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REVIEW

Carbonic anhydrase in relation to higher plants

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Abstract

The review incorporates recent information on carbonic anhydrase (CA, EC: 4.2.1.1) pertaining to types, homology, regulation, purification, *in vitro* stability, and biological functions with special reference to higher plants. CA, a ubiquitous enzyme in prokaryotes and higher organisms represented by four distinct families, is involved in diverse biological processes, including pH regulation, CO_2 transfer, ion exchange, respiration, and photosynthetic CO_2 fixation. CA from higher plants traces its origin with prokaryotes and exhibits compartmentalization among their organs, tissues, and cellular organelles commensurate with specific functions. In leaves, CA represents 1–20 % of total soluble protein and abundance next only to ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCO) in chloroplast, facilitating CO_2 supply to phospho*enol*pyruvate carboxylase in C_4 and CAM plants and RuBPCO in C_3 plants. It confers special significance to CA as an efficient biochemical marker for carbon sequestration and environmental amelioration in the current global warming scenario linked with elevated CO_2 concentrations.

Additional key words: carbon sequestration; compartmentalization; isozymes; phylogeny; regulation.

Introduction: Carbonic anhydrase (CA, EC: 4.2.1.1), a ubiquitous enzyme among living organisms, catalyses the following reversible inter-conversion of HCO_3^- and CO_2:

$$CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$$

CA was discovered in bovine red blood cells by Meldrum and Roughton (1933). The presence of CA in plant tissues was disputed for almost a decade. With the use of sulphydryl protecting agents, Bradfield (1947) demonstrated ubiquitous presence of CA in plants. However, with its identification in *Neisseria sicca* by Veith and Blankeship (1963), the enzyme traces its origin from prokaryotes. Till date, the enzyme has been purified from five different prokaryotes, *e.g*. *Neisseria sicca* (Adler *et al.* 1972), *Rhodospirillum rubrum* (Gill *et al*. 1984), *Anabaena variabilis* (Yagawa *et al*. 1984), *Methanosarcina thermophila* (Alber and Ferry 1994), and *Acetobacterium woodii* (Braus-Stromeyer *et al*. 1997). CA has also been reported in extracts from diverse land plants and algae (Waygood and Clendenning 1950, Waygood 1955, Everson and Slack 1968, Bowen 1969, Chen *et al*. 1970) and subsequently from wide groups of terrestrial plants (Reed and Graham 1981, Graham *et al*. 1984), animals, algae, cyanobacteria, and bacteria (Aizawa and Miyachi 1986, Tsuzuki and Miyachi 1989, Colman 1991, Nimer *et al*. 1997). The present review, however, lays emphasis on recent aspects of CA in higher plants.

Types of CA: No other family of enzymes except CA has been thoroughly characterized at catalytic, cellular, and tissue levels across all life forms. Based on their amino acid sequences, CAs can be categorized into three independent families designated as alpha, beta, and gamma (Hewett-Emmett *et al*. 1996). Plants have all three types of CAs as *Arabidopsis thaliana* genome represents them all. However, animals possess only the alpha type. Recently, CA from *Thalassiosira weissflogei* was isolated and sequenced. But its putative CA cDNA did not match with known CA from any of the three gene families, suggesting existence of an additional CA gene family, *i.e*. delta CA (Roberts *et al*. 1997). Table 1 provides information about different CAs whose detail accounts may be consulted from Karlsson *et al*. (1998), Smith *et al*. (1999), Kimber and Pai (2000), Liljas and Laurberg (2000), Moroney *et al*. (2001), Strop *et al*. (2001), and Tripp *et al*. (2001).

CA homology among various organisms: The amino acid composition of CA from plants and tissues of vertebrate animals is very similar (Pocker and Sarkanen 1978,

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Table 1. Characteristics of alpha, beta, gamma, and delta carbonic anhydrases.

Parameters	Alpha	Beta	Gamma	Delta
Evolution	200-300 million years ago (Hewett-Emmett and Tashian 1996)	Data not available	3.0–4.5 billion years ago (Smith *et al.* 1999, Jiang and Gupta 1999)	Data not available
Occurrence	Humans, animals, higher plants, green algae, eubacteria and viruses (Smith *et al.* 1999)	Angiosperms and green algae (Eriksson *et al.* 1996), archaebacteria, eubacteria (Hewett-Emmett and Tashian 1996)	*Methanosarcina thermophila* - acetate utilizing methanogenic anaerobe (Alber and Ferry 1994)	Marine diatom, *Thalassiosira weissflogei* (Roberts *et al.* 1997)
Structure				
(a) Polypeptide units	Monomer and trimer	Dimer, tetramer, hexamer, octamer	Homotrimer	Monomer
(b) Polypeptide nature	Ten stranded, antiparallel β sheet (Strop *et al.* 2001)	Four stranded, parallel β sheet core with α helices (Mitsahashi *et al.* 2000)	Left handed, parallel β-helix	Data not available
(c) Active site configuration	Zn coordinates with three histidine and one water molecule (Christianson and Cox 1999)	Zn ligates with two conserved cysteines and one conserved histidine (Kimber and Pai 2000)	Zn ligates with three histidine along with two water molecule (Kisker *et al.* 1996)	As in alpha CA (Cox *et al.* 2000)
(d) Metal	Zn (Cox *et al.* 2000)	Zn (Tripp *et al.* 2001)	Zn (Alber and Ferry 1996), Fe and Co (Alber *et al.* 1999)	Cd (Roberts *et al.* 1997) 43 kDa (Roberts *et al.*, 1997)
(e) Molecular mass	29 kDa (Karlsson *et al.* 1995)	22 kDa (Eriksson *et al.* 1998)	Data not available	
Inducibility	High CO_2 inducible protein (Ghoshal and Goyal 2001)	Low CO_2 inducible protein (Eriksson *et al.* 1996)	Data not available	Data not available
Functions	Tissue mineralization, intra-ocular pressure regulation (Kimber and Pai 2000)	pH regulation (Tashian 1989), photosynthesis (Khan 1994), diffusion and transport of inorganic carbon (Smith and Ferry 2000)	Enhancement of dehydration rate in presence of cobalt (Roberts *et al.* 1997)	Data not available
Inhibitor	Highly sensitive to inhibition by sulfonamides (Earnhardt *et al.* 1998)	Data not available	Low inhibition by sulphonamide (Alber and Ferry 1996)	Data not available

Reed and Graham 1981, Graham *et al*. 1984, Burnell *et al*. 1990). However, a remarkably high content of sulphur containing amino acids methionine and cysteine is found only in plants. The amino acid sequence of mature spinach CA shows more than 75 % homology with the pea enzyme, suggesting a high degree of structural homology among CAs from higher plants. Furthermore, the predicted amino acid sequences of the pea and spinach CA share only 22 % similarities with that of CA from the cyanobacterium (Fukuzawa *et al*. 1992). The deduced amino acid sequence from pea CA shows significant homology to the cyanate permease from *E. coli* (Majeau and Coleman 1991). A genomic CA clone was isolated from *A. thaliana* library in λEMBL4 using spinach CA cDNA as a probe (Fawcett *et al*. 1990). Comparison of the genomic sequence with the cDNA sequence reveals nine exons and eight introns (Raines *et al.* 1992). All splicing junctions between exons and introns are well conserved and follow GT-AG rule as laid down by Mount (1982). The intron between first exon and second exon is the largest, consisting of 946 nucleotides. The remaining seven introns are approximately 100 nucleotides in length. The first exon encodes the transit sequence, whereas the second exon represents the largest sequence. The deduced amino acid sequence of the encoded protein has 74 % similarity with that of spinach CA (Kim *et al.* 1994). The deduced amino acid sequence from the corresponding cDNA of extra cellular CA from *Chlamydomonas reinhardtii* exhibits sequence homology especially to the human CAs but relatively low similarity to CA1 alpha types of animals (40.0 % identity) and the bacterium *Neisseria gonorrhoeae* (40.6 % identity). The algal homologue has most of the conserved domains characteristic of alpha CA family, with three histidine residues forming hydrogen bond network with zinc (Fukuzawa *et al*. 1990). In contrast, no such homology has been observed with the CA from spinach and pea (Burnell *et al*. 1990, Fawcett *et al*. 1990, Majeau and Coleman 1991). Nucleotide sequence analysis of CA gene from spinach (Fawcett *et al*. 1990), pea (Majeau and Coleman 1991, 1992), tobacco (Majeau and Coleman 1992), *Arabidopsis* (Kim *et al*. 1994), *Chlamydomonas* (Karlsson *et al*. 1998), *Gossypium* (Moroney and Somanchi 1999), and a diatom (Lane *et al*. 2000) has also been investigated. In *Gossypium*, nucleotide sequence analysis reveals two different CA isoforms (Moroney and Somanchi 1999). Recently, homologous sequences of a beta CA were found in *Methanobacterium thermoautotrophicum* (bacterium), *Saccharomyces cerevisiae* (fungus), and *Caenorhabditis elegans* (nematode). In addition, gamma CA sequences of *Methanosarcina thermophila* are homologous to those of plants and eubacteria (Alber and Ferry 1994).

Gene expression and post-translation processing of CA in plants: Okabe *et al*. (1984) found that the protein translated with poly (A^+) mRNA in soybean reacts with antibody raised against CA, suggesting CA protein to be synthesized in the cytoplasm and then transported to the chloroplasts. The cDNA encoding chloroplast CA in spinach (Burnell *et al*. 1990, Fawcett *et al*. 1990) and pea (Majeau and Coleman 1991) was isolated and characterized. In both cases, enzyme is transcribed in the nucleus and synthesized as transit peptides of 27.6–34.6 kDa in spinach and 35.7 kDa in pea that are transported to plastids and subsequently processed to yield a mature protein of 25.5 kDa (spinach) and 24.2 kDa (pea). Northern blotting of CA cDNA reveals single transcripts of 1.00–1.45 kb pairs, which are visible on being hybridized with mRNA isolated from irradiated leaf and stem tissues but not with mRNA from CA enriched etiolated leaves and roots. These results may indicate that the chloroplast CA is light-regulated and different from non-plastid CA (Majeau and Coleman 1991). Goyal *et al*. (1992) have demonstrated that CA isozymes in *Dunaliella* species are induced by salt and classical sulfonamide inhibitors such as ethoxyzolamide and acetazolamide (a potent inhibitor for periplasmic CA activity). In *Ch. reinhardtii*, five genes encoding CA isozymes were identified. The gene products of *cah1* and *cah2* are directed to the periplasmic space (Fujiwara *et al*. 1990), the *cah3* encodes the chloroplast CA (Funke *et al*. 1997), and the other two genes encode mitochondrial CA (Eriksson *et al*. 1996). Two distinct cDNAs (β-CA1 and β-CA2) encoding mitochondria CAs identified from *Ch. reinhardtii* are likely to be a product of gene duplication as both share 97 % similarity among their amino acid sequences. Low CO_2 conditions and photon flux density (PFD) rather than quality facilitate their expression. Both mitochondria CAs share amino acid sequences with that from *Synechocystis* species (50 % identity and 66 % similarity). The periplasmic CA is synthesized as a 41.6 kDa precursor that is post-translationally cleaved and glycosylated to 35–38 kDa with 4 kDa small subunit. The holoenzyme is heterodimer composed of two large and two small subunits joined together by a disulfide bond (Sultemeyer *et al*. 1998).

Regulation: Biosynthesis of CA is regulated by photon flux density (PFD), CO_2 concentration, and availability of Zn. The 5'-flanking region of the CA gene contains sequences with homology to the G box, GT box, and I box (Kim *et al*. 1994). These motifs play roles in tissue specific and light-modulated expression of the small subunits of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCO) (Green *et al*. 1987, Giuliano *et al*. 1988). High CO_2 concentrations do not affect CA in 5–10-d-old leaves but depress CA activity in 15-d-old (25 % loss) and 25-d-old (75 % loss) cotton plants (Chang 1975a). However, maize and pea plant show a little change of CA activity at 10 % CO_2 in air (Graham *et al*. 1971). But Cerfigni *et al*. (1971) have shown 20 % gain (maize) and 30 % loss (pea) at 0.06 % CO_2. Zinc deficiency causes decrease in CA activity in leaves of navy beans (Edwards and Mohamed 1973), spinach (Randall and Bauma 1973), and rice (Sasaki *et al*. 1998).

Table 2. Purification of carbonic anhydrases from different plants.

Plant	Extent of purification [fold]	Overall yield [%]
Cotton (*Gossypium hirsutum*)	33	-
Lettuce (*Lactuca sativa*)	900	1
Navy bean (*Phaseolus vulgaris*)	3.5	90
Parsley (*Petroselinum crispum*)	133	80
Pea (*Pisum sativum*)	200	32
Spinach (*Spinacia oleracea*)	330	7
Tomato (*Lycopersicon lycopersicum*)	6	40
Tradescantia albiflora	24	30

Table 3. Molecular mass of carbonic anhydrase in different plants.

Taxonomic group	Species	Molecular mass [kDa]
Monocot	*Zantadeschia aethiopica, Cana indica, Triticum vulgare, Dietes iridioides, Chlorophytum comosum, Cymbidium, Chamaedorea erumpens, Typha* spp., *Sagittaria graminea, Amaryllis belladonna, Tradescantia albiflora* (Atkins *et al.* 1972a)	42-45
Dicot	*Spinacia oleracea* (Rossi *et al*. 1969, Pocker and Ng 1973),	148-180
	Petroselinum crispum (Tobin 1970),	180
	Amaranthus hybridus, Beta vulgaris, Helianthus annuus, Convolvulus mauritanicus, Raphanus sativus, Pisum sativum, Passiflora edulis, Rheum raponcicum, Grevillea rosmarinifolia, Fortanella japonica, Hydrangea macrophylla, Lantana camara (Atkins *et al.* 1972a),	140-250
	Pisum sativum (Atkins *et al.* 1972a, Kisiel and Graf 1972)	188-194
	Phaseolus vulgaris (Atkins 1974)	205
	Lactuca sativa (Walk and Metzner 1975)	195

Purification and *in vitro* stability of CA: CA has been isolated and purified to various degrees from a number of plants, *i.e.* parsley (Tobin 1970), pea (Kisiel and Graf 1972), *Tradescantia albiflora* (Atkins *et al*. 1972a), spinach (Pocker and Ng 1973), navy bean (Atkins 1974), tomato (Kositsin and Khalidova 1974), cotton (Chang 1975a,b), and lettuce (Walk and Metzner 1975). The greatest purification was in the order: lettuce>spinach> pea>parsley (Table 2). However, available information about the stability of CA is fairly old (Poincelot 1972). CA is thermostable but inactivated by certain inhibitors such as acetazolamide (Everson 1970), ethoxyzolamide (Tobin 1970), and azide (Atkins *et al*. 1972a) or some other chemical reagents like arsenite, nitrite, nitrate, iodide, chloride, mercury, and magnesium (Rossi *et al*. 1969). CA from peas at pH 8.1 retains 40 % activity on incubation for 5 min at 60 °C (Kisiel and Graf 1972) and that from cotton at pH 8.0 losses 50 % activity on incubation for 5 min at 65 °C but less than 50 % activity on incubation for 20 min at 55 °C (Chang 1975c). CA from peas and *Tradescantia* exhibit maximal stability at pH 8.25 (Atkins *et al*. 1972a,b). In most cases, CA has been investigated in the presence of sulfhydryl reducing agent. CA activity in parsley is rapidly lost without sulfhydryl agent (Tobin 1970). However, the reverse appears to be true for CA from spinach exhibiting prolonged storage life with little loss in activity for 50 h at room temperature or for 1 year at 4 °C (Pocker and Ng 1973) and that from cotton maintaining activity for 20 h at 4 °C (Chang 1975c) without protective sulfhydryl agent. We stored CA leaf extract from teak (*Tectona grandis*) without sulfhydryl reducing agent for one year at 0 °C with periodic checking of activity, which remained stable for initial four months and subsequently albeit gradually declined to 50 % towards the end of the year (unpublished data).

CA characteristics in plants

Molecular mass: CA was found in high concentration in plant leaves (Bradfield 1947, Chen *et al*. 1970, Atkins *et al*. 1972a). Two main forms of CA from leaf extracts of higher plants, *i.e.* monocotyledon type and dicotyledon type (Table 3), were identified based on their behaviour on polyacrylamide gradient gel electrophoresis. More than one band of enzyme was found on gels from most species, suggesting the existence of CA isozymes in higher plants (Atkins 1972a,b). The native plant CA occurs in various oligomeric forms in different species with molecular mass of 42–250 kDa (Reed and Graham 1981, Graham *et al*. 1984), which is much higher than that from prokaryotes (Table 4). The subunits of molecular mass between 4 and 30 kDa (Burnell *et al*. 1990, Fawcett *et al*. 1990, Majeau and Coleman 1991) are probably held together by disulfide bonds (Kamo *et al*.

1990). But considerable difference occurs in the molecular masses of CA holoenzyme between monocotyledons (42–45 kDa) and dicotyledons (140–250 kDa). CA from *Pisum sativum* (Atkins *et al*. 1972a) and *Spinacia oleracea* (Pocker and Ng 1973) has six subunits each and K_m values of 30 and 50 mM CO_2, respectively.

Table 4. Molecular mass of carbonic anhydrase in microbes (Smith *et al.* 1999).

Species	Molecular mass [kDa]		
	Alpha	Beta	Gamma
Archaea domain	–	21–24	17–37
Bacteria domain	22–23	22–26	18–20

Metal content: CA is a metalloenzyme requiring Zn^{2+} for its activity. Wood and Sibly (1952) found that Zn deficiency reduces the content of CA in oats and tomatoes. Zinc deficiency could inhibit the growth by reducing the available content of Zn in the plant, directly affecting metabolism through upsetting the balance of other nutrients in the plants such as iron, phosphorus, and copper (Millikan 1953, Polson 1968). The effect of Zn deficiency on CA may not be selective as it causes a general decrease in protein synthesis. A positive correlation between leaf protein N and CA under Zn deficiency may indicate a general repression of protein synthesis. However, the association of Zn with CA has been reported from pea, lettuce, parsley, *Tradescantia*, and spinach (Tobin 1970, Atkins *et al*. 1972a, Kisiel and Graf 1972, Pocker and Ng 1973, Walk and Metzner 1975). Parsley enzyme contains one atom of Zn per subunit of 30 kDa (Tobin 1970) but Rossi *et al*. (1969) prepared a Zn-free CA extract. On the other hand, the CAs containing Co and Cd were also published (Price and Morel 1990, Morel *et al*. 1994, Lee and Morel 1995, 1996, Yee and Morel 1996). The enzyme containing Co is less active than the native Zn form, and the *in vitro* substitution of Co in place of Zn in alpha CA also results in a significant decrease in activity (Tu and Silverman 1985).

Isozymes: Enzymes often exist in multiple forms, varying in their molecular masses and activities for regulation of metabolism. These forms are called isozymes, which play a great role in adaptation of organisms and are utilized as co-dominant biochemical markers for identification of genotypes and establishment of phylogenic relationship among different groups of taxa. Atkins *et al*. (1972a) demonstrated the presence of CA isozymes in 24 species of monocotyledonous and dicotyledonous plants (Table 5). Subsequently, CA isozymes from pea (Kachru and Anderson 1974) tomato (Kositsin and Khalidova 1974), and lettuce (Walk and Metzner 1975) were isolated. CA isozymes are localized in plasmalemma (Badger and Price 1994), chloroplast (Husic and Markus 1994), mitochondria (Eriksson *et al*. 1996), and cytoplasm (Hiltonen *et al*. 1998). Cytoplasmic and chloroplastic isozymes of CA are present in leaves of C_3 plants with the cytosolic CA having a higher molecular mass than the chloroplastic form (Reed and Graham 1981). The two isozymes of CA detected in pea (Kachru and Anderson 1974) and cotton (Moroney and Somanchi 1999) are possibly localized in cytoplasm and chloroplast, respectively. Pea CA isozymes have iso-electric points of pH 5.75 and 6.30 (Kachru and Anderson 1974). The taxonomic diversity of plant CAs was also demonstrated using antibodies against spinach leaf CA which showed cross-reactivity with the leaf extracts from several C_3 monocotyledons, C_3 and C_4 dicotyledons, and Crassulacean Acid Metabolism (CAM) species (Okabe *et al*. 1984, Burnell 1990). On the other hand, CA extracts from green algae, C_4 monocotyledonous species, and bovine erythrocytes have no cross immunoreactivity with antibodies against spinach leaf CA. Chloroplast CA (alpha type) has a number of isozymic forms (I–VII). Among these, chloroplast CA isozyme-II is the best characterized and occurs in many cell types (Kimber and Pai 2000).

CA in C_3, C_4, and CAM plants: RuBPCO competitively binds with CO_2 or O_2. However, binding with O_2 triggers photorespiration *vis-à-vis* reduction in photosynthesis. Close association of CA with RuBPCO increases the availability of CO_2 at the site of carboxylation (Everson and Slack 1968, Graham and Reed 1971, Poincelot 1972, Werdan *et al*. 1972). Similarly, in C_4 plants, association of CA with phospho*enol*pyruvate (PEP) carboxylase (PEPC) provides continuous supply of HCO_3^- at the site of carboxylation (Rathnam and Das 1975). This is supported by the fact that CA of C_4 leaves is largely or exclusively confined to the cytosol of mesophyll cells while bundle sheath cells contain little or no CA acivity (Burnell and Hatch 1988). Thus, the distribution of CA is similar to PEPC, which uses HCO_3^- rather than CO_2 for the carboxylating reaction at neutral or slightly alkaline conditions. In mesophyll cells, CA rapidly converts diffusing atmospheric CO_2 to HCO_3^- at rates that are compatible with those for photosynthesis. In C_4 plants, CA catalyzes the first critical step of C_4 photosynthesis, the hydration of CO_2 to bicarbonate, which PEPC uses as the substrate for carboxylation of PEP to oxaloacetate in the cytosol of mesophyll cells (Burnell and Hatch 1988, Badger and Price 1989). The inorganic carbon substrate HCO_3^- of this enzyme was recognized as being supplied by CA (Burnell and Hatch 1988, Hatch and Burnell 1990). Burnell *et al*. (1990) indicated closely related mechanism controlling the expression of maize leaf PEPC and CA activities. The maximum activity of CA in C_4 and CAM leaf extracts is similar to that in extracts from C_3 leaves (Burnell and Hatch 1988, Hatch and Burnell 1990). The K_m value ranges from 0.8 to 2.8 mM CO_2 for CAs from both C_3 and C_4 plants (Hatch and Burnell 1990), which otherwise behave similarly against antibodies raised for maize CA. However, CAs from both C_3

Table 5. A comparison of carbonic anhydrase between monocotyledons and dicotyledons. Means ± standard deviation. Monocotyledons (Amaryllideaceae*–Typhaceae**) and Dicotyledons (Proteaceae*–Verbeneaceae**).

Type	Family	Migration on gel [cm]	Activity [U mg^{-1}(chlorophyll)]
Monocotyledons	11	5-6	3 798±2 772 (330*–9 040**)
Dicotyledons	13	3-4	5 509±3 518 (813*–13 850*)

and C_4 plants exhibit apparent differences with respect to their sensitivity to several inhibitors (Burnell 1990) as well as to their isozymic forms based on molecular masses and residual activity in the supernatant after cross reactivity with maize CA antibodies (Okabe *et al*. 1984, Burnell *et al*. 1990).

CA compartmentalization in higher plants: CA exhibits a wide range of distribution patterns among organs, tissues, and cellular organelles commensurate with its diverse physiological roles. The enzyme has been found in high amounts in leaves of plants (Bradfield 1947, Waygood 1955, Chen *et al*. 1970, Atkins *et al*. 1972a), leguminous root nodules (Atkins 1974), and grape and pea roots (Champagnol 1976, Goustiana *et al.* 1998). In leaves of higher plants, CA protein is abundant accounting for 1–2 % of total soluble protein (Okabe *et al*. 1984). However, the enzyme activity follows pattern: leaves>stem>pods but is absent in root tissue (Majeau and Coleman 1994).

In C_3 plants, CA activity was found in mesophyll cells of leaves (Everson and Slack 1968). The location of CA in C_4 plants is unclear. Some reports indicate that the bulk of the activity is confined to the mesophyll cells (Graham *et al*. 1971, Gutierrez *et al*. 1974). Poincelot (1972) describes distribution of CA between the mesophyll cells and bundle sheath cells. Enhanced content of CO_2 in bundle sheath cells leads to a large diffusion gradient between bundle sheath and mesophyll cells, resulting in leakage of CO_2 from bundle sheath cells whereas CA of mesophyll cells recaptures CO_2 by converting it to HCO_3^- and prevents its complete loss to the atmosphere. Furthermore, CA of C_4 plants is largely confined to the mesophyll cells while bundle sheath cells contain little or no activity (Burnell and Hatch 1988).

Mesophyll chloroplasts isolated with aqueous or non-aqueous media from C_3 plants contain the most of the CA activity, whereas a small part of it is associated with the whole leaf (Everson 1970, Poincelot 1972, Chang 1975a). Whether all the activity resides in the chloroplast or a small part of it is associated with the cytoplasm is uncertain. Gutierrez *et al*. (1974) and Ku and Edwards (1975) reported that CA is localized in the cytoplasm of mesophyll cells. However, by using polyacrylamide gel electrophoresis for extracts from normal green leaves, C_3 plants exhibit both chloroplast and cytosol forms of CA (Atkins *et al*. 1972a, Kachru and Anderson 1974, Walk and Metzner 1975). Kachru and Anderson (1974) reported chloroplast and cytosol forms of CA in pea leaves based on separation by isoelectric focusing. Nishimura *et al*. (1976), using spinach protoplast extracts to analyze the compartmentalization of several enzymes by sucrose density centrifugation technique, have made dubious conclusion that major part of CA resides in cytosol, presuming loss of chloroplast CA during preparation of the sample. Tsuzuki *et al*. (1981, 1985), using protoplast isolates from the leaves of wheat and spinach, showed CA to be exclusively located in chloroplasts, facilitating diffusion of inorganic carbon in solution by converting CO_2 to bicarbonate. They also demonstrated localization of CA in chloroplasts but not in the cytosol of mesophyll cells of higher C_3 plants.

Reed (1979) demonstrated CA activity to be associated with two proteins of relatively high but unequal molecular masses. The absence of smaller of the two proteins in etiolated tissue of *Brassica chinensis* and spinach as well as white tissues from variegated leaves of *Hedera canariensis* and *Tradescantia albiflora* suggests that the large protein resides in cytosol and small protein in chloroplast. Tsuzuki *et al*. (1985) observed induction of CA commensurate with greening (chlorophyll synthesis) and expression of RuBPC in light-exposed etiolated tissue of wheat. This is consistent with earlier suggestions made by Okazaki *et al*. (1976) that CA may have role in photosynthesis due to its localization in chloroplast. Northern blot analysis also confirms absence of transcripts homologous to the chloroplast CA in etiolated leaves and roots (Majeau and Coleman 1994).

As for partition within cellular organelles, CA in higher plants has been found in the chloroplast stroma of the leaf mesophyll cells in *Tradescantia* (Atkins *et al*. 1972a), tobacco (Majeau *et al*. 1994, Price *et al*. 1994), and barley (*cf*. Moroney *et al*. 2001). Karlsson *et al*. (1995) reported intracellular alpha CA from *C. reinhardtii* that is located inside the thylakoid lumen. Nevertheless, the evidence for a thylakoid-associated CA in higher plants remains dubious. Stemler (1997) measured CA activity in thylakoids and photosystem 2 (PS2) preparations of maize and pea. However, Western blot test shows that polypeptides from pea PS2 membrane preparations do not cross-react with antibodies raised against the pea stroma CA, indicating that the enzyme activity comes from thylakoid membranes (*cf*. Moroney *et al*. 2001).

CA function in different cellular organelles: The cytoplasm CA, a 110 kDa polypeptide, maintains HCO_3^- pools and compensates leakage of free CO_2 from the cytosol. Multiple isoforms of cytoplasmic CA have been

found in *Dunaliella* species (Goyal *et al*. 1992), *Chlorella* (Karlsson *et al*. 1998), and *Coccomyxa* (Hiltonen *et al*. 1998). They are less sensitive to inhibition by sulfonamide compared to periplasmic CA (Moroney *et al*. 1987, Fukuzawa *et al*. 1990). The chloroplast CA (alpha type family), a 29.5 kDa protein, is associated with the insoluble fraction that could be solubilized with salt (Karlsson *et al.* 1995). Under PAR, the enzyme is highly sensitive to inhibition by sulfonamides (Eriksson *et al*. 1998). The expression of chloroplast CA encoded by *cah 3* gene increases upon transfer of cells to a low CO_2 concentration and is constitutively present even at high CO_2 concentrations. The enzyme contains putative signal peptide with amino acid sequences similar to those of target lumen protein of the thylakoids (Karlsson *et al*. 1998). Based on the sequence data, chloroplastic CA is present in the lumen of thylakoids. The lumen CA works at pH 5.0 and converts HCO_3^- to CO_2 at the inner side of the thylakoid membrane, using acidification of the irradiated thylakoid with HCO_3^- acting as an uncoupler. The HCO_3^- is transported along with light-dependent movement of proton (H^+) across the thylakoid membrane into the lumen, gets converted to CO_2 by CA, and leaks into the stroma. The mitochondrial CA, 22 kDa low CO_2 inducible beta type protein (Eriksson *et al*. 1996), is involved in the pH regulation and/or diffusion and transport of inorganic carbon within mitochondrial matrix (Pesheva *et al*. 1994, Eriksson *et al*. 1998). Two distinct cDNAs (β-CA1 and β-CA2) encoding mitochondria CA were characterized from *Ch. reinhardtii*. The amino acid sequences of these two cDNAs are 97 % identical, sharing amino acid sequences with those of CA from *Synechocystis* species (50 % identity and 66 % similarity).

Role in photosynthesis: CA is involved in a variety of biological processes including pH regulation, CO_2 transfer, ion exchange, respiration, biosynthesis, and photosynthetic CO_2 fixation (Tashian 1989, Badger and Price 1994, Smith and Ferry 2000, Moroney *et al*. 2001). Any change in CA activity directly affects the rate of photosynthetic CO_2 fixation under CO_2 limiting conditions. CA is the only enzyme of photosynthetic carbon metabolism, which is known to fluctuate in activity in a number of species with changes in environmental CO_2 concentration. Its activity rapidly decreases on bubbling air from low CO_2 to high CO_2 and *vice versa* (Imamura *et al*. 1981). The functions of CA in photosynthetic CO_2 fixation are: (*1*) The rapid dehydration of stored HCO_3^-, which is potential CO_2 source as a substrate to RuBPCO. (*2*) The hydration of CO_2 to form HCO_3^-, for PEPC as substrate in C_4 and CAM plants. (*3*) The facilitation of CO_2 diffusion across the plasma membrane and chloroplast envelope by extracellular and intracellular CA. (*5*) Participation of active transport of CO_2 across the plasma membrane by conversion of CO_2 to HCO_3^-, which is the carbon species entering the cell.

Conclusion: Emergence of metallozymes containing zinc (CA and DNA/RNA polymerases) and iron-molybdenum (nitrogenase, nitrate reductase) seems logically to have set stage for origin of life, for they facilitate steady supply of carbon and nitrogen to biological system for its constitution, maintenance, and function. Therefore, there is no wonder that these enzymes are associated with primitive self-sustaining life forms, *i.e*. prokaryotes. CA performs the first step of carbon sequestration in biological systems, which accounts for its ubiquitous evolution and distribution in all life forms. Initially, CA protein appears to be non-specific with respect to acceptance of zinc as has been evident from the existence of CA containing cadmium or cobalt for catalytic purpose (Roberts *et al.* 1997, Alber *et al.* 1999). Further evolution in CA protein seems to have proceeded for selection of zinc as the most suitable catalytic metal. However, evolutionary divergence of CA proteins has initiated among prokaryotes, resulting in at least three independent lines represented by *Neisseria gonorrhaeae*, *Methanobacterium thermoautotrophicum,* and *Methanosarcina thermophila*, which contribute CA sequences to diverse organisms. However, phylogeny of CA of diatom (*Thalassiosira weissflogii*) with prokaryotes remains obscure (Fig. 1). As evolution of CAs has proceeded among different organisms, the protein has gained molecular masses and functional diversity in eukaryotes, employing core catalysis of CO_2/HCO_3^- interconversion. Doing so, CAs have compartmentalized as diverse isozymes in organs, tissues, and organelles as well as come under control of promoters commensurate with tissue specific and light-modulated expression in plants.

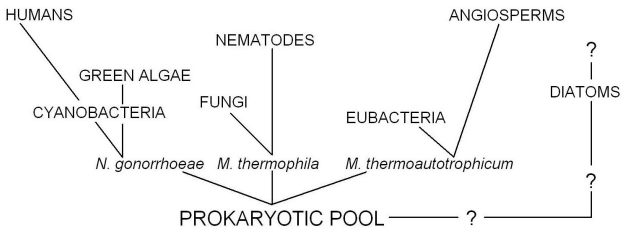

Fig. 1. A possible evolutionary relationship of carbonic anhydrase among different organisms from the findings of Okabe *et al.* (1984), Burnell (1990), Fawcett *et al.* (1990), Fukuzawa *et al.* (1990, 1994), Majeau and Coleman (1991), Alber and Ferry (1994), and Moroney and Somanchi (1999).

Co-existence and feeding of RuBPCO in C_3 plants and PEPC in C_4/CAM plants with carbon source by CA (Hatch and Burnell 1990) assumes special significance for global forests, which sequesters to the tune of 90 % terrestrial carbon pool (Woodwell 1978) responsible for burgeoning global warming (Houghton 1990). This view is consistent with the finding of Khan (1994), who noted significant positive correlation of CA with photosynthesis and dry mass at 50, 70, and 90 d in 12 cultivars of mus-

tard (*Brassica juncea* L.). Recently, we have also established that 47.6 % half-sib families of teak (*Tectona grandis* L. f.), a paragon tropical timber, exhibit a strong significant positive correlation between CA and photosynthesis (unpublished data). Thus, CA qualifies to be recognized as a biochemical marker for selection of plants for high rate of carbon sequestration both to mitigate ill effect of global warming as well as to enhance biomass/-staple food production to cater to the need of people on a sustainable basis.

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