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Review

An Update on the Metabolic Roles of Carbonic Anhydrases in a Model Alga *Chlamydomonas reinhardtii*

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Abstract: Carbonic anhydrases (CAs) are metalloenzymes that are omnipresent in nature. The CAs catalyze the basic reaction of reversible hydration of CO_2 to HCO_3^- and H^+ in all living organisms. Photosynthetic organisms contain six evolutionarily different classes of CAs, namely, α-CAs, β-CAs, γ-CAs, δ-CAs, ζ-CAs, and θ-CAs. Many of the photosynthetic organisms contain multiple isoforms of each CA family. Model alga, *Chlamydomonas reinhardtii* contains fifteen CAs belonging to three different CA gene families. Out of the fifteen CAs, three belong to α-CA gene family, nine to β-CA gene family, and three are γ-CAs. The multiple copies of the CAs in each gene family may be due to gene duplications within the particular CA gene family. The CAs of *Chlamydomonas reinhardtii* are localized in different subcellular compartments of this unicellular alga. The presence of a large number of CAs and their diverse subcellular localization within a single cell suggests the importance of these enzymes in metabolic and biochemical roles they perform in this unicellular alga. In the present review, we update the information on molecular biology of all the fifteen CAs and their metabolic and biochemical roles in *Chlamydomonas reinhardtii*. We also present a hypothetical model showing the known functions of CAs and predicting the functions of CAs for which precise metabolic roles are yet to be discovered.

Keywords: carbonic anhydrases; CA gene family; *Chlamydomonas reinhardtii*; model alga; metabolic role; photosynthesis

1. Introduction

The carbonic anhydrases (EC 4.2.1.1) (CAs) are metalloenzymes that perform basic chemical reaction of reversible hydration of carbon dioxide to bicarbonate ($CO_2 + H_2O \leftrightarrows HCO_3^- + H^+$). The CAs belong to seven evolutionarily unrelated CA-gene families (α-, β-, γ-, δ-, ζ-, η-, and θ-CAs) with no sequence or structural similarity, and therefore, the CAs are excellent examples of convergent evolution [1-5]. The CAs are widespread in nature and are found abundantly in plants, animals and microorganisms, suggesting that the CAs have many diverse metabolic roles in living organisms [6-8]. Vertebrates and mammals have only α-CAs and contain multiple isoforms of the enzyme. In contrast, multicellular plants and unicellular photosynthetic organisms seem to have members of six CA gene families, often multiple isoforms of CAs from each gene family [4, 9]. The *C. reinhardtii* genome analysis has revealed the presence of at least fifteen CA genes encoding three different families of CAs. The number of CAs in *C. reinhardtii* is thus much higher than previously thought for a single cell alga. Interestingly, recent study showed that limiting CO_2-inducible B protein (LCIB) family belongs to the β-CAs [10]. The amino acid sequences of these CA families are different but all these CA families have a Zn^{+2} atom at the active site [11]. In this alga, CAs have been found in the mitochondria, chloroplast thylakoid, cytoplasm, and periplasmic space [12, 13]. A recent study showed that, CAH6 is localized in the flagella instead of the pyrenoid stroma as previously reported [14].

Down regulation of CA activity using molecular techniques and chemical inhibitors showed reduced lipid biosynthesis in chloroplasts compared to chloroplasts from wild type plants [15]. The plastids are double membrane organelles found in algae, and they are the sites of manufacturing and storage for important chemical compounds used by the cells. CAs are involved in lipid synthesis (and perhaps other HCO_3^- requiring pathways in plastids) indirectly, serving to "concentrate" CO_2 in plastids as HCO_3^- and reduce the rate of CO_2 diffusion out of plastids [15]. The CA might indirectly influence fatty acid synthesis in plastids by modulating plastidial pH, as the enzyme fatty acid synthase activity requires optimal pH for fatty acid synthesis [15].

Role of CAs in pH regulation is well known in animal cells. However, the roles of CAs in pH regulation in this model alga are not known and need to be investigated (7). The *C. reinhardtii* has fifteen CAs belonging to three different CA gene families, suggesting that they are involved in several other metabolic functions in addition to CO_2-concentrating mechanism (CCM) that is attributed to the evolutionarily conserved enzymes in plants. In *C. reinhardtii*, CAs are involved in many metabolic functions that involve carboxylation or decarboxylation reactions, including both photosynthesis and respiration. In addition, it has been clearly shown that CAs also participate in the transport of inorganic carbon to actively photosynthesizing cells and away from respiratory cells [12, 16].

In the current article, we will review the information on CAs of *C. reinhardtii*, a unicellular model alga. We describe the information available on molecular biology and present the data on metabolic and biochemical roles of the three CA gene families. For each CA enzyme from the three CA families, we will highlight the current research and questions that were addressed by researchers in the field. We also, present a hypothetical model showing the known functions of CAs and predicting the functions of CAs for which precise metabolic roles are yet to be discovered. Finally, we present future directions in the field of *C. reinhardtii* CA research to study the precise metabolic and physiological roles of CAs from this alga.

2. Carbonic anhydrases

2.1. Carbonic anhydrases in photosynthetic organisms

The photosynthetic organisms contain CAs that belong to six different CA gene families, namely, α-CAs, β-CAs, γ-CAs, δ-CAs, ζ-CAs, and θ-CAs. Each of the at least three gene families of α-CAs, β-CAs, and γ-CAs are represented by multiple isoforms in all of the species. The γ-CAs are also found in photosynthetic bacteria [17, 18] and plants [19]. A θ-CA has been recently discovered

in thylakoid lumen of marine diatom *Phaeodactylum tricornutum [4].* All the four CA gene families of photosynthetic organisms contain zinc as a metal ion at the active site of the enzymes. Due to alternative splicing of CA transcripts the number of functional CA isoforms in many of the species are more than the number of genes that encode a particular CA enzyme. In photosynthetic organisms, CAs are expressed in different cellular compartments, and are most prevalent in chloroplasts, cytosol, and mitochondria. The diversity in location suggests their importance in many physiological and biochemical roles the CAs may play in photosynthetic organisms.

2.2. Carbonic anhydrases in Chlamydomonas reinhardtii

The model alga, *Chlamydomonas reinhardtii* is a unicellular photosynthetic eukaryote and contains multiple genes encoding CAs for three different gene families. The α-CAs were discovered in 1980s and 1990s in *C. reinhardtii* [12, 20, 21]. The β -CAs have been discovered during 1990s, [22-24], and with the sequencing of the complete genome of *C. reinhardtii* three novel γ-CAs were found in the later part of 2000s [25-28].

The alga, *C. reinhardtii* has three α-CAs, nine β-CAs that include recently discovered three homologs of LCIB protein family, and three γ-CAs [10]. Among the CAs that are found in *C. reinhardtii,* the β-CAs are dominating with the highest isozyme number in this organism. Details of all the CAs that have been discovered in *C. reinhardtii* till date are presented in table 1.

Table 1. Details of the fifteen carbonic anhydrases found in *Chlamydomonas reinhardtii* belonging to α, β, and γ gene families.

CA protein	Chr	Gene family	MW (kDa)	Location	Known/predicted physiological roles of the CAs	References
CAH1	4	α	78	Periplasm/late secretory pathway	Supply of Ci in low CO_2	[21, 29-35]
CAH2	4		84	Periplasm/late secretory pathway	Supply of Ci in high CO_2	[14, 21, 36, 37]
CAH3	9		29.5	Chloroplast	Growth in low CO_2,	[14, 38-44]
CAH4*	5	β	21	Mitochondria		[14, 45-47]
CAH5*	5		21	Mitochondria		[40-42[14]]
CAH6	12		31	Flagella	CCM	[14]
CAH7	13		35.79	Periplasm?		[48]
CAH8	9		35.79	Periplasm		[48]
CAH9	5		13.06	Cytosol		[14]
LCIB1			48[a]	Chloroplasts	CO_2, uptake, CCM	[10, 49]
LCIB2			48[a]	Chloroplasts	CO_2, uptake, CCM	[10, 49]
LCIB3			48[a]	Chloroplasts	CO_2, uptake, CCM	[10, 49]
CAG1	9	γ	24.29	Mitochondria	Transport of mitochondrial CO_2 to chloroplast	[14, 26, 27, 50]
CAG2	6		31.17	Mitochondria	Transport of mitochondrial CO_2 to chloroplast	[14, 27, 28, 50]
CAG3	12		32.69	Mitochondria	Transport of mitochondrial CO_2 to chloroplast	[14, 27, 28, 50]

*The amino acid sequences of these two β-CAs are identical but encoded by two separate genes.

[a]Predicted molecular weight. Chr = chromosome.

2.1.1. α-Carbonic anhydrase 1

Among the CA genes, the *α-CA1* was the first gene that was identified in *C. reinhardtii* in 1980s [20, 21, 51] and was named as CA1 in the order of discovery. Several groups have shown that CAH1 is localized in periplasmic space of the alga [20, 21, 51]. *Cah1*, the gene encoding CAH1, has been cloned [34]. The cDNA encodes a polypeptide of 377 amino acid residues. It is composed of a 20 amino acids long signal peptide, the small subunit, the large subunit, and the spacer region between the subunits [32, 34]. Fujiwara et al. discovered that the gene sequence is 93.6% identical with the sequence of *Cah2*, which encodes CAH2. In addition, their insertion sites of introns are identical. These findings advocate that *Cah1* and *Cah2* are originally from the same gene [21].

The production of CAH1 is induced when the cells are transferred from high CO_2 conditions to low CO_2 conditions. However, Kucho et al. conclude that the induction of CAH1 in the changing CO_2 concentration requires light, as it does not happen in the dark and according to their study the expression of CA1 is even downregulated in the dark in a similar manner as in the high CO_2 conditions [31]. Accordingly, mRNA of CAH1 also accumulates when the CO_2 concentration reduces in the presence of light [20, 21]. Fukuzawa et al. inhibited the photosynthesis with 3-(3,4-dichlorophenyl)-1,1-dimethylurea and showed that the accumulation of mRNA requires functioning photosynthesis [20]. CO_2 regulates the induction of CAH1 through various enhancer and silencer sites. At least, a 692-bp region from -651 to +41 relative to the transcription start site was detected to be adequate for full induction of CAH1 in response to light and low CO_2 [31]. Kucho et al. located a crucial regulatory area (63-bp from -293 to -231 relative to transcription start site) which contains two enhancer elements. In addition, they detected DNA-binding proteins that specifically interact with these enhancer elements in the presence of light and low CO_2 conditions [30]. Additionally, other silencers and enhancers have been found, but they are usually responsible for only small changes in the induction or downregulation of CAH1 [31].

The physiological role of CAH1 has already been extensively discussed in the earlier review [12]. CAH1 provides more C_i to the *C. reinhardtii* cell in C_i-deficient environment [12]. Nonetheless, many studies have shown that CAH1 mutant cells are as viable as wild type. On the contrary, the drug inhibition restricted the growth which implicates that other CAs, such as CAH2 and CAH8 might maintain necessary CA activity in CAH1 deficient cells [12].

2.1.2. α-Carbonic anhydrase 2

The CAH2 was discovered at same time as CAH1 by Fukuzawa et al. [20, 36, 37]. CAH2 is a periplasmic protein and is heterotetramer, as CAH1. The CAH2 consists of two identical large and two small subunits [21]. The molecular weight of the holoenzyme is approximately 84.5-87.9 kDa. The large subunit is 38 kDa and the small one is 4.2 kDa. Therefore, they are slightly larger than the corresponding units in CAH1 [36]. The genetic similarity has already been stated but also the similarity of amino acid sequences is 91.8% [21]. Nevertheless, the catalytic activity of CAH2 is approximately 1.6 times that of CAH1, as that of CAH2 is 3300 units per mg protein compared to 2200 units per mg protein with CAH1 [36]. The subunits of CAH2 are bounded to each other with disulfide bonds as in CAH1. CAH2 also has the similar glycosylation sites as CAH1 in the large subunit [36].

The expression of CAH2 is more abundant compared to CAH1, and its expression is greatly induced in low CO_2 conditions as opposed to CAH1 which is moderate in amount and is present in high CO_2 conditions, at least [21]. Furthermore, Tachiki et al. suggest that CAH2 might be present in low CO_2 conditions as well as high ones as *Cah2* mRNA is expressed in both conditions [36]. The function and role has been suggested to be the same as CAH1 and Rawat et al. proposed that *Cah2* could represent agene duplication without a specific own role [37].

2.1.3. α-Carbonic anhydrase 3

Among the α-CAs of *C. reinhardtii*, α-CAH3 was identified in late 1990s by Karlsson et al. and was shown to be localized in thylakoid lumen [38, 40, 47]. CAH3 is a 29.5 kDa polypeptide that was originally isolated by Karlsson et al. in 1995 [40]. The longest cDNA clone obtained from the cDNA library consisted of 1383 bp and contained an open reading frame that encoded a polypeptide of 310 amino acids [38].

CAH3 functions in the thylakoid lumen and it has been suggested to be part of photosystem II (PSII) or CCM [13, 44, 52-54]. Hanson et al. showed that cia3, which is a mutant line of *C. reinhardtii* lacking functioning CAH3, has a limiting effect on the function of Rubisco *in vivo* and perhaps not PSII. [13]. The physiological function of CAH3 is also related to the location within thylakoids and thus, in stromal thylakoids CAH3 is probably associated with light reactions of photosynthesis and in the intrapyrenoid thylakoids CAH3 is presumably connected to the actions of Rubisco [44].

In addition, the actions of CAH3 are connected to the fatty acid composition of the thylakoid membranes [44]. In the low CO_2 conditions, the activity of CAH3 is implicitly related to the increase of relative amount of polyunsaturated fatty acids. The change in the fatty acid composition changes the fluidity of the membranes and, therefore, the ion transport across the thylakoid membrane. The desaturation of fatty acids also provides H^+ ions and hence implies that there is a reaction where H^+ ions are needed [44].

The regulation of CAH3 in different CO_2 conditions differs vastly from the regulation of CAH1 or CAH2. On the contrary to the accumulation of mRNA of CAH1 in low CO_2 conditions, no one has detected the similar effect on CAH3 but the activity and localization of CAH3 changes according to the CO_2 conditions. Blanco-Rivero et al. discovered that the amount of mRNA or the actual protein did not increase significantly during acclimation to low CO_2 conditions [41]. However, the activity of CAH3 increased due to phosphorylation, as did the amount of CAH3 in intrapyrenoid thylakoids in the expense of stromal thylakoids [41].

Additionally, the optimal pH of CAH3 is more acidic [43] compared to other CAs of *C. reinhardtii*. Benlloch et al. measured the activity of CAH3 in different pH values and discovered that the optimum was approximately pH 6.5 compared to the other CAs which function best around neutral pH. The activity also persists higher than the activity of the other CAs at lower pH values [43].

Recent studies has shown that CAH3a associates with TAT2 and TAT3 proteins of the twin arginine translocation (Tat) pathway and delivers substrate proteins to the thylakoid lumen [14]. The study also showed that CAH3 is phosphorylated through its interaction with STT7 and increases its catalytic activity when CO2 is low and converts HCO2 to CO2 an in thylakoid membranes that traverse the pyrenoid, supplying the pyrenoid with high concentration of CO2 essential for CCM.[14, 38]

2.1.4. β-Carbonic anhydrase 4.

The presence of a CA in *C. reinhardtii* that belongs to the β-CA family was reported in 1995 by Eriksson et al [47]. The CAH4 is localized in the mitochondria of *C. reinhardtii* and has a molecular

mass of 20.7-22 kDa. The gene coding CAH4 is called *β-Ca1* of which the whole nucleotide sequence has been examined and found to have 96% identity with another mitochondrial CA (CAH5) coding gene, *β-Ca2* [47]. CAH4 is only present at low CO_2 conditions because *β-Ca1* is induced in low CO_2 conditions but not in the high ones [47].

There have been many theories about the physiological role of CAH4 as well as CAH5. On one hand, Eriksson et al. suggested that they are used in buffering reactions in changing CO_2 conditions [47]. Glycine decarboxylation in photorespiration produces excessive amounts of CO_2 and NH_3 in low CO_2 conditions. H^+ is used because NH_3 forms NH_4^+ at the pH of the mitochondrial matrix. Due to the need of H^+, CAH4 catalyzes the hydration of CO_2 to be faster in order to maintain the pH in the matrix [47]. On the other hand, Raven hypothesized that there might be a HCO_3^- channel in the inner mitochondrial membrane and thus, both CAH4 and CAH5 have a role in preserving the CO_2 [45].

There is also a third hypothesis of the function of CAH4 as well as CAH5; they might provide HCO_3^- for reactions catalyzed by phosphoenolpyruvate carboxylase where N is combined to C skeletons that can be later used in protein synthesis, for instance [45]. It has also been shown that because of this assumed function, external NH_4^+ concentration is an essential regulator for the expression and function of CAH4. In low CO_2 conditions, the expression of mitochondrial CAs (mtCAs) decreases if the external NH_4^+ concentration decreases. This also operates vice versa: if external NH_4^+ concentration is high, the CO_2 concentration can also be higher and mtCAs are still expressed at levels of CO_2 at which they would not normally be expressed [45].

2.1.5. β-Carbonic anhydrase 5

CAH5 in *C. reinhardtii* was identified simultaneously with CAH4 by Eriksson at al. [47]. The two clones that code for CAH4 and CAH5 differ only slightly in their nucleotide sequences. In the coding area, the difference is only seven nucleotides, leading to one amino acid change at position 53 where serine is replaced by alanine [47]. In addition, the upstream regulating sites of *β-Ca1* and *β-Ca2* are very similar. Due to the striking similarity of *β-Ca1* and *β-Ca2,* the genes are likely to be duplicates that were formed simply to increase the quantity of mtCA [46]. CAH4 and CAH5 lack any known functional difference, which also supports the gene-duplication assumption [46].

2.1.6. β-Carbonic anhydrase6

CAH6, the third β-CA was discovered in 2004 by Mitra et al. and localized in the chloroplast stroma [55, 56]. In contrast, localization studies performed by Mackinder et al recently showed that CAH6 is expressed in flagella and showed no detectable signal in chloroplasts [14]. To validate their findings the authors analyzed the presence of CAH6 in proteomic datasets and showed it in the flagellar proteome and in intraflagellar transport (IFT) cargo [14].

The cDNA of *Cah6* is 2,886 bp long and it encodes a 264-amino-acid-long polypeptide, CAH6. It has a calculated molecular mass of 26 kDa, but experimentally it was 28.5 kDa in SDS-polyacrylamide gel [55]. The activity of CAH6 is also slightly induced in low CO_2 conditions, but it is expressed constantly even in high CO_2 conditions similar to the many other CA isoenzymes in this

alga. The CAH6 is believed to be involved in trapping CO_2 that is leaking out of pyrenoid by converting it to HCO_3^- and thus, preventing C_i from leaving chloroplast [55].

However, recent study showing its localization to be in the flagella suggested that the CAH6 is not required in the chloroplast as its presence in the chloroplast may short-circuit the CCM by converting CO_2 from HCO_3 and its subsequent release away from Rubisco[14]. Indeed, this is the case at least in cyanobacterium, where presence of CA disrupts the CCM [49]. Chlamydomonas are known to show chemotaxis toward $HC0_3$, and CAs have been implicated in C_i sensing and hence may be directly involved in sensing of Ci [14, 57, 58].

2.1.7. β-Carbonic anhydrase 7

The CAH7 was identified in 2008 by Ynalvez et al. by examining the sequences of two genes that code for CAs, namely CAH7 and CAH8 [48]. The identified gene sequence of *Cah7* contains 5077 bp. The protein product of the gene *Cah7*, has 399 amino acids including 23 amino acids that are well conserved in β-CAs and also two cysteines and one histidine which coordinate Zn^{2+}. In addition, they predicted that CAH7 has a transmembrane domain, so it might be attached to a membrane [48].

CAH7 is present in low and high CO_2 conditions, although it is slightly more abundant in low CO_2 conditions than in the high. All in all, CAH7 is expressed in lower amounts than most of the other CAs in *C. reinhardtii*. The location and physiological role of CAH7 in the cell is yet to be resolved [48].

2.1.7. β-Carbonic anhydrase 8

C. reinhardtii CAH8 was identified by Ynalvez et al. in 2008 with CAH7, and both sequences were found to be closely related to each other [48]. The cDNA coding for CAH8 contains 2649 bp corresponding to 333-amino-acid-long polypeptide. Furthermore, CAH8 has the same β-CA characteristics as CAH7, except that CAH8 has 22 of the 23 well-conserved amino acid residues. The molecular mass of CAH8 is approximately 40 kDa. Additionally, CAH8 has the same transmembrane domain near the C-terminus as CAH7, even though immunolocalization has located CAH8 in the periplasmic space with CAH1 and CAH2. However, CAH8 appears closer to the cell membrane than CAH1 [48].

CAH8 is present in slightly higher amounts in high CO_2 conditions than in low ones but nevertheless, it is constantly present. The overall expression of CAH8 resembles the one of CAH6 as it is moderate among the CAs in *C. reinhardtii*. There are some theories of the function of CAH8. Firstly, it has been suggested that, as CAH8 is closely related to the cell membrane, it would ensure the presence of CO_2 near the membrane despite the external pH conditions. Secondly, it has been proposed to be a part of C_i delivery system as a carbon-binding protein. Thirdly, the association with a pore or a channel has been proposed [48].

2.1.9. β-Carbonic anhydrase 9

The presence of CAH9 in *C. reinhardtii* was first reported in 2005 by Cardol et al. from the genome sequencing project to analyze the proteome of the mitochondrial oxidative phosphorylation [27]. The RNA-Seq data that is available suggest that CAH9 is expressed at low levels (http://genomes.mcdb.ucla.edu/Cre454/) under the growth conditions that were used in the experiment at the time [12]. No further studies have been done since then on the CAH9 expression and its role in *C. reinhardtii.*

2.1.10. Limiting CO_2 inducible-B protein/ β-carbonic anhydrase family

Limiting CO_2 inducible-B protein (LCIB) is a key player in the eukaryotic algal CCM function in *Chlamydomonas reinhardtii [59]*. The *LCIB* genes encode for a novel chloroplast protein that consists of 448 amino acids with a predicted MW of 48 kDa, and forms a heteromultimeric complex with its close homolog LCIC and the complex may be tightly regulated or may require additional factors for proper functioning [14, 56, 57, 59]. Interestingly, a recent study involving a double mutant analysis of LCIB/CAH3 showed that LCIB functions downstream of CAH3, a low carbon inducible-B protein. It has been hypothesized that LCIB captures CO_2 leaked from the pyrenoid, possibly by unidirectionally hydrating CO_2 back to HCO_3 [58]. Recently, to study function of LCIB, phylogenetically diverse set of recombinant LCIB homologs were produced in *E. coli* and purified [10]. Structural characterization of the purified proteins showed three of the six homologs structurally similar to the β-CAs at the level of overall fold, zinc binding motif and active site architecture. However, none of the three proteins showed CA enzymatic activity and the lack of CA activity could be due to widening of the intersubunit cleft which affects active site integrity by causing disordering of the important His162/161 and Arg194/193 residues in the protein [10].

Based on the results of the study, it is proposed that LCIB in association with LCIC acts as noncatalytic structural barrier for CO_2 [10]. However, to elucidate the precise role of LCIB further studies involving characterization of LCIB-LCIC complex purified from native source are needed.

2.1.11. γ- Carbonic anhydrase

The gene *Glp1* that encodes γ-CAH1 was discovered in 2005 using γ-CA protein sequence of *M. thermophila* and expressed sequence tag (EST) databases [26]. Similarly, the presence of three γ-CAs in *C. reinhardtii* was also shown by two other groups [27, 28] and were reported as CAG1, CAG2, and CAG3, predicted to be localized in mitochondrial matrix.

The *Glp1* gene that codes for γ-CAH has seven exons and six intros and encodes a putative protein of 312 amino acids [26]. The localization studies using prediction programs showed that this enzyme is localized in cytoplasm or is secreted outside the cell. The γ-CAH1 has about 40% similarity with γ-CAH of *M. thermophila* and has three histidine residues coordinating zinc at the active site of the enzyme. The recombinant proteins expressed in *E. coli* showed no CA activity in either crude cell extracts or purified fusion protein [26].

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The presence of two additional γ-CAHs that are located on scaffolds 16 and 19 and have been annotated as submits of mitochondrial NADH dehydrogenase complex [26]. The sequence analysis showed that these γ-CAHs do not contain three histidine residues that are required for the catalytic activity of the CAs [26]. Based on the available studies the γ-CAHs of *C. reinhardtii* are localized in mitochondrial matrix, and a part of mitochondrial complex I, the complex I of the mitochondrial electron transport chain (mETC) in *Arabidopsis thaliana* also contains three different protein domains that are homologous to γ-CAs [60]. Double mutants of *Arabidopsis thaliana* lacking γ-CAH1 and γ-CAH2 were analyzed for their role in development and physiology. The analysis of mutant strains of *A. thaliana* showed developmental delay and upregulation of complex II and complex IV with increased oxygen consumption in mitochondrial respiration [60]. Based on this study it can be speculated that the three γ-CAHs in *C. reinhardtii* may perform similar functions. The studies on γ-CAHs are few and have been done a decade ago, and therefore the information on physiological roles of these CAs is incomplete. We need more studies using bioinformatic and molecular tools on structural and functional analysis of these γ-CAHs to know their precise roles in *C. reinhardtii*

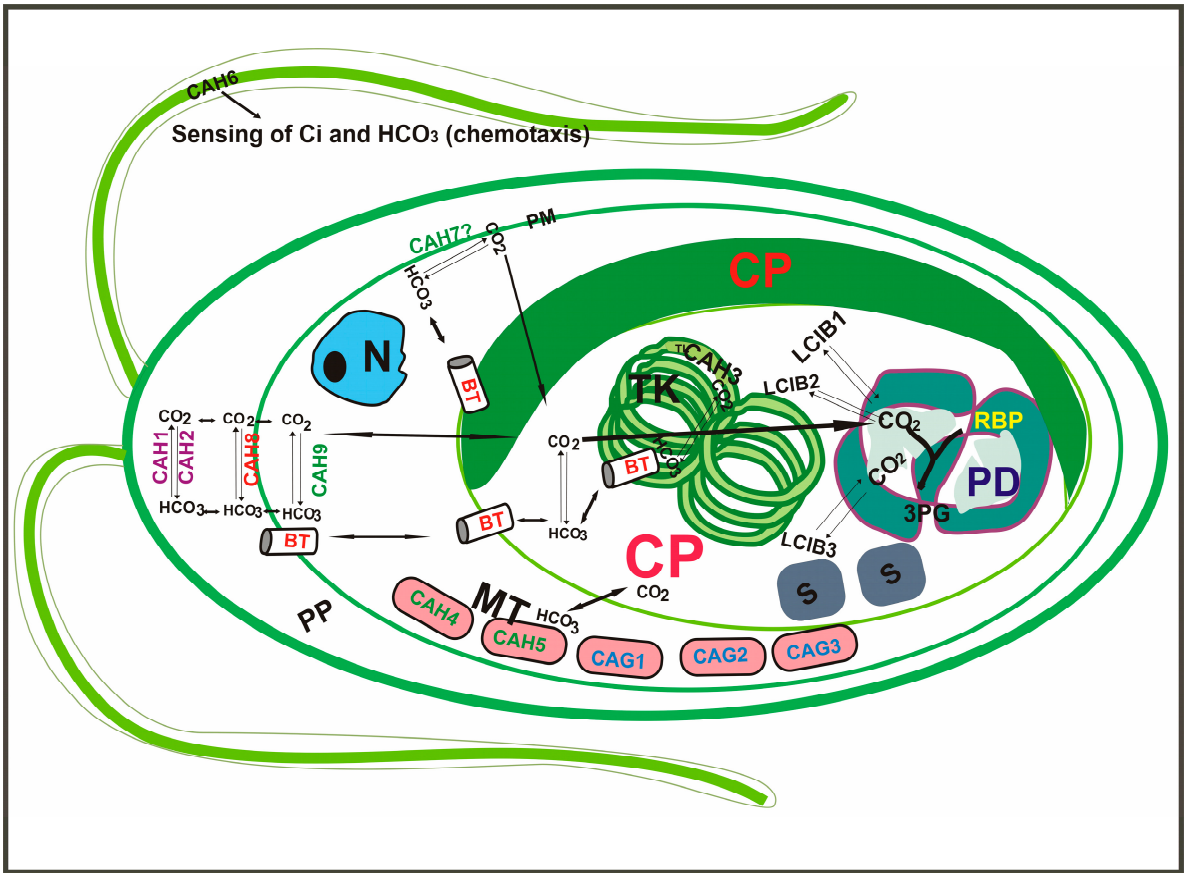

Figure 1. Schematic presentation of *C. reinhardtii* model showing roles of CAS in the cell and subcellular organelles. PM-Plasma membrane, PP-Periplasmic space, N-Nucleus, CP-Chloroplast, TK-Thylakoids, TL-Thylakoid Lumen S-Starch, MT-Mitochondria, and PD-Pyrenoid. Cah1, Cah2, Cah3-α-Carbonic anhydrases, Cah4, Cah5 Cah6 Cah7 Cah8 and Cah9- β-

Carbonic anhydrases, LCIB1, LICB2 and LCIB3 Low CO_2 inducible proteins (β-CAs). CAG1, CAG2, and CAG3- γ- Carbonic anhydrases, BT-Bicarbonate transporters. *RuBisCO*-Ribulose-1,5-bisphosphate carboxylase oxygenase, RuBP-d-ribulose 1 5-bisphosphate, 3PG-3-phosphoglycerate.

3. Conclusions and future directions

The CA enzymes belonging to different classes of CA gene families are found in vertebrates, invertebrates, plants, unicellular marine and fresh water algae, bacteria, and archaea. The CAs are localized in almost all the tissues of higher animals and subcellular organelles of eukaryotic cells and perform variety of metabolic and physiological roles. Several classes of CAs are found in plants that are localized in subcellular organelles and are involved in CCM for photosynthesis and perform other metabolic functions. Researchers in plant biology have used marine and fresh water unicellular photosynthetic model organisms to study the precise metabolic roles of CA enzymes. Fresh water alga, *C. reinhardtii* is one such model organism, which has emerged as an important model organism and has answered many questions on the metabolic and physiological roles of CAs mainly on CCM. However, the precise metabolic roles of most of the CA enzymes in this alga remain to be studied.

There has been a continuous interest in CA research in unicellular photosynthetic organisms as the genomes of these algae are available. Availability of bioinformatic and molecular tools have helped to study the precise metabolic roles of CAs in the photosynthetic model organisms. In *C. reinhardtii*, researchers have attempted to study the localization and metabolic roles of three α-CAs. There have emerged contradictory reports on the precise localizations of CAs, and only limited information is available on the physiological roles of six β-CAs and newly reported LCIB protein family that belongs to β-CA group. No studies are available on γ-CAs except the presence of three forms of this enzyme and their predicated localization in mitochondrial matrix. The challenge for future researchers will be to determine the precise localization and biochemical roles of all the twelve CAs and newly discovered three LCIB family proteins.

It is important to identify precise physiological roles for all the CAs found in *C. reinhardtii*. Chlamydomonas is an important model organism to study the fundamental processes such as photosynthesis. It is the most commonly studied species of Chlamydomonas and has a relatively simple genome, which has been sequenced in many different strains, including non-motile strains. More importantly, various strains of *C. reinhardtii* have been developed for specific research purposes. The role of CAs in pH regulation of this alga needs to be investigated. Future studies focusing on the role of CAs in lipid biosynthesis will give us information which CAs are involved in the synthesis and accumulation of lipids in this alga.

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