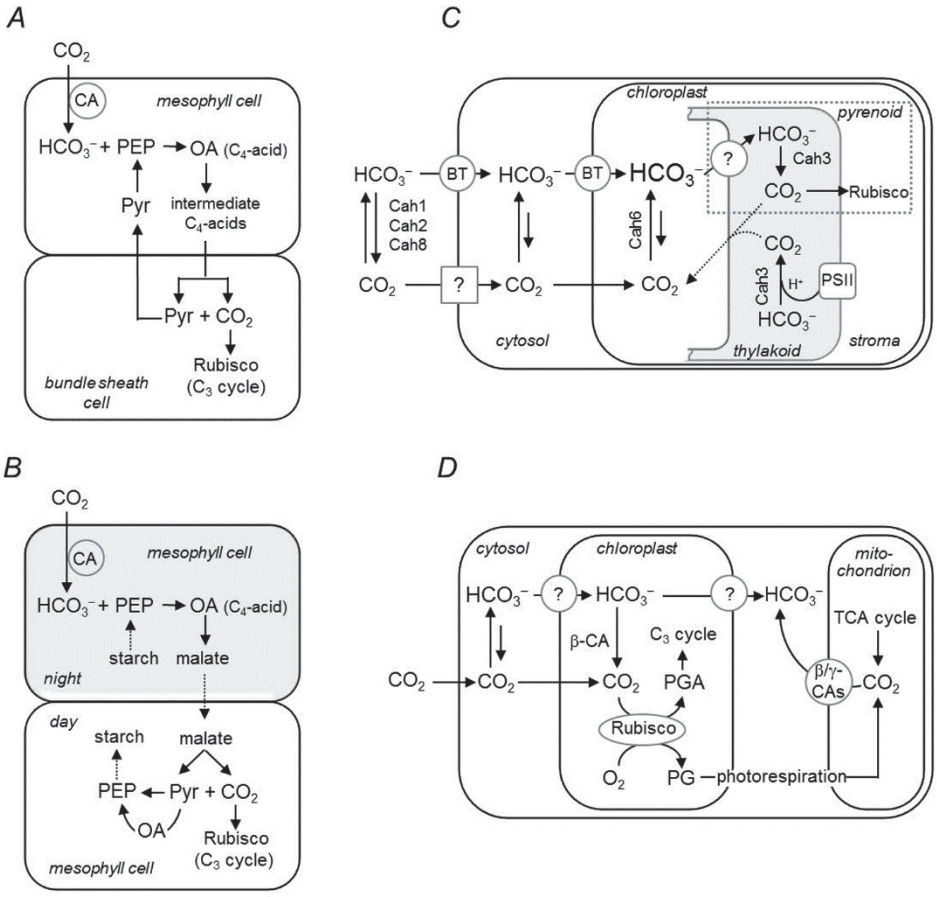

Fig. 6. Principle schemes of carbon fixation pathways in eukaryotic photoautotrophs and their relationship to CA systems. (*A*) C_4 pathway; (*B*) CAM photosynthesis; (*C*) C_3 pathways in aquatic photoautotrophs (microalgae) with CCM; (*D*) C_3 carbon fixation in higher plants and proposal basal CCM. PEP – phospho*enol*pyruvate; OA – oxaloacetate; Pyr – pyruvate, BT – bicarbonate transporter; PGA – 3-phosphoglyceric acid; PG – 2-phosphoglycolate; TCA – tricarboxylic acid (cycle).

cyanobacteria, eukaryotic algae (*e.g. C. reinhardtii*) have an additional barrier for C_i in its way to Rubisco –chloroplast membrane (Fig. 6*C*). In *Chlamydomonas*, there are 12 currently known genes that encode CAs of α-, β-, and γ-class with different subcellular localization (Moroney *et al*. 2011). The most studied is α-CA, Cah3, which operates in lumen of thylakoids. This enzyme supplies CO_2 for Rubisco in pyrenoids (Pronina and Borodin 1993, Markelova *et al*. 2009, Sinetova *et al*. 2012), as well as stabilizes the water oxidizing complex of PSII by protecting it from the excess of protons (Shutova *et al*. 2008). Other CAs of *C. reinhardtii* are much less studied. Extracellular enzymes (α-CAs, Cah1 and Cah2, as well as β-CA, Cah8) are expected to participate in C_i uptake. β-CA, Cah6, is located in a stroma of chloroplasts, and it can prevent a leakage of CO_2 out of a chloroplast. The possible roles of other CAs in *C. reinhardtii* are described in reviews of Moroney *et al*. (2011) and Jungnick *et al*. (2014).

Mechanisms of C_i concentration operating in diatoms are best explored in two model organisms – *Phaeodactylum tricornutum* and *Thalassiosira pseudonana* (Hopkins *et al*. 2016). CCM of *P. tricornutum* is generally similar to that of *Chlamydomonas*. The 'chloroplast-pump' transports bicarbonate into stroma of a chloroplast. Then it further diffuses into the pyrenoid, where it is converted by the CA into CO_2 – the substrate for Rubisco. It seems that in *T. pseudonana*, a modified C_4 pathway operates, in which the cytoplasmic CA plays the role of HCO_3^- provider for phospho*enol*-pyruvate carboxylase, while the chloroplast CA delivers CO_2 molecules to Rubisco (Kustka *et al.* 2014, Hopkins *et al*. 2016).

Macroalgae often possess C_3-based biochemistry of photosynthesis, except certain species that feature a C_4-like pathway (Raven and Hurd 2012). It is noteworthy that some species of macroalgae with C_3-type of carbon fixation have a mechanism for the C_i concentration, which differs from that of microalgae. Thus, Characeae generate the acid zone in a cell wall, which facilitates the entry of exogenous bicarbonate. An extracellular CA operating in that zone promotes the conversion of HCO_3^- into CO_2 followed by the absorption of CO_2 into a cell *via* passive diffusion. This allows to maintain the intracellular concentration of C_i higher than that in the environment. Such mechanism is also employed by some freshwater angiosperms (Raven and Beardall 2016).

Similar mechanism of C_i concentration is thought to appear in many marine macrophytes, although the presence of characteristic acid zones in their cell walls was not directly demonstrated (Raven and Hurd 2012). At the same time, the participation of CAs in photosynthetic C_i assimilation in seaweeds is well-documented. It has been shown that the extracellular CA, located at the cell wall of seaweeds, can promote the formation of CO_2 from HCO_3^- (basic form of C_i in seawater) for subsequent absorption by the cell (Larsson and Axelsson 1999). Simultaneously, intracellular isoforms of CA help to maintain the pH balance and perform HCO_3^- dehydration to supply CO_2 to Rubisco (Fernández *et al.* 2014b). Recently, the presence of CCM components similar to that of *C. reinhardtii* was found in green macroalgae *Ulva rigida*; however, its activity and the mechanism of action is still to be assessed (Rautenberger *et al.* 2015).

In contrast to C_4, CAM-plants, and algae, C_3 higher plants possess a significant amount of Rubisco with high affinity for CO_2. Thus they can actively photosynthesize even under CO_2 limitation. In the genome of the model organism, *Arabidopsis thaliana*, 19 genes for CAs have been identified; they encode the enzymes of α-, β-, and γ-classes with different cellular localization (Rudenko *et al*. 2015, Fromm *et al*. 2016). The most important role in the assimilation of C_i apparently belongs to β-CA located in stroma of chloroplasts of mesophyll cells (Fig. 6*D*). It is believed that this enzyme converts bicarbonate ion into CO_2, a substrate for Rubisco. However, suppression of this stromal CA, the cellular amount of which is comparable to Rubisco, did not result in the expected reduction of CO_2 fixation (Price *et al.* 1994). Thus, unambiguous evidence, which confirms the function of this enzyme, is absent (Ludwig 2016). Recent data suggest that cells of C_3 higher plants are likely to operate with so-called "basal CCM", which employs mitochondrial β- and γ-CAs (Zabaleta *et al.* 2012). These enzymes convert CO_2 released during respiration to bicarbonate, which is then transported into the chloroplast for refixation (Fig. 6*D*).

Conclusion: Carbonic anhydrase, which catalyzes a very simple reaction of interconversion of CO_2 and bicarbonate ion, in fact, is the key enzyme that integrates all currently known living organisms of carbon-based life. Wide spreading of CAs is determined by their participation in the carbon cycle of the planet, where these enzymes play the principle role in interaction of organic and inorganic carbon cycles. At present, CAs have been found in all the organisms, examined for its presence, indicating the vital function of this enzyme for all living beings. This is why CA was apparently "invented" by nature several times, representing an example of convergence in the evolution of the catalytic function. The biological significance of CAs is very diverse and determined by structural features of each organism – a mode of nutrition, metabolism, *etc*. At the same time, CAs cover a number of important biological processes, including global processes of CO_2/HCO_3^- exchange during animal respiration and plant photosynthesis. Here, we briefly refreshed the history of discovery of CAs, and summarized recent advances in CAs field – from their diversity and classification to the mechanisms of catalysis and their physiological roles at different organismal levels. We proposed further specification in CAs classification: the extensive conserved family of the inactive forms of γ-CAs should be referred to as γCA-related proteins or γCA-RPs.

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