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Prokaryotic carbonic anhydrases

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Abstract

Carbonic anhydrases catalyze the reversible hydration of CO_2 [$CO_2+H_2O \rightleftharpoons HCO_3^-+H^+$]. Since the discovery of this zinc (Zn) metalloenzyme in erythrocytes over 65 years ago, carbonic anhydrase has not only been found in virtually all mammalian tissues but is also abundant in plants and green unicellular algae. The enzyme is important to many eukaryotic physiological processes such as respiration, CO_2 transport and photosynthesis. Although ubiquitous in highly evolved organisms from the Eukarya domain, the enzyme has received scant attention in prokaryotes from the Bacteria and Archaea domains and has been purified from only five species since it was first identified in *Neisseria sicca* in 1963. Recent work has shown that carbonic anhydrase is widespread in metabolically diverse species from both the Archaea and Bacteria domains indicating that the enzyme has a more extensive and fundamental role in prokaryotic biology than previously recognized. A remarkable feature of carbonic anhydrase is the existence of three distinct classes (designated α, β and γ) that have no significant sequence identity and were invented independently. Thus, the carbonic anhydrase classes are excellent examples of convergent evolution of catalytic function. Genes encoding enzymes from all three classes have been identified in the prokaryotes with the β and γ classes predominating. All of the mammalian isozymes (including the 10 human isozymes) belong to the α class; however, only nine α class carbonic anhydrase genes have thus far been found in the Bacteria domain and none in the Archaea domain. The β class is comprised of enzymes from the chloroplasts of both monocotyledonous and dicotyledonous plants as well as enzymes from phylogenetically diverse species from the Archaea and Bacteria domains. The only γ class carbonic anhydrase that has thus far been isolated and characterized is from the methanoarchaeon *Methanosarcina thermophila*. Interestingly, many prokaryotes contain carbonic anhydrase genes from more than one class; some even contain genes from all three known classes. In addition, some prokaryotes contain multiple genes encoding carbonic anhydrases from the same class. The presence of multiple carbonic anhydrase genes within a species underscores the importance of this enzyme in prokaryotic physiology; however, the role(s) of this enzyme is still largely unknown. Even though most of the information known about the function(s) of carbonic anhydrase primarily relates to its role in cyanobacterial CO_2 fixation, the prokaryotic enzyme has also been shown to function in cyanate degradation and the survival of intracellular pathogens within their host. Investigations into prokaryotic carbonic anhydrase have already led to the identification of a new class (γ) and future research will undoubtedly reveal novel functions for carbonic anhydrase in prokaryotes.

Keywords: Carbonic anhydrase; Zinc; CO_2; HCO_3^-; Sulfonamide; CO_2-concentrating mechanism

Contents

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1. Introduction

The environmental concentration of CO_2, a key metabolite in all living organisms, is seldom high. In addition, CO_2 is in equilibrium with HCO_3^-, carbonic acid and carbonate of which HCO_3^- is the most physiologically important. HCO_3^- is negatively charged and highly soluble in aqueous solution but poorly soluble in lipids, while CO_2 is highly soluble in both aqueous solution and lipids. Therefore, although CO_2 can freely diffuse in and out of the cell, HCO_3^- must be transported across the cell membrane. Above pH 6.3, the equilibrium between the two species shifts toward HCO_3^-, thus posing problems in the maintenance of required intracellular CO_2 and HCO_3^- concentrations. Not surprisingly, the interconversion of the planar CO_2 and the pyramidal HCO_3^- is slow at physiological pH and requires enzymatic catalysis. Conversion of HCO_3^- to CO_2 may facilitate its transport into the cell while conversion of CO_2 to HCO_3^- may be important for trapping CO_2 in the cell. Thus, enzymatic conversion of CO_2 and HCO_3^- not only allows the cell to concentrate CO_2 to the levels required for cellular enzymes but also helps the cell maintain the proper intracellular levels of CO_2 and HCO_3^- to carry out cellular processes.

Carbonic anhydrase is a Zn-containing enzyme that catalyzes the interconversion of carbon dioxide and bicarbonate:

$$CO_2 + H_2O \rightleftharpoons HCO_3^- + H^+ \quad (1)$$

This enzyme has been found in virtually all mammals as well as plants and algae and has been shown to be fundamental to many eukaryotic biological processes such as photosynthesis, respiration, CO_2 and ion transport, calcification and acid–base balance. Three evolutionarily distinct classes of carbonic anhydrase have been identified and designated α, β and γ [1]. No significant sequence similarities are observed between representatives of the different classes; thus, the carbonic anhydrase classes are excellent examples of convergent evolution of catalytic function.

The enzymology [2–6] and physiology [7–13] of carbonic anhydrases from the Eukarya domain, particularly those from humans, have been well studied. Although ubiquitous throughout the Eukarya domain, the enzyme has received less attention in prokaryotes from the Bacteria and Archaea domains, having been purified from only five species. The availability of antisera to enzymes from each class and the emergence of genome sequencing have recently been utilized to perform a comprehensive search for carbonic anhydrase in metabolically and phylogenetically diverse prokaryotes [14]. The enzyme was shown to be widespread in both the Bacteria and Archaea domains with the β and γ classes predominating. All of the mammalian isozymes belong to the α class; however, very few α class carbonic anhydrase genes have thus far been found in the Bacteria domain and none in the Archaea domain. The β class is comprised of enzymes from the chloroplasts of both plants and algae as well as enzymes from phylogenetically diverse species from the Archaea and Bacteria domains. Although the only γ class carbonic anhydrase that has thus far been isolated and characterized is from the methanoarchaeon *Methanosarcina thermophila* [15,16], additional sequences which could encode γ class enzymes have been identified.

Previous reviews have focused on mammalian or plant carbonic anhydrases with little attention given to the prokaryotic enzymes. The subject of this review is to examine the physiology, enzymology and structure of enzymes from both the Bacteria and Archaea domains. Many prokaryotes contain carbonic anhydrase genes from two or even all three known classes, or, as is the case in mammals, some contain multiple genes from the same class. The presence of multiple carbonic anhydrase genes suggests this enzyme has a major role in prokaryotic physiology; however, the roles are still largely unknown. Investigations into prokaryotic carbonic anhydrase have

already led to the identification of a new class (γ) and future research will undoubtedly reveal novel functions for this enzyme in prokaryotes. Thus, the purpose of this review is to alert the reader to the broad importance of this enzyme in prokaryotic physiology.

2. Background

2.1. Historical perspective

Carbonic anhydrase was independently discovered in 1933 by Meldrum and Roughton [17] and by Stadie and O'Brien [18]. The enzyme was first characterized as a result of a search for a catalytic factor that had been theoretically determined as necessary for the rapid transit of HCO_3^- from the erythrocyte to the pulmonary capillary. Meldrum and Roughton purified the erythrocyte carbonic anhydrase to a relatively high degree [17] and complete purification from bovine erythrocytes was achieved in the late 1930s [19–21]. Keilin and Martin [21] demonstrated a specific role for Zn in catalysis by finding that activity was directly proportional to the Zn content; thus, carbonic anhydrase was the first Zn metalloenzyme identified. The enzyme has subsequently been found in all animals examined for its presence. Initially, there was some doubt about its existence in plants; however, the use of sulfhydryl protectants to preserve activity during purification [22] confirmed an earlier report of a plant carbonic anhydrase by Neish [23] in 1939. Carbonic anhydrases in plants were shown to differ from those isolated in animals by size and decreased sensitivity to the sulfonamide family of inhibitors.

In 1963, Veitch and Blankenship [24] reported the first carbonic anhydrase from a prokaryote. They noted carbonic anhydrase activity in the nasal exudates of patients suffering from respiratory infections. *Lactobacillus*, nine strains of *Neisseria*, and a strain of *Streptococcus salivarius* were examined for activity to test the possibility that the carbonic anhydrase was of microbial origin. Significant activities were detected in strains of several species of *Neisseria* and in *S. salivarius*. The enzyme from *Neisseria sicca* strain 6021 was purified and found to share many of the same properties as the human carbonic anhydrases [25,26]. Carbonic anhydrase activity in the Archaea domain was first detected in the methanoarchaeon *Methanosarcina barkeri* [27], and an enzyme was first purified and characterized from the closely related *M. thermophila* in 1994 [15].

2.2. Three evolutionarily distinct classes of carbonic anhydrase

The first carbonic anhydrase (CA) sequences were obtained from the 260-residue low activity CA I [28,29] and the 259-residue high activity CA II [30,31], both isolated from the human erythrocytes. The complete amino acid sequences of the CA I and CA II isozymes from bovine, equine and other animals have followed over the years [32–35]. In fact, 10 isozymes have now been identified in humans that clearly arose from a common ancestral enzyme [1,36,37]. The crystal structures have been determined for human CA I [38], human CA II [39–41], bovine CA III [42], human CA IV [43] and a truncated form of murine CA V [44]. The overall folds of the monomeric human isozymes are highly similar with mainly antiparallel β-sheet as the dominating secondary feature.

The amino acid sequence of a single short peptide fragment of a spinach chloroplast carbonic anhydrase was obtained in 1984 and could not be aligned with the amino acid sequence of any mammalian enzyme [45]. Subsequently, the cDNA sequences encoding several plant chloroplast carbonic anhydrases were determined and shown to belong to a genetically distinct class. This was a somewhat surprising result since the amino acid sequences of two periplasmic carbonic anhydrases from the green alga *Chlamydomonas reinhardtii* had previously been shown to be most similar to those of animal carbonic anhydrases [46,47]. There are no significant sequence similarities between representatives of the animal and plant classes of carbonic anhydrase. A three-dimensional structure has yet to be reported for a plant enzyme, although CD spectra suggest a domination of α-helix structure that is in stark contrast to that of human isozymes that are composed mainly of β-sheet structures [48]. The plant class also appears to be more diverse in their quaternary structure in that the enzymes from dicotyledonous plants are presumed to be octamers in which two tetramers are linked by disulfide bridges [49], while the monocotyledonous enzymes have been suggested to be dimers [7]. Therefore, all carbonic anhydrases identified in the animal kingdom belong to a single gene class that is now referred to as α class carbonic anhydrases, while the plant enzyme class is referred to as the β class carbonic anhydrases [1].

The carbonic anhydrase from *N. sicca* was originally purified in 1972 [25,26], but the first sequence of a carbonic anhydrase from a prokaryote was not reported for another 20 years. In 1992, the product of the *cynT* gene in *Escherichia coli* was found to have carbonic anhydrase activity [50]. When the amino acid sequence was compared with those of other known carbonic anhydrases, CynT was found to be most similar to members of the plant-type or β class. Shortly thereafter, a gene showing identity to β class genes was found to be essential for photosynthetic CO_2 fixation by *Synechococcus* PCC7942 [51]. Thus, it appeared that all prokaryotic carbonic anhydrases would belong to the β class.

Alber and Ferry [15] reported the first isolation of a carbonic anhydrase from the Archaea domain in 1994. When the deduced amino acid sequence of the enzyme from *M. thermophila* was compared to the amino acid sequences of known carbonic anhydrases, no significant similarity was detected. Furthermore, the crystal structure

revealed a trimer with a novel left-handed β-helix fold [52]. These results indicated that this carbonic anhydrase represents a third distinct class, which was designated γ [1]. It would have been tempting to speculate that all carbonic anhydrases from the Bacteria domain would belong to the β class and all carbonic anhydrases from the Archaea domain would belong to the γ class. However, the deduced sequence of the *M. thermophila* enzyme showed 35% sequence identity to the deduced amino acid sequence of the *ccmM* gene from the cyanobacterium *Synechococcus* [15]. Subsequently, the deduced amino acid sequence of a purified carbonic anhydrase from *Neisseria gonorrhoeae* indicated that this enzyme belongs to the α class [53]. More recently, a putative β class carbonic anhydrase gene was identified [54] from the methanoarchaeon *Methanobacterium thermoautotrophicum* and the enzyme was characterized and shown to have carbonic anhydrase activity [55]. Thus, the β class has now been identified in all three domains of life. Therefore, although the α and β classes clearly predominate in the eukaryotes, all three genetically distinct classes of carbonic anhydrase are represented in the prokaryotes. A summary of the specific properties of the characterized prokaryotic carbonic anhydrases is shown in Table 1.

2.3. Proposed mechanism of carbonic anhydrase

The kinetic properties of human isozymes CA I, CA II and CA III from the α class have been extensively investigated and follow a common 'Zn-hydroxide' mechanism for catalysis [4,6]. The catalytically active group is the Zn-bound water which ionizes to a hydroxide ion. According to the proposed mechanism, the enzyme-catalyzed reaction occurs in two mechanistically distinct reactions (where E = enzyme and B = buffer). The first half-reaction is the interconversion between CO_2 and HCO_3^- (Eqs. 2a and 2b) in which the rate is related to the steady-state parameter k_{cat}/K_m. The second half-reaction is the regeneration of the active form of the enzyme (Eqs. 2c and 2d), involving the rate-determining intramolecular and intermolecular proton transfer events which are reflected in the steady-state parameter k_{cat}.

$$E - Zn^{2+} - OH^- + CO_2 \rightleftharpoons E - Zn^{2+} - HCO_3^- \quad (2a)$$

$$E - Zn^{2+} - HCO_3^- + H_2O \rightleftharpoons E - Zn^{2+} - H_2O + HCO_3^- \quad (2b)$$

$$E - Zn^{2+} - H_2O \rightleftharpoons {}^+H - E - Zn^{2+} - OH^- \quad (2c)$$

$${}^+H - E - Zn^{2+} - OH + B \rightleftharpoons E - Zn^{2+} - OH + BH^+ \quad (2d)$$

Most of the kinetic properties observed for the β class carbonic anhydrases and the γ class enzyme from *M. thermophila* are also consistent with this mechanism [48,56–59]; therefore, all three classes likely have a Zn-hydroxide mechanism.

3. Carbonic anhydrase is widespread in prokaryotes

The sporadic reports of the presence of carbonic anhydrase in prokaryotes would lead one to expect that this enzyme is not prevalent in the Bacteria and Archaea domains. In the past 20 years, several groups have screened certain prokaryotes for the presence of carbonic anhydrase [27,60–65]. Nafi et al. examined heterotrophic prokaryotes with antisera raised against the α class *N. sicca* enzyme [66]. By gel immunodiffusion, they detected cross-reactive material in several species of *Neisseria* and a few other prokaryotes. Enzyme activity was only detected in *N. sicca* and in several strains of *N. gonorrhoeae*; however, all *Neisseria* strains were sensitive to acetazolamide, a carbonic anhydrase inhibitor. Activity was also detected in several phototrophic bacteria and was found to decrease with a change from photoautotrophic to photoheterotrophic conditions [63], a result consistent with a function for carbonic anhydrase in CO_2 fixation. Carbonic anhydrase activity was recently detected in prokaryotes that oxidize acetate [27] and those that produce acetate as an end product of fermentation [65]. In addition, carbonic anhydrases have also been identified in cyanobacteria [51,67] and *E. coli* [50] through studies of prokaryotic CO_2 fixation and cyanate metabolism, respectively.

The recent discovery of the novel γ class in the Archaea domain [15] and the finding that an enzyme from the β class was present in thermophilic chemolithotrophic methanoarchaea [55] led to the hypothesis that these enzymes are more central to prokaryotic metabolism than previously recognized. This was tested by performing a survey of metabolically diverse species from both the Archaea and Bacteria domains for the presence of carbonic anhydrase utilizing both activity assays and Western blotting [14]. Using antisera derived against prokaryotic enzymes from each of the α, β and γ classes of carbonic anhydrase now available, Smith et al. [14] determined the extent to which each of the carbonic anhydrase classes is represented within the prokaryotes. In addition, the advent of genome sequencing allowed both completed and unfinished prokaryotic genome sequences to be searched for the presence of open reading frames encoding putative carbonic anhydrases. BLASTp and tBLASTn searches [68] using the deduced amino acid sequences from the *Anabaena ecaA* gene (α class) [67], the *M. thermoautotrophicum cab* gene (β class) [69] and the *M. thermophila cam* gene (γ class) [15] as queries identified six putative α class, 26 putative β class and 25 putative γ class carbonic anhydrase sequences. Evidence for the presence of carbonic anhydrase was obtained for freshwater, marine, mesophilic, thermophilic, aerobic, anaerobic, pathogenic, symbiotic, methylotrophic, acetotrophic, methanogenic, aceto-

genic, autotrophic, heterotrophic and photosynthetic species. These results demonstrated that carbonic anhydrases are not only far more prevalent in prokaryotes and distributed among far more metabolically diverse species than previously recognized, but that the β and γ classes are predominant [14]. In fact, α class carbonic anhydrases were detected only in a few of the microbes examined from the Bacteria domain and none from the Archaea domain.

4. Properties of isolated carbonic anhydrases from the Bacteria domain

Although now known to be nearly ubiquitous in prokaryotes, carbonic anhydrase has been purified from only five species in the Bacteria domain since it was first identified in *N. sicca* in 1963 [15,25,65,70,71]. Interestingly, all three classes of carbonic anhydrase are represented in the Bacteria domain and enzymes from the α and β class have been purified and characterized. An overview of the properties of these enzymes is shown in Table 1.

4.1. Neisseria

Carbonic anhydrase activity was first reported in *N. sicca* by Veitch and Blankenship and this enzyme was the first carbonic anhydrase purified from a prokaryote [24]. The gene encoding this enzyme has not been cloned; however, antisera derived against the purified enzyme cross-reacted with the *N. gonorrhoeae* α class carbonic anhydrase [66]. Furthermore, circular dichroism (CD) spectra of the two neisserial enzymes were nearly identical indicating a similar secondary structure [26,53]. Thus, it is highly likely that the *N. sicca* carbonic anhydrase belongs to the α class.

The *N. sicca* enzyme is a monomer with a molecular mass of 28.6 kDa [26]. It contains approximately one mole of Zn, a result consistent with a role for this metal in catalysis. As is the case with the human α class carbonic anhydrases, aromatic and heterocyclic sulfonamides act as strong inhibitors of the *N. sicca* enzyme. The *N. sicca* carbonic anhydrase also has a weak esterase activity (10% of the human CA II activity) with 4-nitrophenyl acetate as a substrate, a property characteristic of all α class mammalian enzymes.

Adler et al. [25] found that several factors were important for obtaining optimal expression of the *N. sicca* enzyme. Maximal activity was observed when the pH of the growth medium was maintained at 7.1 and growth was at low pCO_2 as opposed to a high pCO_2 (10% CO_2) suggesting the enzyme is optimally expressed under low pCO_2 conditions. The sulfonamides acetazolamide and ethoxyzolamide were shown to inhibit growth of *N. sicca* [72,73]. This inhibition was reversed with progressive increases in the concentration of CO_2 (3, 5 and 10%) or excess HCO_3^- in the growth medium indicating that carbonic anhydrase facilitates the exchange of CO_2 and HCO_3^- which enhances growth. Carbonic anhydrase activity was associated with the inner membrane and was released by mild sonication or solubilization with detergents. Although additional research is needed, these results are consistent with a role for carbonic anhydrase in *N. sicca* for the acquisition and transport of CO_2 by converting a freely dif-

Table 1
Distribution and properties of carbonic anhydrases

Enzyme	Class	Phylogenetic domain	Holoenzyme (kDa)[a]	Subunit composition	I_{50} (μM)[d]		Kinetic parameter[e]			Refs.
					AZA	AZI	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} M^{-1})	
Anabaena sp. strain PCC7120	α	Bacteria	30	?	ND	ND	ND	ND	ND	[67]
Neisseria gonorrhoeae	α	Bacteria	25	monomer	ND	ND	1.1×10^6	20	5.5×10^7	[53]
Neisseria sicca	α	Bacteria	29	monomer	0.05	ND	ND	ND	ND	[25,26]
Human CA II	α	Eukarya	30	monomer	0.015	1100	1.0×10^6	8.3	1.2×10^8	[16,142,188]
Methanobacterium thermoautotrophicum	β	Archaea	90	tetramer	60	2100	1.7×10^4	2.9	5.9×10^6	[55]
Escherichia coli	β	Bacteria	90	tetramer[c]	5	ND	ND	ND	ND	[50]
Synechocystis sp. strain PCC6803	β	Bacteria	?	?	ND	ND	ND	ND	ND	[132]
Pisum sativum (pea)	β	Eukarya	180	octamer	28	6	2.3×10^5	2.5	9.2×10^7	[48,189]
Coccomyxa sp.	β	Eukarya	100	tetramer	ND	ND	3.8×10^5	4.7	8.1×10^7	[137,164]
Methanosarcina thermophila	γ	Archaea	74	trimer	400	3400	7.7×10^4	14.8	5.5×10^6	[16,58]
Acetobacterium woodii	?	Bacteria	20 or 30[b]	?	4.4	ND	ND	ND	ND	[65]
Anabaena variabilis	?	Bacteria	48	dimer	0.25	ND	ND	ND	ND	[71]
Rhodospirillum rubrum	?	Bacteria	28	dimer	0.0042	12	ND	ND	ND	[70]

ND, not determined.

[a] Holoenzyme molecular masses were determined by gel filtration.

[b] Two protein bands were visible by SDS–PAGE but further efforts to obtain pure protein by gel filtration were unsuccessful.

[c] The presence of 5 mM HCO_3^- dissociated the tetramer into a dimer.

[d] I_{50}s were determined for the inhibitors AZA (acetazolamide) and AZI (azide).

[e] k_{cat} and K_m for CO_2 in the direction of CO_2 hydration were determined between pH 8.5 and pH 9.0 at 25°C.

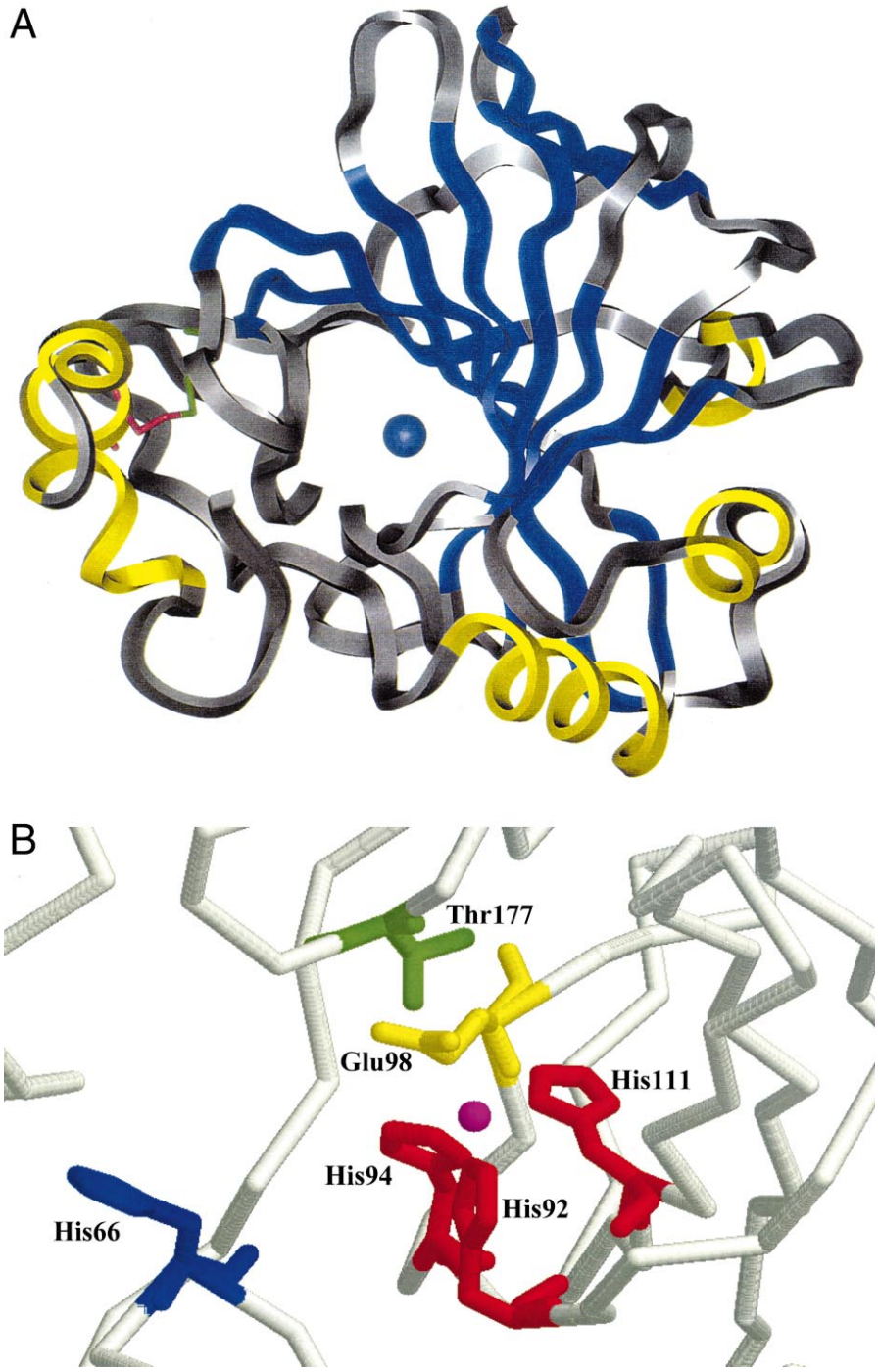

Fig. 1. Crystal structure of the *N. gonorrhoeae* α class carbonic anhydrase. A: View of the monomeric *N. gonorrhoeae* structure. The active site Zn is shown in blue. The α-helices are shown in yellow and the β-strands are shown in blue. Residues Cys-28 (shown in red) and Cys-181 (shown in green) are completely conserved in prokaryotic α class carbonic anhydrase sequences and form a disulfide bond. The crystal structure model was produced by Insight II (Molecular Simulations, San Diego, CA, USA) using the atomic coordinates of the 1.78-Å structure [78]. B: View of the active site. The active site Zn is shown in magenta and the coordinating residues (His-92, His-94 and His-111) are shown in red. His-66, thought to act as a proton shuttle analogously to His-64 of human CA II, is shown in blue. Glu-98 and Thr-177 of the hydrogen bond network are shown in yellow and green, respectively. The active site model was produced by Rasmol (Biomolecular Structure, Glaxo Research and Development; Middlesex, UK) using the atomic coordinates of the 1.78-Å structure [78].

fusable uncharged gas to a charged species and releasing it on the cytoplasmic side of the membrane.

Although the first reported prokaryotic carbonic anhydrase was from *N. sicca*, no sequence for a neisserial enzyme was available until the nucleotide sequence of a gene fragment in *N. gonorrhoeae*, designated ORF2 [74], was detected by homology searches of the sequence databases. Chirica et al. [53] cloned the entire gene and found it to

encode a protein of 252 residues having a molecular mass of 28.1 kDa. This carbonic anhydrase was heterologously produced in *E. coli* as a periplasmic protein lacking the N-terminal 26 residues suggesting that the *E. coli* processing machinery recognized this fragment as a signal sequence and cleaved it. Although the location of the enzyme in *N. gonorrhoeae* has yet to be ascertained biochemically, these results are consistent with a location at or outside the cytoplasmic membrane; therefore, this carbonic anhydrase could potentially have the same function in CO_2 transport as hypothesized for the *N. sicca* enzyme.

The CO_2 hydration activity of the *N. gonorrhoeae* enzyme is similar to that of the high activity human CA II [53] (Table 1). The k_{cat} values at pH 9.0 are almost identical, but the K_m for the *N. gonorrhoeae* enzyme is nearly 2.5 times greater than reported for human CA II (Table 1). The rapid CO_2 hydration turnover of approximately 10^6 s^{-1} for human CA II requires the participation of His-64 as a proton shuttle [75–77] and this residue (His-66) is also conserved in the amino acid sequence of the neisserial carbonic anhydrase. The *N. gonorrhoeae* enzyme also has esterase activity, hydrolyzing 4-nitrophenyl acetate, but the specific activity is only 5% that of human CA II [53].

The crystal structure of the *N. gonorrhoeae* enzyme (Fig. 1A) was solved to a resolution of 1.78 Å by molecular replacement using human CA II as a template [78]. The dominating secondary structure is a 10-stranded, twisted β-sheet that divides the molecule into two halves. The α-sheet is antiparallel with the exception of two pairs of parallel strands. The fold of the neisserial enzyme is nearly identical to that of human CA II [40] and most of the secondary structure elements present in the human isozyme are retained in the bacterial enzyme, but there are differences in the few helical regions of the protein [78]. Long deletions in the neisserial carbonic anhydrase relative to human CA II have resulted in the considerable shortening of three surface loops. One of these deletions corresponds to residues 128 to 139 of human CA II and leads to a widening of the entrance to the hydrophobic region of the active site cavity. Approximately the same regions have also been deleted in the sequences of the cyanobacterial α class enzymes from *Anabaena* and *Synechococcus* (Fig. 2). The structure of this region has also been shown to vary in other human isozymes as well. For example, in the membrane-associated human CA IV, an extended loop is observed rather than a helix [43]. Also, the helix in the mouse mitochondrial CA V is shifted about 2 Å from its position in human CA II [44].

The active site of the *N. gonorrhoeae* enzyme is located in a large cone-shaped cavity that stretches into the center of the molecule (Fig. 1A). The active site Zn is coordinated by three histidine residues (Fig. 1B) in a tetrahedral geometry with a water molecule serving as the fourth ligand. Most of the residues in the active site of the neisserial enzyme are conserved with the human isozymes including the three Zn ligands, His-92, His-94 and His-111 (Fig. 2). In addition, some of the residues of the neisserial enzyme that are hydrogen bonded to the histidine Zn ligands are also conserved with the human isozymes including Gln-90 and Glu-98 (Fig. 2). Nearly all of the residues in both the hydrophilic and hydrophobic parts of the active site are conserved and have similar structural positions.

X-ray crystallographic analysis of human CA II has shown that the Zn-bound hydroxide is hydrogen bonded to the hydroxyl oxygen of Thr-199 (Thr-177 of the neisserial enzyme), which is in turn hydrogen bonded to the side chain carboxyl of Glu-106 (Glu-98 of the neisserial enzyme) [79]. Both Glu-106 and Thr-199 are completely conserved among the α class carbonic anhydrases (Figs. 1B and 2) [1]. Glu-106/Thr-199 has been proposed to play two mechanistic roles. In addition to orienting the lone pair of electrons on the Zn-bound hydroxide for nucleophilic attack on CO_2 [80], these residues also have a 'gate-keeper' function in selecting only protonated molecules to bind to the active site [41,75,81].

His-66, which is thought to act as a proton shuttle analogously to His-64 of human CA II, is located between the active site Zn and the mouth of the active site cavity (Fig. 1B). In the structure of the neisserial carbonic anhydrase, the imidazole ring of His-66 interacts with an extraneous entity, thought to be 2-mercaptoethanol of the crystallization buffer, and is locked in the 'out' position. When this entity was removed from the crystallization buffer, the side chain of His-66 became extremely mobile and would presumably facilitate the proposed function as a proton shuttle. Whether 2-mercaptoethanol is an inhibitor of proton transfer remains to be determined.

The only two cysteine residues (Cys-28 and Cys-181) present in the neisserial carbonic anhydrase (Fig. 2) form a disulfide bond (Fig. 1A) that connects the N-terminal domain of the protein with a loop containing several active site residues [78]. Disulfide bonds have also been demonstrated for membrane-associated human isozymes such as CA IV [11,82] and secreted human isozymes such as CA VI [83], as well as the periplasmic enzyme from the unicellular green alga *Chlamydomonas reinhardtii* [46,47].

4.2. E. coli

Cyanase is an inducible enzyme that catalyzes the conversion of cyanate into ammonia and carbon dioxide [84,85]:

$$NCO^- + 3H^+ + HCO_3^- \rightleftharpoons NH_4^+ + 2CO_2 \quad (3)$$

Cyanate can be toxic to *E. coli* under some growth conditions, but can also be used as a sole source of nitrogen for growth as a result of the cyanase-catalyzed decomposition to ammonia [86]. The gene encoding cyanase, *cynS*, is transcribed as part of an operon [87] along with genes encoding a carbonic anhydrase (*cynT*) and a protein of unknown function (*cynX*) [88]. The cyanase operon is in-

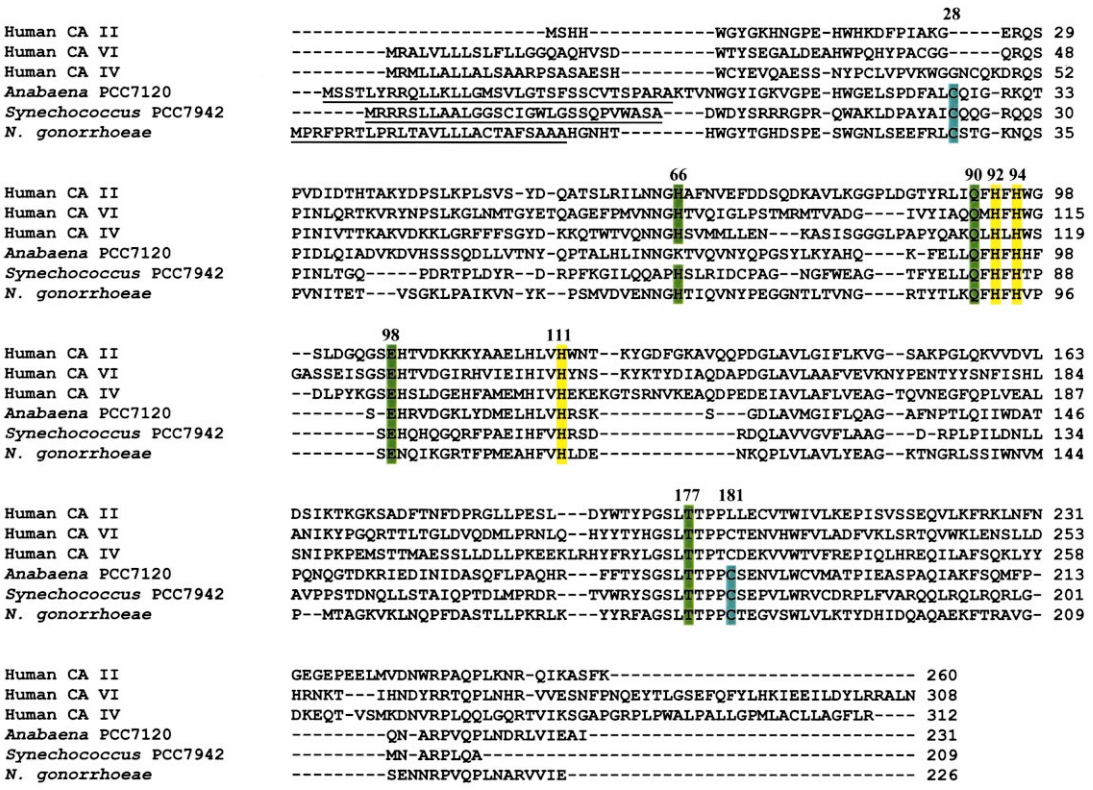

Fig. 2. Alignment of α class carbonic anhydrase sequences. The sequences of the documented prokaryotic α class carbonic anhydrases were aligned with the sequences of the cytoplasmic CA II, the secreted CA VI and the membrane-bound CA IV human isozymes using Clustal X [190]. The conserved Zn ligands His-92, His-94 and His-111 are shown in yellow. The prokaryotic disulfide bond-forming residues Cys-28 and Cys-181 are shown in blue. Other conserved residues important for enzyme activity (His-66, Gln-90, Glu-98 and Thr-177) are shown in green. Prokaryotic signal sequences are underlined. Numbers indicating residue positions refer to the position in the *N. gonorrhoeae* sequence.

duced in the presence of cyanate and CynT is found localized in the cytosol [87,88].

The deduced amino acid sequence of CynT shows significant identity to the spinach and pea sequences, thereby making it the first β class carbonic anhydrase sequence identified in the prokaryotes. The subunit molecular mass of the CynT carbonic anhydrase was determined to be 24 kDa and one Zn is present per subunit [50]. The isolated enzyme is relatively unstable in the absence of ethylene glycol and appears to aggregate irreversibly. The native molecular mass of CynT is approximately 90 kDa indicating that this enzyme is a tetramer, a property unlike any plant carbonic anhydrase. When the purified carbonic anhydrase is stored in 15% ethylene glycol at 26°C, a slow cleavage occurs yielding an active fragment of 22 kDa. Whether this is the result of a contaminating protease or an autocatalytic activity has yet to be determined. Cyanate and acetazolamide confer 50% inhibition at 0.1 and 0.005 mM, respectively [50]. Like the plant carbonic anhydrases, 4-nitrophenyl acetate does not serve as a substrate [50]. However, carbamate, a product of the breakdown of cyanate catalyzed by cyanase, is decarboxylated by the enzyme [50]. This decarboxylation reaction has also been shown to be catalyzed by a carbonic anhydrase from the yeast *Saccharomyces cerevisiae* [89].

Two pieces of experimental evidence suggest that enzymatic activity of CynT may be regulated within the cell. The first mechanism for regulation is protein degradation. After cyanate is depleted from the culture, CynT is degraded very rapidly at high pCO_2 (3% CO_2) but very slowly at low pCO_2 (0.03% CO_2) [88]. The processes for increasing the rate of degradation in the presence of high pCO_2 is not yet known. Whether the 22-kDa proteolytic fragment observed upon storage of the purified enzyme represents the first step in the degradation of CynT in the cell has not been reported. A second regulatory mechanism may be enzyme dissociation in the presence of HCO_3^-. Both sucrose density gradient experiments and gel filtration chromatography have shown that the molecular mass of the CynT is approximately 50 kDa in the presence of HCO_3^-, suggesting that the tetramer dissociates into a dimer in the presence of HCO_3^- [50]. By this mechanism, when the intracellular levels of HCO_3^- reach a certain level, the active CynT tetramer would dissociate into dimers which would presumably be inactive although this has yet to be shown. Subunit dissociation in the presence of HCO_3^- has not been reported for multimeric plant enzymes of the β class or any enzyme from the α or γ class. Whether this property is unique to CynT or to prokaryotic enzymes from the β class remains to be determined.

The discovery that *cynT* encodes a carbonic anhydrase

and that cyanase catalyzes the formation of two molecules of CO_2 from cyanate and HCO_3^- suggests that the role of CynT is to catalyze the hydration of the CO_2 generated by cyanase into HCO_3^-, thereby preventing depletion of the HCO_3^- required for further degradation of cyanate or for other metabolic processes [90]. Under low pCO_2, it would be expected that CO_2 would diffuse out of the cell faster than it is hydrated nonenzymatically resulting in depletion of cellular HCO_3^- levels. This hypothesis was tested by the construction of a mutant with a non-polar deletion mutation within *cynT* [90]. In the presence of low pCO_2 (0.03% CO_2), the *cynT* mutant was extremely sensitive to inhibition of growth by cyanate. Even though the cyanase wasstill induced, the mutant could not catalyze the decomposition of cyanate or use cyanate as a sole source of nitrogen. The addition of cyanate to the growth media of the *cynT* deletion mutant resulted in the depletion of the cellular HCO_3^-, catalyzed by cyanase, which prevented further degradation of cyanate and led to an inhibition of growth. When the mutant was grown under higher pCO_2 (3% CO_2), the above growth inhibition disappeared. Thus, high pCO_2 serves the same role as the carbonic anhydrase in providing sufficient levels of cellular HCO_3^- for growth with cyanate as the nitrogen source.

Two experimental results indicated that the role of CynT may be more than just to supply HCO_3^- for the decomposition of cyanate. First, growth of a strain in which the entire cyanase operon was deleted was also susceptible to cyanate inhibition at low pCO_2 [91]. Growth inhibition was relieved when the *cynTSX* deletion mutant was grown in the presence of high pCO_2. Second, growth inhibition by cyanate of a *cynS* deletion strain which still expressed carbonic anhydrase activity was the same as the effect on growth of the wild-type strain. The growth inhibition was less than that observed for the *cynTSX* deletion mutant and considerably less than the effect on the *cynT* deletion mutant. This result indicates that carbonic anhydrase alone provided some protection against the inhibition of growth by cyanate. Thus, depletion of HCO_3^- has more serious consequences for growth than does the inhibitory effect of cyanate, implying some essential HCO_3^--dependent function in *E. coli*.

The dependence of bacteria on the presence of CO_2 for growth or for overcoming long lag times (the so-called sparking phenomenon) has been known for 25 years [92,93]. At low pCO_2 and low rates of metabolism, CO_2 is expected to diffuse out of the cell faster than it is hydrated to HCO_3^-, thereby depleting the intracellular HCO_3^- needed for carboxylation reactions. Previous studies have shown that either a large inoculum or the presence of succinate, aspartate or oxaloacetate can overcome the long lag times observed during growth at low pCO_2 by increasing the citric acid cycle rates and the concentration of CO_2 and HCO_3^- [92,93]. Kozliak et al. [91] showed that the growth of wild-type *E. coli* and the *cynS*, *cynT* and *cynTSX* deletion mutants is negligible when CO_2 is absent from the atmosphere. When succinate, fumarate, aspartate, α-ketoglutarate or malate are added to the media, growth of both the wild-type and deletion mutant strains are restored to near normal rates [91]. If growth inhibition by cyanate of the *cynT* deletion mutant is due to the depletion of HCO_3^- required for replenishing the citric acid cycle intermediates, these intermediates would be expected to relieve the inhibition of growth by cyanate. However, the presence of citric acid cycle intermediates in the growth media failed to overcome the cyanate inhibition of growth [91]. Another explanation for cyanate inhibition is that HCO_3^- is required for other carboxylation reactions besides those of the citric acid cycle. Kozliak et al. [91] attempted to relieve the inhibition of growth by adding end-product metabolites of other carboxylation reactions such as succinate, histidine, adenine, oleate and palmeate [91]. Although some effect was observed, the growth inhibition could not be completely overcome.

The above results suggest that CO_2 and HCO_3^- are important for additional cellular functions that are required for growth and inhibited by cyanate at low pCO_2. Unfortunately, effects on these functions would be observed only at low pCO_2 when the carboxylation reactions are also limiting [94] which will make identification of these putative functions difficult. Isocyanic acid (HN=C=O), a reactive tautomer of cyanate and a structural and electronic analogue of CO_2 (O=C=O), has been used to demonstrate a competition between cyanate and CO_2 at some unknown site thus causing growth inhibition [91]. This inhibition could be the result of cyanate either competing with CO_2 or HCO_3^- for a regulatory or catalytic binding site or carbamoylating a nucleophilic group (such as –SH, $–NH_2$, –OH and –COOH) that might normally undergo nonenzymatic carboxylation by CO_2.

4.3. *Salmonella typhimurium*

Little is known about what genes are required for intracellular growth and survival of pathogens. Valdivia and Falkow devised a selection strategy on the basis of differential fluorescence induction for identifying genes whose expression is induced when the facultative intracellular pathogen *S. typhimurium* associates with its host cell [95]. Green fluorescent protein was used as a selectable marker in conjunction with fluorescence-activated cell sorting. Host macrophages infected with a bacterium having a transcriptionally active *gfp* gene fusion were separated by a fluorescence-activated cell sorter and lysed. The recovered bacteria were then grown in vitro and those that had little to no fluorescence were isolated. Fourteen promoters with intracellular-dependent activity were identified and the genes present downstream of these promoters were then isolated. One of the genes identified was *mig-5* (for macrophage-inducible gene), whose expression is induced 24-fold in macrophages [95]. The predicted amino acid

sequence of *mig-5* is 24% identical to that of the *E. coli* CynT β class carbonic anhydrase.

Gene disruptions were constructed for each of the *mig* genes to determine if the macrophage-inducible proteins have a role in virulence [95]. Each mutant was tested not only for the ability to colonize the spleen of BALB/c mice in competition assays against wild-type *S. typhimurium* but also for the ability to survive in the macrophage-like cell line RAW 264.7. Only 13% of the colony forming units present in the spleen were derived from the *mig-5* insertion mutants, but the *mig-5* mutant showed no growth defect in the RAW 264.7 cells. These results suggest that the *mig-5* gene product is not essential for survival in tissue culture but is important for survival of *S. typhimurium* within the host.

4.4. Cyanobacteria

As compared to plants, cyanobacteria have a much higher affinity for environmental CO_2 [96–100] and their growth is dependent on both photosynthesis and the fixation of CO_2 via the Calvin cycle [10]. The CO_2-fixing enzyme ribulose 1,5-biphosphate carboxylase (Rubisco) has a very low affinity for CO_2 with a K_m of approximately 100–150 μM [101]; however, cyanobacteria thrive at CO_2 concentrations even as low as 10 μM [102]. The ability to survive at low pCO_2 is assumed to be the consequence of a CO_2-concentrating mechanism (CCM) (Fig. 3) [96–100] whose essential features are: (i) active transport of inorganic carbon, C_i (CO_2 and HCO_3^-), into the cell by energy-dependent transporters with resultant accumulation of HCO_3^- within the cell and (ii) generation of elevated CO_2 levels for efficient CO_2 fixation by Rubisco within the carboxysomes. Carbonic anhydrases are thought to play a central role in the CCM and in fact activities are found both at the plasma membrane and in the carboxysomes [103–105].

Cyanobacteria are able to utilize both CO_2 and HCO_3^-; however, only HCO_3^- accumulates within the cytoplasm [106–109], suggesting CO_2 is converted to HCO_3^-. Recent studies of C_i transport in *Synechococcus* species have identified multiple transporters for CO_2 and HCO_3^- [99,100]. CO_2 uptake is constitutive under both low and high pCO_2 and shows saturable kinetics indicating that CO_2 uptake is mediated by a membrane-bound active transport system [106,107,110]. Since CO_2 transport is inhibited by the lipid-soluble carbonic anhydrase inhibitor ethoxyzolamide, a membrane-associated carbonic anhydrase was assumed to function in transport [111,112], possibly by converting CO_2 to HCO_3^-; however, it has yet to be determined if this CO_2 hydration is due to a carbonic anhydrase or a carbonic anhydrase-like moiety [99,100,107]. The CO_2 hydration associated with the plasma membrane is half that of the carboxysomal activity and has a 20-fold higher I_{50} for ethoxyzolamide [111,112]. The concentrations of ethoxyzolamide required to inhibit C_i transport are high

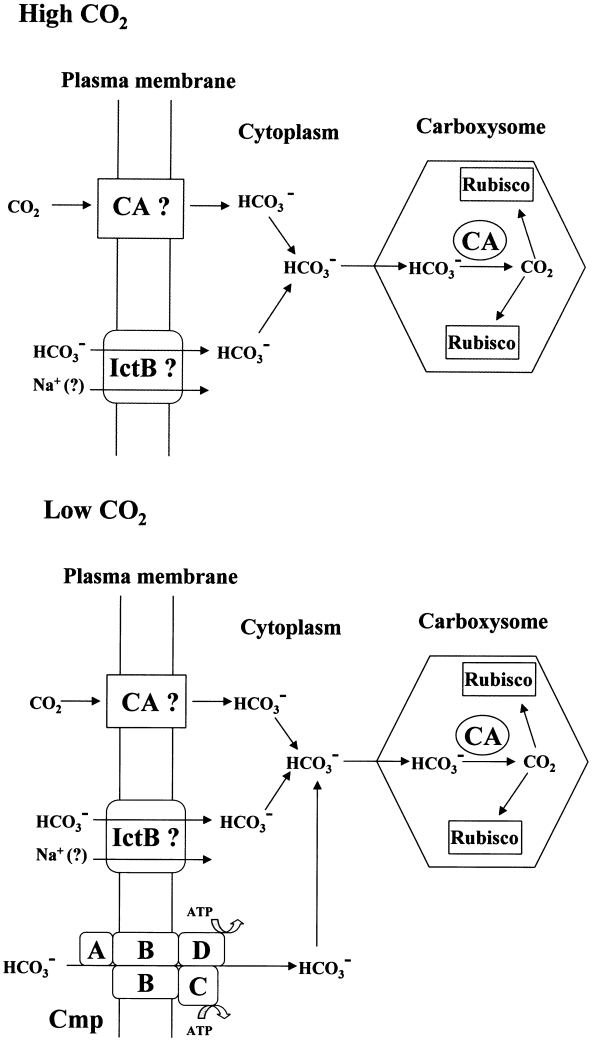

Fig. 3. Models showing the role of carbonic anhydrase in the CCM of cyanobacteria.

(100 μM) compared with purified CA enzymes (1 nM–1 μM). The fact that the less lipophilic acetazolamide is not as effective an inhibitor suggests that the site of inhibition is buried within a hydrophobic membrane environment. Membrane-bound carbonic anhydrases, in which the active site of the enzyme is located within the membrane, have not been identified in either eukaryotes or prokaryotes. Although there is strong evidence for a carbonic anhydrase-like moiety, the protein(s) involved in the transport of CO_2 has not yet been identified. Mediated transport of CO_2 might be considered unlikely since the lipid bilayer of the membrane should be permeable to CO_2. A recent model [113] postulates passive diffusion of CO_2 into the cell and subsequent energy-dependent conversion of CO_2 to HCO_3^- by the carbonic anhydrase-like moiety.

Although a carbonic anhydrase-like moiety is the most favored model, two other mechanisms for the uptake of CO_2 which could lead to its conversion to HCO_3^- during transport have been proposed [107]. First, CO_2 can undergo reversible reactions with free amino acid groups to

form carbamino adducts or carbamates [114]. An OH^--catalyzed decarboxylation of carbamate would result in the release of HCO_3^-. A second possible mechanism is the binding of CO_2 to a transition metal. Synthetic transition metal complexes have been shown to bind CO_2 and studies indicate the coordinated CO_2 molecule is in a bent configuration [115]. This strained configuration enhances susceptibility of the electrophilic C atom of CO_2 to nucleophilic attack by OH^- [116]. In both mechanisms, the generation of the OH^- is the energy-requiring step and could occur by raising the pH of the immediate surroundings either by photosynthetic or respiratory electron transport. In fact, CO_2 transport has previously been shown to be dependent on electron transport [117].

Experiments in *Synechococcus* PCC7942 have identified two HCO_3^- transporters [99]. High pCO_2-grown cells utilize a Na^+-dependent HCO_3^- uptake system, while cells grown under low pCO_2 possess both Na^+-dependent and Na^+-independent mechanisms for uptake (Fig. 3). The constitutive Na^+-dependent transporter is inhibited by monensin, an ionophore that mediates Na^+/H^+ exchange, suggesting a Na^+/HCO_3^- symport mechanism [118]; however, further experiments are needed to confirm this mechanism. A candidate for this transporter is IctB [119], a protein with nine or 10 membrane spanning domains whose sequence has identity to a Na^+-panthothenate symporter (PanF) from *E. coli*. Disruption of the *ictB* gene results in a strain requiring high levels of CO_2 for growth [119].

CmpABCD is a high affinity HCO_3^- transporter that is induced during CO_2 limitation and appears to be Na^+-independent [120]. This transporter belongs to the diverse subfamily of ABC (ATP binding cassette) transporters found in prokaryotes, also known as traffic ATPases [121]. Expression of CmpABCD alone is not sufficient for full induction of the high affinity state of the CCM. During induction of the CCM, high affinity HCO_3^- transport occurs within a few minutes of CO_2 limitation [122]. This fast induction is unaffected by inhibitors of protein synthesis, but does not occur in the presence of inhibitors of protein kinase [122]. These results suggest that fast induction involves post-translational phosphorylation of existing proteins rather than de novo synthesis [122,123]. If IctB is the Na^+-dependent HCO_3^- transporter and its expression is constitutive, it would be a likely target for the kinase-mediated fast induction.

As a result of C_i transport, the steady-state equilibrium between CO_2 and HCO_3^- is shifted towards HCO_3^-. The substrate for the CO_2-fixing enzyme Rubisco is CO_2 and not HCO_3^- [101]; therefore, an intracellular carbonic anhydrase would be expected to be present to catalyze the dehydration of HCO_3^- to CO_2. Attempts to express human CA II within the cytoplasm resulted in a decreased rate of CO_2 fixation and a requirement for a high pCO_2 (5%) for growth [124]. This led Price and Badger [125] to theorize that the intracellular carbonic anhydrase is localized within organelles termed carboxysomes [126]. These polyhedral protein bodies which contain most if not all the cellular Rubisco are thought to serve as a permeability barrier for CO_2 to prevent its leakage out of the cell. HCO_3^- in the cytoplasm freely diffuses into the carboxysome where the carbonic anhydrase could catalyze its dehydration into CO_2 for consumption by Rubisco. Price et al. [105] isolated carboxysomes and identified an associated carbonic anhydrase activity with some unusual properties, including inactivation by dithiothreitol and a requirement for 20 mM Mg^{2+} for maximal activity. Bedu and Joset [103,104] had previously indicated that the properties of the carbonic anhydrase activity associated with the plasma membrane were distinct from those of the intracellular enzyme(s) based on the differential effects of a carbonic anhydrase inhibitor. These results suggest that different carbonic anhydrases have specific and distinct functions in the transport and fixation of CO_2.

All three classes of carbonic anhydrase have been identified in the cyanobacteria. In fact some cyanobacteria (i.e. *Synechococcus* PCC7942) have genes encoding enzymes from all three classes. Each enzyme class and the potential roles they play are discussed below.

4.4.1. Alpha class carbonic anhydrase

A gene *ecaA* (external carbonic anhydrase alpha class) was identified in *Anabaena* sp. strain PCC7120 which encodes a protein of approximately 29 kDa [67] with a deduced amino acid sequence showing identity to several human α class isozymes. Although heterologous expression of EcaA in *E. coli* resulted in the formation of inclusion bodies with no detectable carbonic anhydrase activity, antisera derived against the chicken CA II cross-reacted with the heterologously produced EcaA. Polyclonal antisera derived against the heterologously produced EcaA also cross-reacted with a 29-kDa protein in whole cell lysates of *Anabaena*.

Expression of EcaA appears to be regulated by the level of CO_2 in the growth medium [67]. High levels of EcaA are present in cells grown at elevated CO_2 levels (1% CO_2) and low but still detectable levels are observed following 24 h of low CO_2 (0.01%) exposure. Transcription of *ecaA* follows a similar pattern of regulation with high levels of mRNA being detected only in high CO_2-grown cells. Immunoelectron microscopy studies with antisera derived against EcaA showed that this protein is found outside the cell and associated with the cell wall, periplasmic space or cytoplasmic membrane [67]. Unfortunately, under optimal conditions for EcaA expression, neither whole cell lysates nor membrane preparations exhibited carbonic anhydrase activity; thus, rigorous evidence for the location of this carbonic anhydrase is not available.

Using primers based on the *Anabaena ecaA* sequence, a homologous gene was identified and isolated from the cyanobacterium *Synechococcus* sp. strain PCC7942 [67]. Interestingly, a search of the entire genome sequence of *Sy-*

nechocystis PCC6803 indicated that this microbe lacks a gene encoding an α class carbonic anhydrase. The *Synechococcus ecaA* gene encodes a 26-kDa protein with a large number of positively charged residues in the amino-terminal domain suggesting the presence of a signal sequence for membrane targeting. *Synechococcus* exhibited the same pattern of EcaA expression as displayed by *Anabaena* with increased levels of EcaA present in cells grown at elevated levels of CO_2 [67].

In an effort to identify the role of EcaA in the CCM of cyanobacteria, deletion mutants of the *Synechococcus ecaA* were constructed [67]. No detectable difference was observed between the mutant and the wild-type cells in carbonic anhydrase activity measured at the cell surface as determined by the ^{18}O exchange method [127]. Active CO_2 transport and Na^+-dependent and Na^+-independent HCO_3^- transport were unaffected in the *ecaA* mutant. The *ecaA* mutant initially grew slower than the wild-type on both low and high C_i, but the rates were nearly indistinguishable during steady state. The maximal rates of C_i-dependent photosynthesis (measured by O_2 evolution) were also identical; however, the mutant grown in low pCO_2 displayed a more rapid onset of O_2 evolution (29% faster) upon addition of limiting concentrations of CO_2 than the wild-type [67]. Both wild-type and mutant cells exhibited similar patterns of photosynthetic O_2 evolution when limiting concentrations of HCO_3^- were added. An earlier onset of photosynthesis in the *ecaA* mutant upon the addition of limiting concentrations of CO_2 suggests that the immediate external concentration of CO_2 available for transport is higher than that for the wild-type. Since CO_2 transport is far more rapid than HCO_3^- transport, a rapid accumulation of C_i would result. In the wild-type, the activity of EcaA would quickly hydrate much of the added CO_2; thus, a lower amount of CO_2 would be available and HCO_3^- would be the major transported species.

These results imply that EcaA does not have a role in the CCM; however, the possibility that EcaA may play a role under more acidic and alkaline growth conditions cannot be ruled out. Several other potential roles for EcaA in cyanobacteria can be envisaged [67]. EcaA may be required to fully equilibrate C_i speciation at the site of transport in order to maximize substrate availability for either the CO_2 or HCO_3^- transporter. Alternatively, EcaA may serve as a sensor, either detecting or signaling changes in the level of CO_2 in the environment [127]. Recent evidence suggests that HCO_3^- is the signal for fast induction of the high affinity state of the CCM and therefore detection of the level of CO_2 is essential [122,128,129].

4.4.2. Beta class carbonic anhydrase

Synechococcus mutants that were able to accumulate C_i inside the cell but were unable to utilize it for CO_2 fixation were isolated [51,130,131]. These mutants required a high level of pCO_2 (4–5%) for growth and one also exhibited a 30-fold reduction in carboxysomal carbonic anhydrase activity [51], the complementation of which resulted in the identification of the gene *isfA* [51]. This gene encodes a protein of 272 amino acids whose sequence is 31% identical to that of the CynT, the β class carbonic anhydrase from *E. coli*.

Inactivation of the *isfA* gene by insertional mutagenesis resulted in a mutant that accumulated C_i to wild-type levels internally, but was unable to use the internal C_i pool for CO_2 fixation [51]. This observation led Fukuzawa et al. [51] to propose that IsfA is the carboxysomal carbonic anhydrase whose function is to dehydrate HCO_3^- to CO_2 for the CO_2-fixing enzyme Rubisco. Neither physiological studies confirming the localization of IsfA within *Synechococcus* cells nor biochemical characterization of the purified enzyme have been reported.

A DNA fragment was isolated from a *Synechocystis* sp. strain PCC6803 subgenomic plasmid library that strongly hybridized to a fragment of the *isfA* gene from *Synechococcus* sp. strain PCC7942 [132]. The isolated gene, denoted as *ccaA* (cyanobacterial carboxysome localized), encodes a 274-amino acid polypeptide with a predicted molecular mass of 30.7 kDa. The deduced amino acid sequence is 55% identical to that of the sequence of the *Synechococcus isfA* gene product. Heterologous expression of *ccaA* in *E. coli* resulted predominantly in the formation of inclusion bodies. However, carbonic anhydrase activity was detected in the soluble fraction due to a small amount of soluble CcaA that was sensitive to ethoxyzolamide.

So and Espie [132] used an improved method of subcellular fractionation [105] to obtain intact carboxysomes free of both soluble enzymes and thylakoid membranes. Immunoassays using polyclonal antisera derived against CcaA revealed that 97% of CcaA was associated with the isolated intact carboxysomes. These results indicate that CcaA in *Synechocystis* is the carboxysomal carbonic anhydrase and provides CO_2 for Rubisco. Whether the *Synechococcus* IsfA is a functional homolog of CcaA and also localized in the carboxysome remains to be determined. Previous studies with *Synechococcus* have indicated that the carboxysomal carbonic anhydrase activity is inactivated by dithiothreitol and requires 20 mM Mg^{2+} for maximal activity [105]; however, these properties were not examined [132] with the purified CcaA.

No cross-reactive proteins to antisera derived against CcaA were detected with the soluble fraction or associated with the thylakoid membranes [132] and neither were examined for carbonic anhydrase activity. Whether other carbonic anhydrases exist in the cytoplasm or are associated with the thylakoid membrane remains to be resolved [133]. No cross-reactive proteins were detected in the partially purified plasma membrane; thus, the carbonic anhydrase activity present in the plasma membrane is mediated by an immunologically distinct activity.

An additional open reading frame (slr0051) in *Synechocystis* PCC6803 with a high degree of sequence similarity

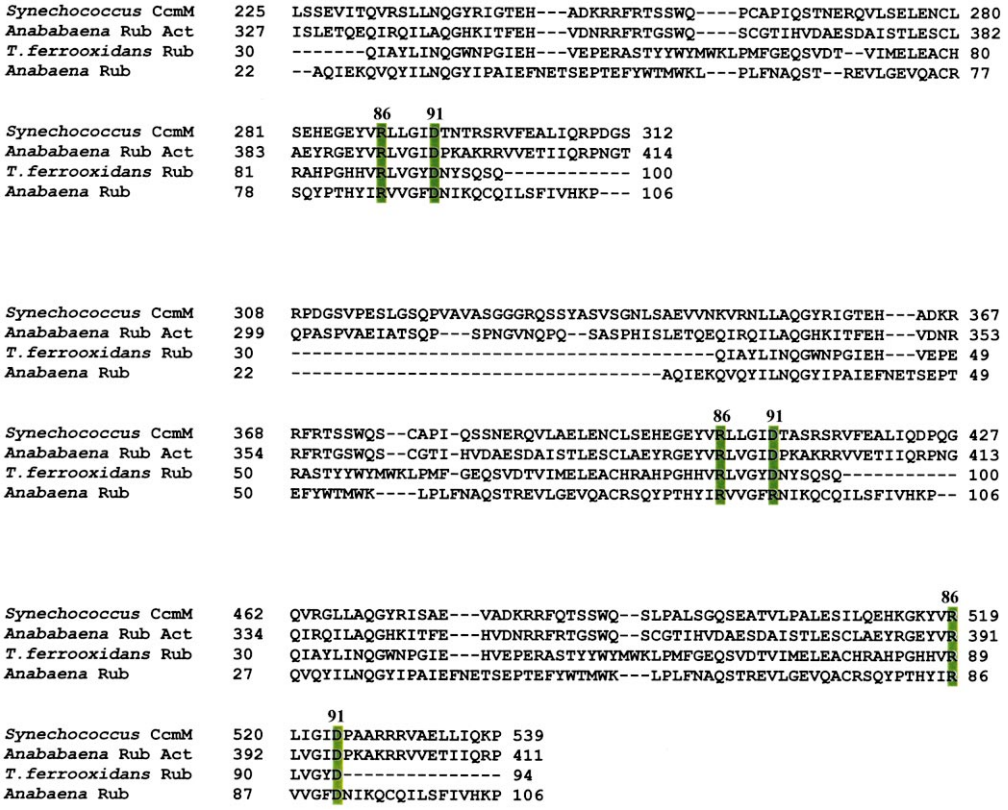

Fig. 4. Alignment of the *Synechococcus* CcmM sequence with the *Anabaena* Rubisco activase sequence and the Rubisco small subunit sequences from *T. ferrooxidans* and *Anabaena* sp. Alignments between the CcmM internal repeat regions with the homologous regions of the Rubisco activase (Rub Act) and Rubisco small subunit (Rub) sequences are shown. Arg-86 and Asp-91 (referring to the position in the *Anabaena* Rubisco small subunit sequence), thought to be involved in binding of the small Rubisco subunit to the large subunits, are highlighted in green.

to the *E. coli* CynT β class carbonic anhydrase has been identified [127]. The gene, designated *ecaB* (external carbonic anhydrase beta class), encodes a 263-amino acid polypeptide that has a putative prokaryotic membrane lipoprotein lipid attachment site indicating that the polyprotein may either be associated with the membrane or located in the periplasm; however, the location of EcaB in *Synechocystis* PCC6803 has not yet been ascertained. Heterologous expression of EcaB in *E. coli* resulted in the formation of inclusion bodies with no detectable carbonic anhydrase activity [127]. The phenotype of a *Synechocystis* PCC6803 *ecaB* mutant appeared to be identical to that of the *Synechococccus* PCC7042 *ecaA* mutant in that no detectable difference was observed between the mutant and wild-type cells in enzyme activity, in CO_2 and HCO_3^- transport or in steady state growth [127]. Like the α class enzyme EcaA, EcaB does not play an essential catalytic role in the functioning of the CCM. Thus, the *Synechocystis* PCC6803 β class carbonic anhydrase EcaB may have the same function as the *Synechococcus* PCC7942 α class enzyme EcaA. Therefore, these two different genera of cyanobacteria may use different classes of carbonic anhydrase for the same function.

4.4.3. Gamma class carbonic anhydrase

A number of genes named *ccm* (for carbon concentrating mechanism) have been identified in a region upstream of the *rbcLS* operon in *Synechococcus* PCC7942, which encodes genes for the subunits of Rubisco [99,134]. Each of the *ccm* genes (*ccmK*, *ccmL*, *ccmM*, *ccmN* and *ccmO*) is essential for growth at low pCO_2 and for correct assembly of the carboxysome [134]. *ccmM* encodes a bifunctional gene product of 539 amino acids of which the first 219 are 35% identical to those of Cam, the prototypical γ carbonic anhydrase from *M. thermophila*. Three internal repeats present in the C-terminal 320 amino acids show 45–51% identity to the C-terminal region of the *Anabaena* Rubisco activase (Fig. 4). Rubisco activase [135] catalyzes the ATP-dependent activation of the Rubisco–ribulose 1,5-biphosphate complex which is energetically coupled to the light reactions of photosynthesis. These internal repeats also show similarity to the small subunit of Rubisco (RbcS). The homologous region includes amino acids Arg-86 and Asp-91 of the small subunit of the *Anabaena* Rubisco (Fig. 4), which are thought to be involved in binding the small and large subunits of Rubisco together [134]. Genes with similar deduced amino acid sequences, but with four RbcS domains, have also been found in *Synechocystis* sp. PCC6803 and *Synechococcus* sp. PCC7002 [99].

Using antisera derived against the 58-kDa CcmM protein from *Synechococcus* PCC7942 overexpressed in *E. coli*,

CcmM was found to be a moderately abundant protein localized to the carboxysomes although it is not yet known if it is located inside or on the peripheral shell of the carboxysome [99]. The CcmM antisera also cross-reacts with a 35-kDa protein in the carboxysome-enriched preparation, but it is not known if this polypeptide is a processed form of CcmM, a second site translation product or an artifact. Carbonic anhydrase activity has not yet been reported for the heterologously expressed CcmM in *E. coli* and its role in CO_2 fixation remains to be determined. In addition to a structural role, CcmM may also prevent leakage of CO_2 out of the carboxysome. Any CO_2 diffusing out of the carboxysome would be converted to HCO_3^-, which can then be dehydrated by the carboxysomal β class carbonic anhydrase to supply CO_2 for Rubisco.

4.4.4. Unclassified

Yagawa et al. [71] found carbonic anhydrase activity in cell extracts from *Anabaena variabilis* M-2 and M-3 in addition to *A. variabilis* ATCC29413. Activity was significantly higher in cells grown under low pCO_2 than in those grown under higher pCO_2 (2–4% CO_2). The soluble carbonic anhydrase from *A. variabilis* ATCC29413 was purified greater than 200-fold and major (48 kDa) and minor peaks (25 kDa) of active carbonic anhydrase were detected after gel filtration chromatography of the enzyme. Native polyacrylamide gel electrophoresis (PAGE) of both peaks showed only a single band of approximately 48 kDa, suggesting that the major peak of carbonic anhydrase was a dimer and the minor peak was the dissociated monomer. The activity of the enzyme from *A. variabilis* ATCC29413 was inhibited by the presence of 5 mM dithiothreitol similar to the previously described *Synechococcus* carboxysomal carbonic anhydrase activity [105]. These similarities suggest this enzyme may be the carboxysomal carbonic anhydrase of *A. variabilis* ATCC29413, but further experiments are clearly required to confirm this. The determination of which class of carbonic anhydrase the *A. variabilis* enzyme belongs to awaits the cloning and sequencing of the gene encoding this enzyme.

4.5. Rhodospirillum rubrum

Carbonic anhydrase activity was detected in anaerobically and photosynthetically grown *R. rubrum* cells [70]; however, no enzyme activity was detected in cells grown aerobically in the dark. Initial attempts at purification of this soluble enzyme were difficult due to a loss of enzyme activity during the purification; however, inclusion of Zn in the buffers largely resolved the loss of enzyme activity, consistent with a role for this metal in catalysis. The molecular mass of the enzyme was determined to be approximately 30 kDa by gel filtration chromatography and sucrose density gradient studies. A single protein band of approximately 14 kDa was detected on denaturing SDS–PAGE indicating that the enzyme is a dimer. In addition to having CO_2 hydration activity, the *R. rubrum* carbonic anhydrase was also shown to have a weak esterase activity; however, this activity was only detectable at enzyme levels 80 times greater than the levels used for the CO_2 hydration assay. Thus far, only α class carbonic anhydrases have been shown to have esterase activity suggesting the *R. rubrum* carbonic anhydrase may belong to this class. The cloning and sequencing of the gene encoding this carbonic anhydrase has not been reported, thus it is impossible to determine in which class this enzyme belongs.

4.6. Acetobacterium woodii

The acetogens *A. woodii*, *Acetohalobium arabaticum*, *Clostridium formicoaceticum* and *Sporomusa silvacetica* were all found to contain carbonic anhydrase activity [65]. Of the acetogens tested, *A. woodii* had the highest specific carbonic anhydrase activity (14 U mg^{-1}) and activity was detected in cell extracts of either glucose- or H_2-CO_2-grown cells. The carbonic anhydrase was found to be cytoplasmic and was purified 300-fold to a specific activity of 5236 U mg^{-1}. After purification, two prominent bands (approximately 20 and 30 kDa) were visible on SDS–PAGE gel, but further attempts to obtain pure carbonic anhydrase by gel filtration were unsuccessful.

The physiological functions of carbonic anhydrase in acetogens is unknown, but several can be envisioned [65]. Acetogens have a high demand for CO_2 in both energy-yielding metabolism and pathways for CO_2 fixation; thus one function may be to increase the intracellular CO_2 levels. Many acetogens cannot grow acetogenically under heterotrophic or autotrophic conditions in the absence of CO_2. A CCM may be essential during autotrophic growth when CO_2 is used as both a carbon source and an electron acceptor. Acetazolamide (2 mM) had no apparent affect on the growth of *A. woodii* during heterotrophic conditions [65]; however, growth on H_2-CO_2 was completely inhibited suggesting an important role for carbonic anhydrase in autotrophic growth. Other potential roles for the *A. woodii* carbonic anhydrase include the regulation of intracellular pH due to the large amount of acetate being produced during growth and the coupling to an acetate/HCO_3^- antiporter [65].

5. Properties of isolated carbonic anhydrases from the Archaea domain

Although only two archaeal carbonic anhydrases have been documented [16,55], the enzyme appears to be widespread in the two kingdoms of the Archaea domain [14]. Interestingly, a gene encoding a putative α class carbonic anhydrase has not yet been identified in the domain, consistent with the relatively recent invention of this class.

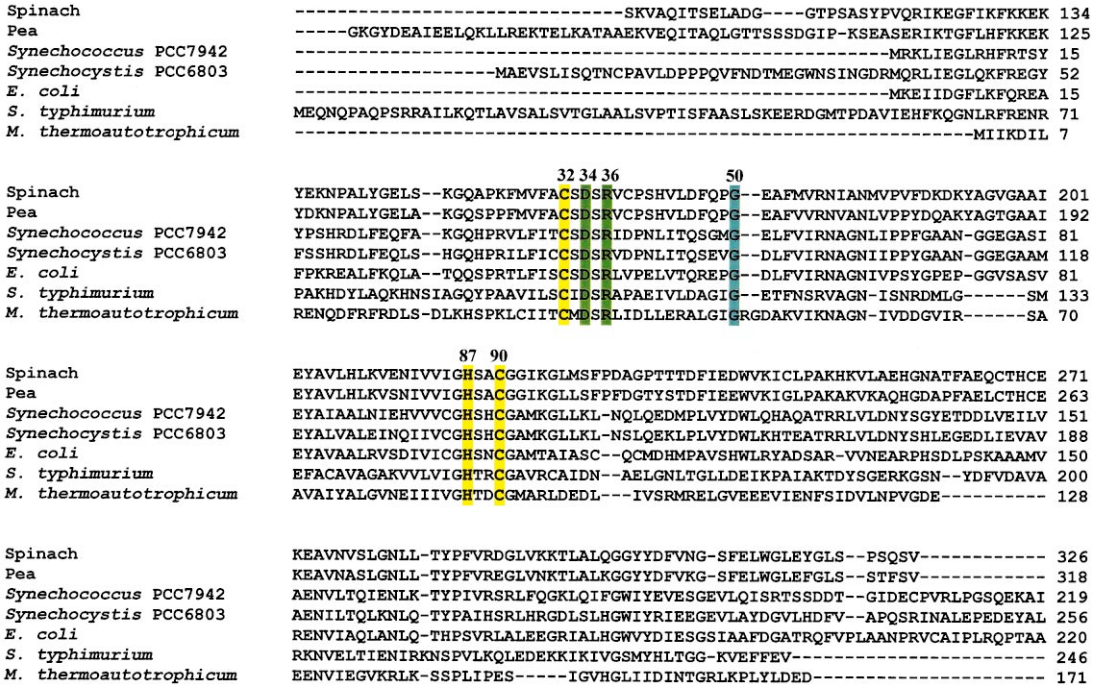

Fig. 5. Alignment of β class carbonic anhydrase sequences. The sequences of documented prokaryotic β class carbonic anhydrases were aligned with the sequences of the spinach and pea enzymes using Clustal X [190]. The spinach and pea chloroplast transit peptides (amino acids 1–98 for the spinach enzyme and 1–70 for the pea enzyme) were not included in the alignment. The conserved Zn ligands Cys-32, His-87 and Cys-90 are indicated in yellow. The completely conserved residues Asp-34 and Arg-36 are shown in green and the highly conserved Gly-50 is shown in blue. Numbers indicating residue positions refer to the position in the *M. thermoautotrophicum* sequence.

The two documented archaeal enzymes are discussed in detail below.

5.1. *M. thermoautotrophicum ΔH*

Recently, genome sequencing of the thermophilic, obligately chemolithoautotrophic methanoarchaeon *M. thermoautotrophicum* ΔH revealed an open reading frame with a deduced sequence 34% identical to CynT, the β class carbonic anhydrase of *E. coli* [54,69]. The ORF-encoded protein, Cab (carbonic anhydrase beta), was expressed in *E. coli*, purified to electrophoretic homogeneity and found to have carbonic anhydrase activity [55]. Native gel filtration chromatography estimates the molecular mass of Cab to be approximately 90 kDa [55]. The calculated subunit molecular mass is 18.9 kDa suggesting that native Cab is a tetramer in agreement with the *E. coli* CynT. Analytical ultracentrifugation studies confirm that Cab is a tetramer [59]. Thus, the first two documented prokaryotic β class carbonic anhydrases are tetramers [50] differing from those characterized from spinach and pea (dicotyledonous plants) which are octameric [49]. CD analysis and secondary structure predictions of Cab and the β class spinach enzyme indicate a domination of α-helical structure that is in stark contrast to that of the α and γ class carbonic anhydrases which are composed mainly of β-sheet structures [59].

Purified Cab retains full activity after incubation for at least 15 min at temperatures up to 75°C; thus, Cab is the most thermostable carbonic anhydrase yet characterized. Iodide, nitrate and azide are potent inhibitors; however, anions such as chloride and sulfate, which inhibit plant β class carbonic anhydrases, have no effect on the activity of Cab. The insensitivity to chloride and sulfate has also been observed for the γ class carbonic anhydrase from *M. thermophila* [15,16], the only other known methanoarchaeal carbonic anhydrase. Like other characterized carbonic anhydrases, Cab is also susceptible to inhibition by the sulfonamides acetazolamide and ethoxyzolamide. As with other characterized β class carbonic anhydrases, Cab shows no detectable esterase activity.

X-ray absorption spectroscopy (XAS) analysis clearly indicates that the Zn coordination of β class Cab is distinct from that of the α and γ classes of carbonic anhydrase [59]. Extended X-ray absorption fine structure (EXAFS) results suggest that the active site Zn is coordinated by two cysteine residues and two oxygen/nitrogen ligands [59]. One oxygen/nitrogen ligand may derive from histidine while the other most likely results from a water molecule which is deprotonated and serves as the Zn-hydroxide attacking CO_2. Residues Cys-32, His-87 and Cys-90 of Cab are completely conserved in the β class carbonic anhydrase sequences (Fig. 5). Although site-directed mutagenesis studies with the spinach enzyme indicate that alterations to these three residues result in variants lacking Zn [136], definitive proof that these residues serve as ligands awaits the first crystal structure for any β class carbonic anhydrase.

Cab is the first prokaryotic β class carbonic anhydrase for which kinetic parameters have been obtained and the results suggest a Zn-hydroxide mechanism for Cab [55,59]. The K_m value for CO_2 is very similar to that of the β class enzymes isolated from higher plants [48,57] and the unicellular green alga *Coccomyxa* [137]; however, the k_{cat} for Cab is at least 10-fold less than those of other β class enzymes (Table 1). One possible reason for the difference in k_{cat} and catalytic efficiency (k_{cat}/K_m) is that the assay temperature was more than 40°C below the optimal growth temperature of *M. thermoautotrophicum*, which is between 65 and 75°C. The optimal temperature for Cab activity is expected to be near the growth temperature; however, the decreased solubility of CO_2 at these temperatures under atmospheric pressure precludes the determination of accurate kinetic parameters above 25°C.

Carbonic anhydrase activity (0.8 U mg^{-1}) was detected in *M. thermoautotrophicum* cell extract [55]. A cross-reacting protein of the correct size for Cab was detected in Western blots using antisera derived against Cab. In addition, a cross-reacting protein to antisera derived against Cam, the γ class carbonic anhydrase from *M. thermophila*, was also detected. Analysis of the genome sequence of *M. thermoautotrophicum* revealed an ORF with 31% identity to Cam; however, it is not yet known if this ORF encodes a protein with carbonic anhydrase activity. Thus, the carbonic anhydrase activity detected in *M. thermoautotrophicum* may be due to the presence of both β and γ carbonic anhydrases. CO_2-reducing chemolithotrophic methanoarchaea have a high demand for CO_2 in both catabolic and anabolic reactions suggesting carbonic anhydrase may be essential to deliver CO_2 to the cell and concentrate it in the vicinity of CO_2-utilizing enzymes. For example, a carbonic anhydrase located at the cell wall may facilitate CO_2 transport into the cell by converting it to a charged species while another carbonic anhydrase in the cytoplasm may convert HCO_3^- to CO_2, the substrate for formylmethanofuran dehydrogenase [138] which catalyzes the first committed step in the reduction of CO_2 to methane during methanogenesis.

5.2. *M. thermophila*

Cam (carbonic anhydrase methanosarcina) from *M. thermophila* is the prototype of the γ class carbonic anhydrase and the only enzyme from this class that has been characterized. Sequencing of the cloned gene revealed an open reading frame encoding 247 amino acids with an additional 34 N-terminal residues not present in the purified enzyme [15]. This 34-amino acid region has many properties that are characteristic of signal sequences in secretory proteins from both the Bacteria and Eukarya domains; the central hydrophobic region (positions 7–26) is flanked by two hydrophilic regions with the amino-terminal one containing a positively charged amino acid [139–141]. Thus, it is proposed that Cam is translocated across the cytoplasmic membrane to the exterior of the cell. Cam lacking the 34-amino acid N-terminal sequence was heterologously produced in *E. coli* as a soluble protein and characterized [16]. Similar to the β class carbonic anhydrases, no detectable esterase activity is observed for Cam when 4-nitrophenyl acetate is the substrate. The heterologously-produced and authentic Cam are equally susceptible to inhibition by sulfonamides and anions. Anions are about 10-fold less effective compared to the α class human CA II. The catalytic mechanism of Cam appears to resemble that of the α class human isozymes [58]. The K_m value for CO_2 is slightly higher (1.8-fold) than that of the α class human CA II [142,143] but is nearly six times greater than that of the β class pea enzyme [48] (Table 1). The k_{cat} and catalytic efficiency (k_{cat}/K_m) for Cam are approximately 10–100-fold less than the eukaryotic β and α class enzymes, respectively (Table 1).

The active site Zn of the heterologously-produced Cam can be isomorphously replaced with cobalt (Co) by denaturation and renaturation followed by addition of the metal. EXAFS indicates the active site metal is coordinated by three histidines and two or three water molecules [58]. Thus, the γ class carbonic anhydrase resembles α carbonic anhydrases in that the active site Zn is coordinated by three histidine residues; however, in contrast to Cam a single water molecule completes the tetrahedral coordination of the active site Zn in human CA II [40,41]. The specific activity of the Co-substituted Cam (2020 U mg^{-1}) is greater than twice that of the Zn enzyme (910 U mg^{-1}) [58]. This result differs with those for the α class carbonic anhydrases. Co substitution of the active site Zn in human CA II resulted in an enzyme with approximately 50% of the CO_2 hydration activity [144,145] even though X-ray crystallography showed that the metal ion in the Co-substituted human CA II has a tetrahedral coordination similar to Zn [146]. The possibility that the active site metal of authentic Cam from *M. thermophila* is Co instead of Zn remains to be determined.

The crystal structure of Cam (Fig. 6) determined at 2.8 Å [52] shows that it is a homotrimer with the monomers adopting a novel left-handed β-helix fold. As a consequence of the fold, only crossover connections between the neighboring parallel β-strands are observed. Left-handed crossovers have been observed infrequently, with isolated examples in subtilisin, class 1 human leukocyte antigen and KP4 toxin [52]. At nearly the same time the structure of Cam was solved, a left-handed β-helix motif was discovered in the structure of the UDP-*N*-acetylglucosamine acyltransferase [147]. More recently, left-handed parallel β-helices were reported for tetrahydrodipicolinate *N*-succinyltransferase (THDP succinyltransferase) [148] and the hexapeptide xenobiotic acetyltransferase [149]. Cam, UDP-*N*-acetylglucosamine acyltransferase, THDP succinyltransferase and xenobiotic acetyltransferase are trimeric molecules belonging to a family of proteins which display a tandemly-repeated hexapeptide sequence known

as a 'hexapeptide repeat', characterized as [LIV]-[GAED]-X-X-[STAV]-X [150,151], which is essential for the left-handed fold. Most, though not all, enzymes of this group are acyltransferases that utilize a phosphopantothenyl-based substrate donor, either acyl-CoA or the acyl carrier protein, whose prosthetic group resembles CoA [148]. Each of the short β-strands is composed of one hexapeptide repeat with the conserved aliphatic residue (Leu, Ile, Val) projecting into the interior of the left-handed β-helix at triangular corners of each coil [52]. The sequence differences found between members of this family are not in the structurally constrained residues of the hexapeptide repeat but are instead in the loop residues which are less restricted. The residues forming the active site of Cam and those in the inhibitor binding site of THDP succinyltransferase are in these loop regions. The capacity of THDP succinyltransferase to bind metals such as Co as an inhibitor and the fact that Cam binds Zn may be a shared ancestral trait; however, the residues which coordinate the Zn in Cam are not found in the THDP succinyltransferase [52,148]. As compared to Cam, the associations of the three monomers are somewhat different in UDP-*N*-acetylglucosamine acyltransferase and THDP succinyltransferase [52]. Of the three enzymes, only Cam contains a metal ion and the location of the active site Zn at the subunit interface (Fig. 6A) leads to more extensive monomer–monomer interactions than found in the other two enzymes [52]. Thus, Cam belongs to a very diverse family of acetyl and acyl transferases that most likely arose through successive duplication events.

The three active-site Zn atoms located at the interfaces between two monomers are each ligated with three histidines, two (His-81 and His-122) from one subunit and the third (His-117) from an adjacent subunit (Fig. 6) [52]. Apart from the histidine residues ligating Zn, the active-site residues of Cam are significantly different from those of the α class carbonic anhydrases. Nonetheless, the catalytic mechanism of Cam at high pH appears similar to human CA II suggesting essential residues in Cam and the human enzyme will have analogous functions [58]. Based on their proximity to the active site, Glu-62, Gln-75 or Asn-202 of Cam (Fig. 6B) may function analogously to Glu-106 and Thr-199 of the α class human CA II which participate in a hydrogen bond network with the Zn-bound hydroxide to orient the lone pair of electrons for attack on CO_2 [79]. Site-directed mutagenesis studies indicate Glu-62 is important for CO_2 hydration activity though its function in the Zn hydroxide mechanism has not yet been determined [152]. Investigations into the roles of Gln-75 and Asn-202 have not yet been reported.

The left-handed β-helix motif of Cam is interrupted by three protruding loops [52], one of which (residues 83–90) contains residues Glu-84, Glu-88 and Glu-89 that form an acidic 'valley' extending to Glu-62 which is adjacent to the active site Zn [152]. The high resolution crystal structure of Cam [153] reveals that Glu-84 has three discrete conformations, analogous to the two discrete conformations of His-64 previously seen for the α class human CA II [154–156], suggesting a role for Glu-84 in proton transfer. The ability of imidazole to rescue the k_{cat} of human CA II His-64 variant is considered strong evidence for involvement of this residue in the proton transfer step of catalysis (Eqs. 2c and 2d) [76,77]. Similarly, imidazole rescues the k_{cat} of a Glu-84 variant of Cam, consistent with a role for this residue in proton transfer [152]. Other ionizable residues, such as histidine and aspartate, also function at this position in Cam [152].

M. thermophila is one of the most metabolically diverse methanoarchaea in that it obtains energy for growth by converting the methyl groups of either acetate, methanol or methylamines to methane [157]. Carbonic anhydrase activity is elevated approximately 10-fold when *M. thermophila* was switched from methanol to acetate as the growth substrate [16]. Using antisera derived against Cam, it was shown that the amounts detected in extracts of methanol- and trimethylamine-grown cells are several fold less than in acetate-grown cells corresponding to the decrease in carbonic anhydrase activity suggesting Cam has an important role for growth on acetate [15,16]. The energy yield for the methanogenic fermentation of acetate is quite low ($\Delta G^{\circ\prime} = -36$ kJ mol^{-1}) [157]; therefore, efficient removal of cytoplasmic CO_2 could improve the thermodynamics of this pathway. The proposed location of the enzyme outside the cytoplasmic membrane could facilitate the efficient removal of cytoplasmic CO_2 by conversion to HCO_3^-. This hypothesis is further supported by experiments showing that the catalytic efficiency (k_{cat}/K_m) of Cam for CO_2 hydration is several fold greater than that for HCO_3^- dehydration over the physiological pH range of 6.5 to 7.5, with a 10-fold difference at pH 7.0 [58]. Another possible function for Cam is in the transport of acetate into the cell. Using the H^+ generated from the Cam-catalyzed hydration of CO_2 into HCO_3^- (Eq. 1), acetate could be transported into the cell via a H^+/acetate symport mechanism.

6. Phylogeny of prokaryotic carbonic anhydrases

With the extraordinary number of genome sequencing projects underway, BLASTp and tBLASTn searches [68] of the nonredundant sequence database at NCBI and the finished and unfinished microbial genome sequences were used to identify α, β and γ class enzymes. Smith et al. [14] had previously identified six putative α class carbonic anhydrase, 26 putative β class carbonic anhydrase and 25 putative γ class carbonic anhydrase sequences. Additional putative carbonic anhydrase sequences from the β and γ classes have been identified in both the Bacteria and Archaea domains, while the α class still appears to be limited to the Bacteria domain. To understand the phylogenies of each carbonic anhydrase class, trees were constructed by

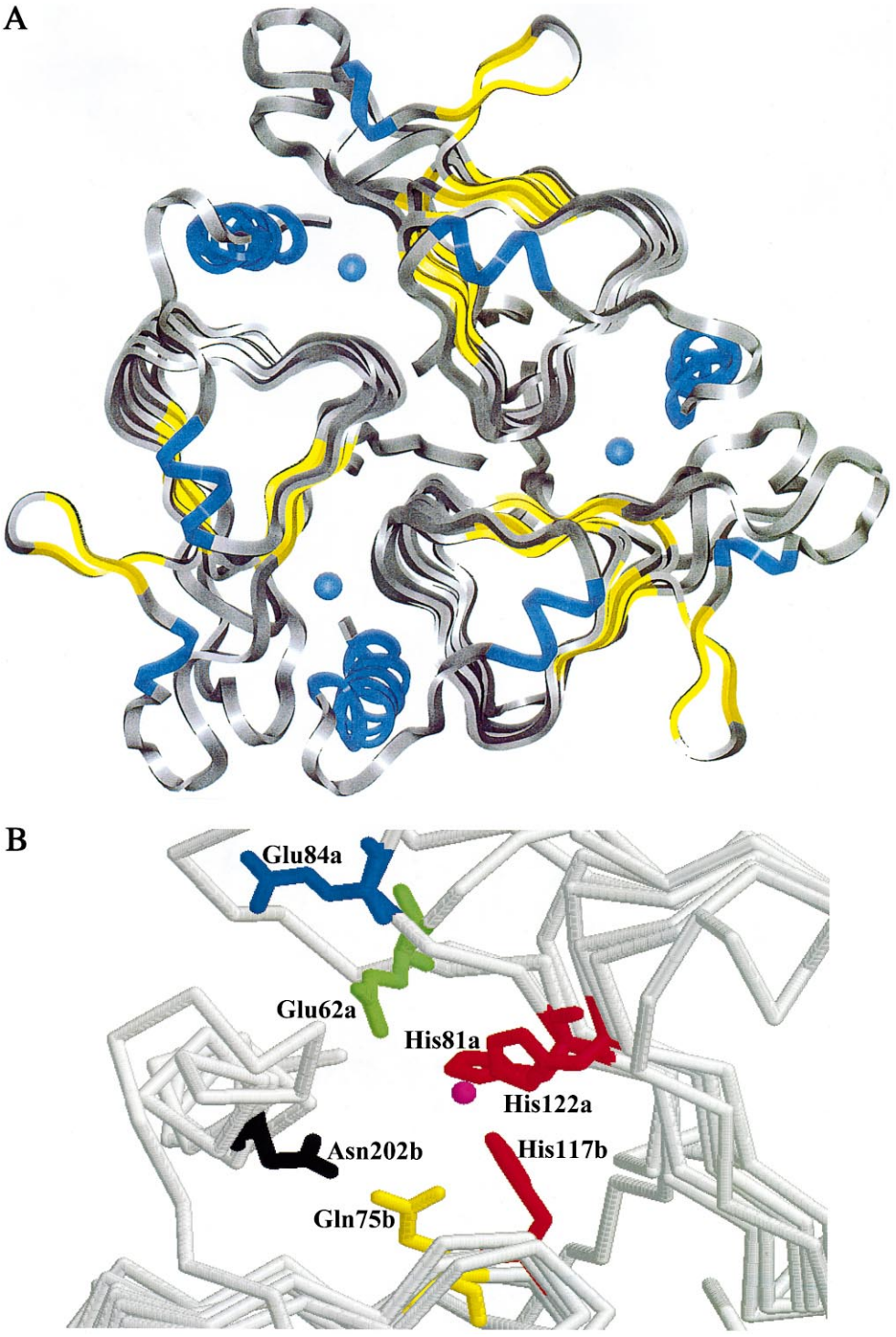

Fig. 6. Crystal structure of the *Methanosarcina thermophila* γ class carbonic anhydrase. A: View of the trimeric *M. thermophila* structure. The active site Zn is shown in blue. The α-helices are shown in yellow and the β-strands are shown in blue. The crystal structure model was produced by Insight II (Molecular Simulations, San Diego, CA, USA) using the atomic coordinates of the 2.8-Å structure [52]. B: View of the active site. The active site is at the interface of two neighboring subunits (designated a and b). The active site Zn is shown in magenta and the coordinating residues (His-81a, His-122a and His-117b) are shown in red. Glu-62a (shown in green) and Glu-84a (shown in blue), conserved among Cam and the cyanobacterial CcmM sequences, are necessary for CO_2 hydration and proton transfer, respectively. Gln-75b (shown in yellow) and Asn-202b (shown in black) may function analogously to Glu-98 and Thr-177 of the α class neisserial enzyme which participate in a hydrogen bond network with the Zn-bound hydroxide to orient the lone pair of electrons for attack on CO_2. The active site model was produced by RasMol (Biomolecular Structure, Glaxo Research and Development, Middlesex, UK) using the atomic coordinates of the 2.8-Å structure [52].

the neighbor-joining algorithm of the MEGA program [158].

6.1. Alpha class

The α class of carbonic anhydrases in mammals is comprised of 10 isoenzymes (CA I–VII, IX, XII and XIV) with defined carbonic anhydrase activity, three carbonic anhydrase-related proteins (CA-RP VIII, X and XI) and two subtypes of receptor-type protein tyrosine phosphatases (RPTPβ and RPTPγ) [1,11,35,37]. Subcellularly, the isozymes are cytostolic (CA I, II, III and VII), membrane-bound (CA IV, IX, XII and XIV), mitochondrial (CA V) or secreted (CA VI) [1,6,11,35,37]. The carbonic anhydrase-related proteins, two subtypes of receptor-type protein tyrosine phosphatases and two pox virus (Vaccinia and Variola) transmembrane proteins do not catalyze the reversible hydration of CO_2 because of the absence of one or more of the three histidine residues that coordinate the active site Zn [1]. Two point mutations (Arg-117 to His and Glu-115 to Gln) can convert the inactive CA-RP VIII into a protein that binds Zn and catalyzes the hydration of CO_2 [159].

Thus far, no functional α class carbonic anhydrases have been found in higher plants (although two putative genes have been found in *Arabidopsis thaliana*) [1]; however, the unicellular alga *C. reinhardtii* contains α class enzymes in the periplasm as well as the chloroplast [46,47,160]. This class was first discovered in the prokaryotes with the cloning of the gene from *N. gonorrhoeae* [53]. Since this discovery, only two additional enzymes from this class have been identified in prokaryotes and subsequently characterized [67,127]. An alignment of the deduced amino acid sequences of the cloned prokaryotic α class genes with three human isozymes representing cytostolic, membrane-bound and secreted forms is shown in Fig. 1 with relevant residues and features highlighted.

Searches with the *Anabaena ecaA* deduced amino acid sequence produced only nine sequences from the Bacteria domain and no archaeal sequences (Fig. 7). The α class is composed of nine prokaryotic sequences, 10 mammalian isozymes (CA I–VII, IX, XII and XIV), several related mammalian sequences which encode proteins lacking carbonic anhydrase activity and several sequences from vertebrates, plants, nematodes and algae that are expected to encode carbonic anhydrase or carbonic anhydrase related proteins. The three histidine residues (His-92, His-94 and His-111 of the neisserial enzyme) coordinating the active site Zn in the human isozymes are completely conserved in all of the prokaryotic α class carbonic anhydrase sequences (Fig. 2). Other residues that have been shown to be important for catalysis in the human isozymes such as Gln-92, Glu-117, Ala/Val-121 and Val-143 of human CA II are also present in the prokaryotic enzymes as well (Fig. 2). Residues Glu-98 and Thr-177 which form the Zn-bound hydroxide hydrogen bond network are also conserved among the prokaryotic enzymes (Fig. 2). His-64, which has been shown to be involved in intramolecular proton transfer in human CA II [75–77], is conserved in all of the α class prokaryotic sequences with the exception of that from *Anabaena* which has a lysine residue at that position. Studies of human CA II variants indicate lysine, aspartic acid and glutamic acid can also function as proton transfer groups at this position [161]. Preliminary kinetic experiments [78] suggest that the neisserial His-66 has the same catalytic function as His-64 of human CA II.

The heterologously produced *N. gonorrhoeae* carbonic anhydrase is missing the N-terminal 26 residues suggesting that the *E. coli* processing machinery recognized this fragment as a signal sequence, cleaved it and exported the enzyme to the periplasm of *E. coli* [53]. The amino-termini of the other prokaryotic enzymes also appear to have signal sequences (Fig. 1).

The only two cysteine residues, Cys-28 and Cys-181, present in the neisserial carbonic anhydrase form a disulfide bond [78] and are highly conserved among the other prokaryotic α class carbonic anhydrases (Fig. 2). The presence of a disulfide bond has also been demonstrated for the membrane-associated human isozymes such as CA IV [11,162] and the secreted human isozymes such as CA VI [83]. From the positions in the phylogenetic tree of the sequences encoding extracellular (secreted or membrane-bound) carbonic anhydrases (Fig. 7), one could speculate that the ancestral α class carbonic anhydrase was attached to a cell membrane or located outside the plasma membrane and that the intracellular cytoplasmic forms of the enzyme evolved later. Furthermore, all of the prokaryotic α class carbonic anhydrases appear to have evolved from this extracellular ancestral form (Fig. 6). The evolution of the α carbonic anhydrases from a common ancestral gene 0.5–0.6 billion years ago [1] is consistent with the evidence that few prokaryotic genomes encode α class enzymes.

6.2. Beta class

Until recently, the β class was thought to be composed solely of carbonic anhydrases from monocotyledonous and dicotyledonous plants. A mitochondrial β class carbonic anhydrase was discovered in *C. reinhardtii* [163] and other enzymes belonging to this class have since been identified in other algae [137,164]. This class was extended into the Bacteria domain upon the isolation of the gene encoding the CynT enzyme from *E. coli* [50]. The identification of CynT has been followed by the identification of several more bacterial enzymes that belong to this class [51,127,132]. Very recently, the β class was found to extend into the Archaea domain with the discovery of Cab in the methanoarchaeon *M. thermoautotrophicum* [55]. Thus, the β class of carbonic anhydrase is composed of enzymes from all three domains of life.

A search of the databases revealed 76 open reading frames with deduced sequences having significant identity

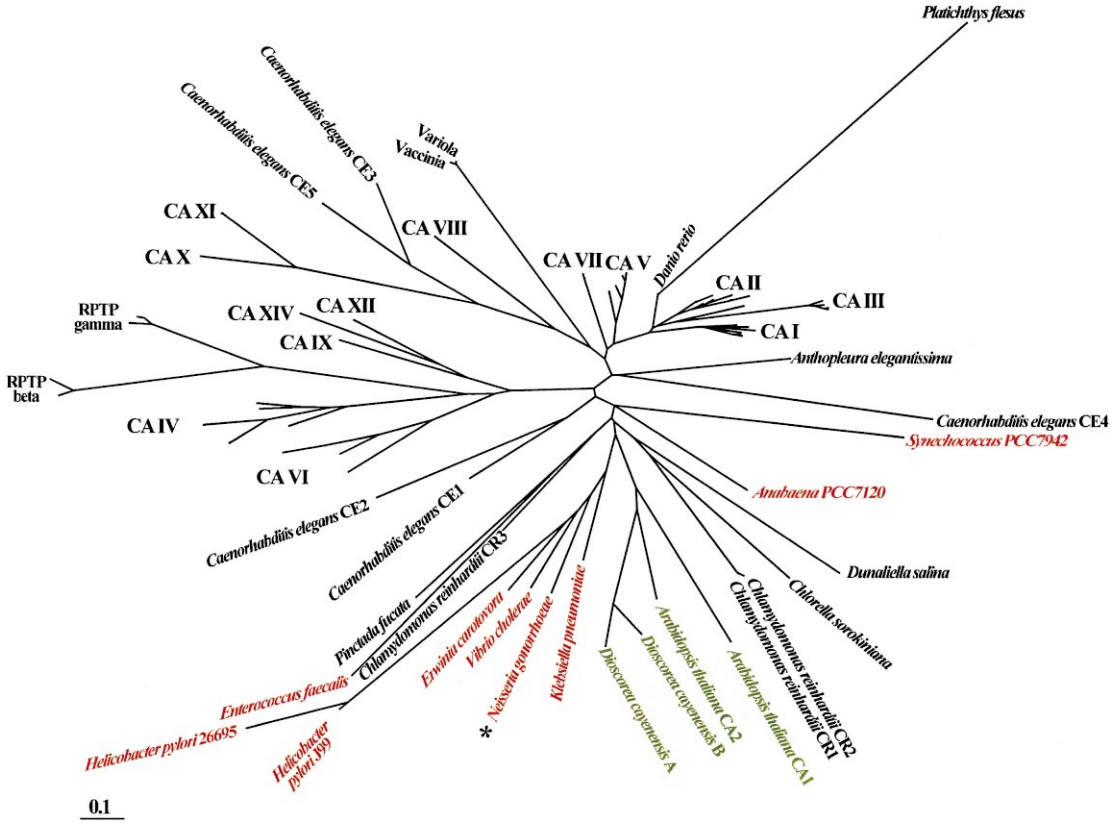

Fig. 7. Phylogenetic tree of α class carbonic anhydrase sequences. Putative α class carbonic anhydrase sequences were aligned using Clustal X [190] and analyzed with the MEGA program [158] using a neighbor joining algorithm with a gamma distance estimation ($\alpha = 2$). Phylogenetic trees were constructed based on pairwise distance estimates of the expected number of amino acid replacements per site (0.1 in the scale bar). One thousand bootstrap replicates were performed. Bacterial sequences are shown in red, plant sequences in green and non-plant eukaryotic sequences in black. Clustered, unlabeled branches indicate closely related sequences from multiple mammalian species and only the generic isozyme name is shown for the cluster. The documented α class carbonic anhydrase from *N. gonorrhoeae* is indicated by an asterisk (*).

to Cab. An alignment of the spinach and pea sequences with the deduced amino acid sequences of the prokaryotic β class carbonic anhydrase genes is shown in Fig. 5 with relevant residues highlighted. Five amino acid residues are completely conserved among the 76 sequences. Cys-32, His-87 and Cys-90 (Fig. 5) are expected to serve as the ligands of the active site Zn [57,59,136]. Asp-34 and Arg-36 (Fig. 5) are also completely conserved and site-directed mutagenesis studies with Cab suggest an important structural or catalytic role for these residues (Smith et al., unpublished results). Gly-50 (Fig. 5) is conserved in all but one (*Sulfolobus solfataricus*) of the 76 sequences.

Forty-five of the open reading frames identified (Fig. 8) are from species of the Bacteria and Archaea domains and of these only three (*E. coli* CynT, *Synechocystis* sp. strain PCC6803 CcaA and *M. thermoautotrophicum* Cab) have thus far been characterized and shown to encode proteins with carbonic anhydrase activity [50,55,132]. Distinct from all other β class carbonic anhydrase sequences, the plant sequences form two monophyletic groups representing monocotyledons (six sequences) and dicotyledons (16 sequences) (Fig. 8). The sequences from *Thiobacillus ferrooxidans*, *Bordetella pertussis* and *Rhodobacter capsulatus* are more closely related to the monocot and dicot sequences than are other prokaryotic sequences (Fig. 8). The remaining sequences can be separated into four clades, one of which (clade D) is 100% supported by bootstrapping.

Both clades B and C contain a mixture of sequences from the Eukarya and Bacteria domains (Fig. 8). The first (clade A) of the two exclusively prokaryotic clades consists primarily of sequences from Gram-negative species in the Bacteria domain and is more closely related to the plant sequences than are the other clades (Fig. 8). The second exclusively prokaryotic clade (clade D) is distantly related to the other clades and consists primarily of sequences from the Archaea domain and Gram-positive species from the Bacteria domain (Fig. 8). This clade contains 10 sequences from the Bacteria domain and three from the Archaea domain, including Cab. The presence of sequences in *Methanobacterium* species and *S. solfataricus* indicates that β class carbonic anhydrases are present in both the Euryarchaeota and Crenarchaeota kingdoms of the Archaea domain. The two exclusively prokaryotic clades A and D both contain documented prokaryotic β class carbonic anhydrases, CynT from *E. coli* [50] and CcaA from *Synechocystis* sp. strain PCC6803 [132] in clade A and Cab from *M. thermoautotrophicum* [55] in clade D. This suggests that the remaining sequences in

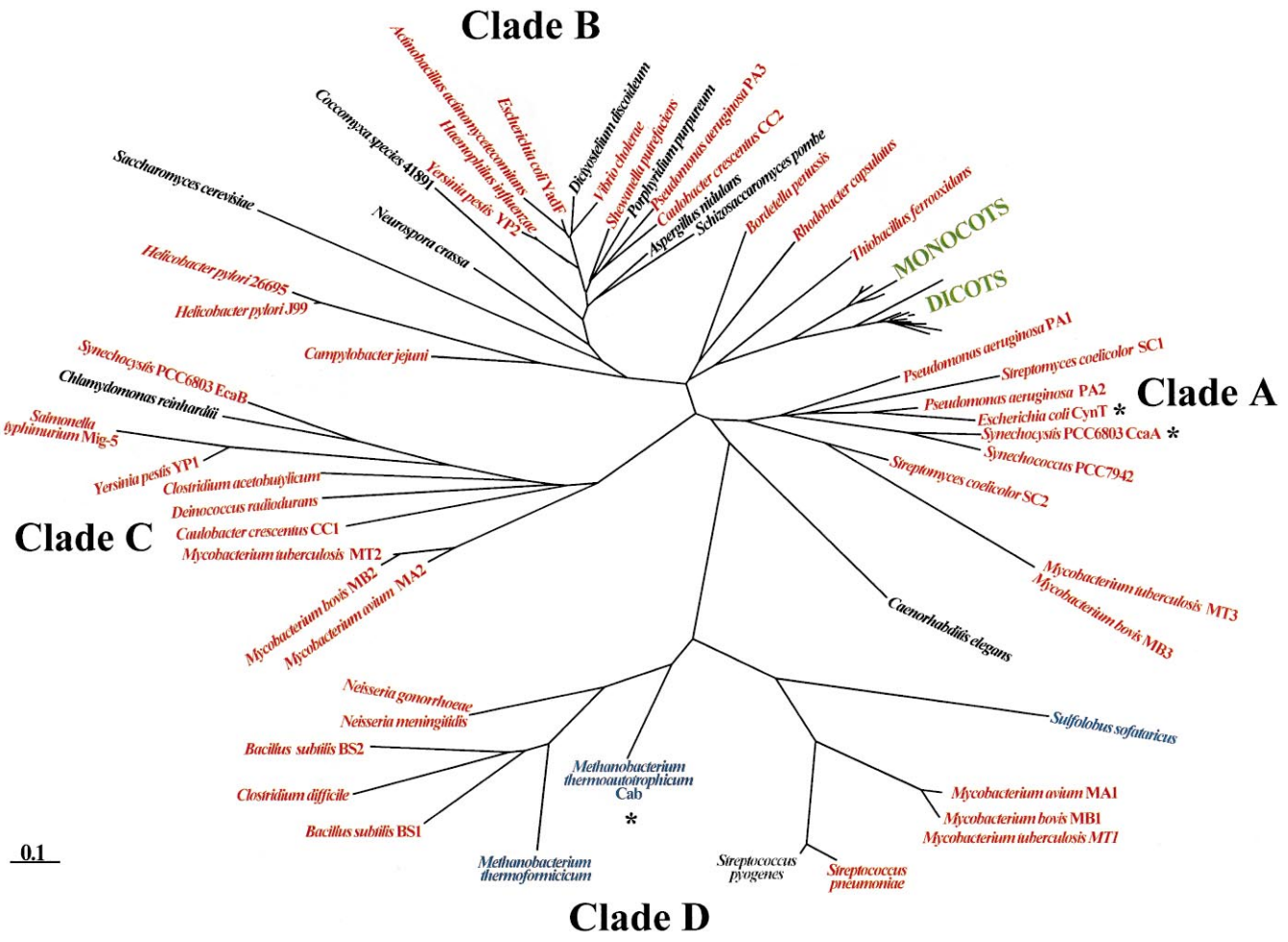

Fig. 8. Phylogenetic tree of β class carbonic anhydrase sequences. Putative β class carbonic anhydrase sequences were aligned using Clustal X [190] and analyzed with the MEGA program [158] using a neighbor joining algorithm with a gamma distance estimation ($\alpha = 2$). Phylogenetic trees were constructed based on pairwise distance estimates of the expected number of amino acid replacements per site (0.1 in the scale bar). One thousand bootstrap replicates were performed. Bacterial sequences are shown in red, archaeal sequences in blue and non-plant eukaryotic sequences in black. The monophyletic monocot and dicot plant groups are indicated in green. The documented prokaryotic β class carbonic anhydrases from *M. thermoautotrophicum*, *E. coli* and *Synechocystis* sp. PCC6803 are indicated by asterisks (*). Clades A–D, discussed in the text, are indicated.

these two clades represent valid β class carbonic anhydrases. Therefore, phylogenetic analysis bolsters the previous results of Smith et al. [14] and provides further support for the conclusion that the β class is widespread in metabolically and phylogenetically diverse prokaryotes.

Phylogenetic analysis of the β class carbonic anhydrase sequences suggests several potential horizontal gene transfer events (Fig. 8). One example is observed in clade C between *Yersinia pestis* and *Salmonella typhimurium*. The *S. typhimurium mig-5* gene product [95] shows 61.3% identity with the *Y. pestis* YP1 β class carbonic anhydrase sequence. The *mig-5* gene is flanked by inverted repeats that are also present in virulence plasmids from other pathogenic species suggesting the gene could have been acquired from a completely unrelated species by gene transfer. Whether *S. typhimurium* acquired this gene from *Y. pestis* or another pathogen remains to be determined. A second example of possible horizontal gene transfer is observed in clade B between the YadF sequence from the Gram-negative *E. coli* and the lower eukaryotic cellular slime mold *Dictyostelium discoideum*. A bootstrap value of 91% strongly supports a close relationship between these two sequences. Analysis of the *yadF* gene revealed standard *E. coli* codon usage, indicating that this gene was not recently acquired from another organism.

Some prokaryotes contain multiple β class carbonic anhydrase sequences (Fig. 8 and Table 2). For example, *Bacillus subtilis*, *Caulobacter crescentus*, *E. coli*, *Mycobacterium avium*, *Streptomyces coelicolor*, *Synechocystis* sp. strain 6803 and *Y. pestis* each contain two sequences while *Mycobacterium bovis*, *Mycobacterium tuberculosis*, and *Pseudomonas aeroginosa* each contain three sequences. Although the physiological significance of multiple genes is unknown, a simple interpretation is that carbonic anhydrases have multiple functions requiring several enzymes as exemplified by the 10 human isozymes.

Previous analysis of β class carbonic anhydrase sequences indicated extensive divergence in this class and a strong

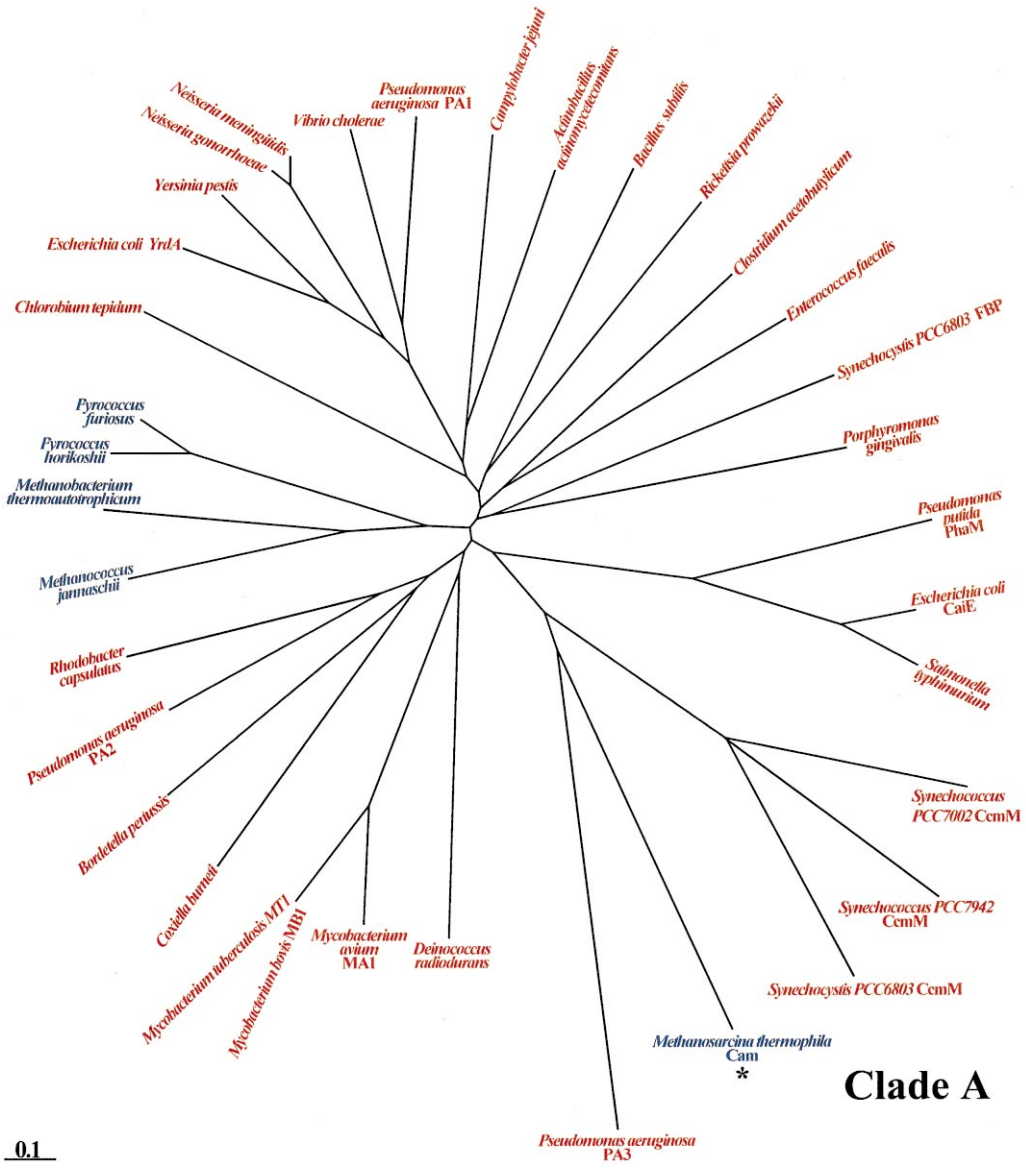

Fig. 9. Phylogenetic tree of γ carbonic anhydrase sequences. Putative γ carbonic anhydrase sequences were aligned using Clustal X [190] and analyzed with the MEGA program [158] using a neighbor joining algorithm with a gamma distance estimation ($\alpha = 2$). Phylogenetic trees were constructed based on pairwise distance estimates of the expected number of amino acid replacements per site (0.1 in the scale bar). One thousand bootstrap replicates were performed. Bacterial sequences are shown in red and archaeal sequences in blue. The documented γ carbonic anhydrase from *M. thermophila* is indicated by an asterisk (*). Clade A, discussed in the text, is indicated.

departure from the molecular clock suggesting that there has been accelerated divergence in primary structure in the different clades [14]. This divergence in primary structure between different clades is correlated with changes in the quaternary structure (Table 1). The dicot enzymes have been shown to be octamers composed of two tetramers linked by disulfide bonds, while the monocot enzymes are dimeric [49]. The *E. coli* CynT [50], the enzyme from the alga *Coccomyxa* [137,164] and the *M. thermoautotrophicum* Cab [55] have been shown to be tetrameric. It will be interesting to determine whether other prokaryotic β class enzymes are also tetrameric. Although deviations from the molecular clock preclude an estimate of divergence times, the extensive sequence diversity suggests the β class has ancient origins [14]. Additional support for an ancient origin is the presence of the β class carbonic anhydrase sequences in thermophiles representing deep branches of the universal tree of life (*M. thermoautotrophicum* and *S. solfataricus*).

6.3. Gamma class

The carbonic anhydrase (Cam) from *M. thermophila* is the prototype of a novel γ class [15]. Sequences with identity to Cam have been previously identified in all three domains of life, but the proteins encoded by these sequences have yet to be examined for carbonic anhydrase activity [14]. A new search of the databases shows 35 open

Table 2
Distribution of putative carbonic anhydrase genes in prokaryotes

Organism	Class[a]		
	α	β	γ
Archaea[b]			
Methanobacterium thermoautotrophicum[c]	0	1	1
Methanobacterium thermoformicicum[c]	–	1	–
Methanococcus jannaschii[c]	0	0	1
Methanosarcina thermophila[c]	–	–	1
Pyrococcus furiosus[c]	0	0	1
Pyrococcus horikoshii[c]	0	0	1
Sulfolobus solfataricus[d]	–	1	–
Bacteria[b]			
Actinobacillus actinomycetecomitans[g]	–	1	1
Anabaena sp. strain PCC7120[i]	1	–	–
Bacillus subtilis[l]	0	2	1
Bordetella pertussis[f]	–	1	1
Caulobacter crescentus[e]	–	2	–
Campylobacter jejuni[h]	–	1	1
Chlorobium tepidum[j]	–	–	1
Clostridium acetobutylicum[l]	0	1	1
Clostridium difficile[l]	–	1	–
Deinococcus radiodurans[k]	–	1	1
Enterococcus faecalis[l]	1	–	1
Erwinia carotovora[g]	1	–	–
Escherichia coli[g]	0	2	2
Haemophilus influenzae[g]	0	1	0
Helicobacter pylori 26695[h]	1	1	0
Helicobacter pylori J99[h]	1	1	0
Klebsiella pneumoniae[g]	1	–	–
Mycobacterium avium[l]	–	2	1
Mycobacterium bovis[l]	–	3	1
Mycobacterium tuberculosis[l]	0	3	1
Neisseria gonorrhoeae[f]	1	1	1
Neisseria meningitidis[f]	–	1	1
Porphyromonas gingivalis[j]	–	–	1
Pseudomonas aeruginosa[g]	–	3	3
Pseudomonas putida[g]	–	–	1
Rhodobacter capsulatus[e]	–	1	1
Rickettsia prowazekii[e]	0	0	1
Salmonella typhimurium[g]	–	1	1
Shewanella putrefaciens[g]	–	1	–
Streptococcus pneumoniae[l]	–	1	–
Streptococcus pyogenes[l]	–	1	–
Streptomyces coelicolor[l]	–	2	–
Synechococcus sp. strain PCC7002[i]	–	–	1
Synechococcus sp. strain PCC7942[i]	1	1	1
Synechocystis sp. strain PCC6803[i]	0	2	2
Thiobacillus ferrioxidans[f]	–	1	–
Vibrio cholerae[g]	1	1	1
Yersinia pestis[g]	–	2	1

[a]Numbers indicate the number of putative carbonic anhydrase genes present. Zero represents no putative genes were identified in a completed genome while – indicates no putative genes have been identified thus far.

[b]Species from the Archaea domain belong to the following kingdoms: euryarchaeota[c], and crenarchaeota[d]. Species from the Bacteria domain belong to the following kingdoms: proteobacteria (α class)[e], proteobacteria (β class)[f], proteobacteria (γ class)[g], proteobacteria (ε class)[h], cyanobacteria[i], cytophagales/green sulfur[j], thermus/deinococcus[k], firmicutes (Gram-positive)[l].

reading frames (Fig. 9) having deduced sequences with significant identity and similarity to Cam. Thirty are from the Bacteria domain and five are from the Archaea domain. An alignment of the putative γ class carbonic anhydrase sequences from Clade A is shown in Fig. 10. The essential histidines required for ligating the active site Zn of Cam (His-81, His-117 and His-122) [52] are completely conserved among all of the sequences suggesting they are valid carbonic anhydrases. Arg-59, Asp-61 and Gln-75 (Fig. 10) are also perfectly conserved in all of the sequences. In the structure of Cam, the side chain of Arg-59 forms a salt bridge to Asp-61 of the same monomer and Asp-76 of the neighboring monomer suggesting that the side chain of Arg-59 is important [52]. Gln-75 may function analogously to Glu-106 and Thr-199 of human CA II in participating in a hydrogen bond network with the Zn-bound hydroxide [79]. Both Glu-62, which is important for CO_2 hydration in Cam, and Glu-84, a residue involved in proton transfer, are conserved among Cam and the cyanobacterial CcmM sequences (Fig. 10). These two residues are not conserved among the other γ class carbonic anhydrase sequences, suggesting that alternative residues must serve these roles.

Two proteins outside of clade A, the *E. coli caiE* and the *Pseudomonas putida phaM*, have been studied. *E. coli* CaiE is encoded by a gene in the *caiABCDE* operon which encodes proteins involved in carnitine metabolism [165]. Concomitant overexpression of *caiE* with *caiD* (encoding the carnitine racemase) was shown to stimulate carnitine racemase activity 10-fold versus expression of *caiD* alone and also dramatically increased the basal level of carnitine dehydratase activity [165]. Concomitant overexpression of *caiE* with *caiB* (encoding the carnitine dehydratase) resulted in a nearly three-fold increase in carnitine dehydratase activity. Crude extracts of cells in which CaiE was overproduced were added to extracts of cells in which CaiB was overproduced to confirm that both CaiB and CaiE were required for maximal carnitine dehydratase activity. The carnitine dehydratase activity from this experiment was nearly equal to that observed when both *caiB* and *caiE* were expressed together [165]. These experiments suggest that CaiE is involved in either the synthesis or activation of a cofactor required for both carnitine dehydratase and carnitine racemase activities. In fact, a cofactor essential for the activity of carnitine dehydratase activity was separated from the enzyme after the first step in purification [166]. Incubation of the cofactor in the presence of DNase, RNase, trypsin or pepsin had no effect on the enzyme-reactivating ability. The cofactor was stable at 80°C for up to 1 h and at pH values down to 1, but became unstable at pH values above 10. The cofactor, with a molecular mass of less than 2 kDa, could not be substituted for by pyridoxal phosphate, ATP, coenzyme A, bivalent cations or thiol compounds. Thus, the identity of the cofactor as well as the role of CaiE in the synthesis or activation of the cofactor are unknown [167].

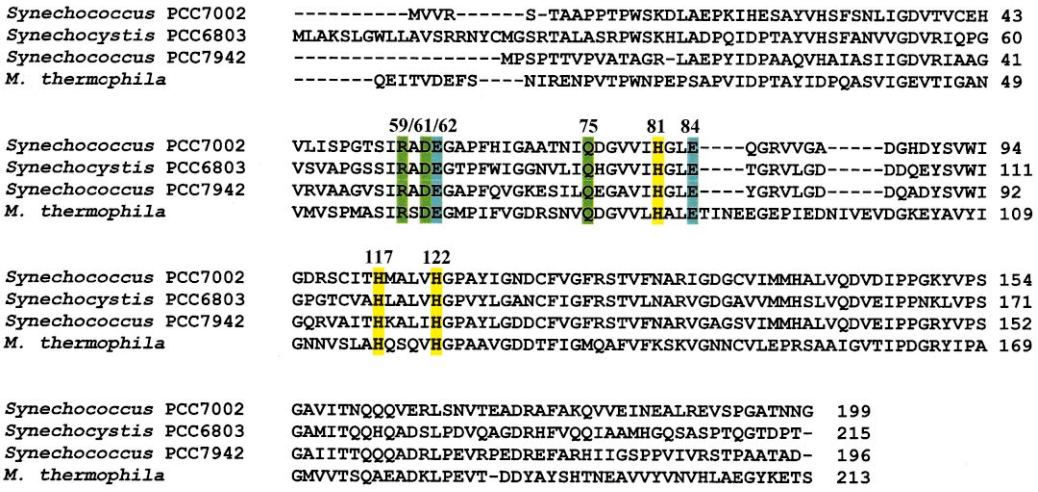

Fig. 10. Alignment of γ class carbonic anhydrase sequences. The sequence of Cam, the documented γ class carbonic anhydrase from *M. thermophila*, was aligned with the cyanobacterial CcmM sequences using Clustal X [190]. The conserved Zn ligands His-81, His-117 and His-122 are indicated in yellow. Arg-59, Asp-61 and Gln-75, shown in green, are completely conserved in all γ class carbonic anhydrase sequences. Glu-62 and Glu-84, necessary for CO_2 hydration and proton transfer, respectively, are shown in blue. Numbers indicating residue positions refer to the position in the *M. thermophila* sequence lacking the signal sequence.

P. putida PhaM is encoded by a gene found within a catabalon involved in the aerobic degradation of phenylacetic acid [168]. The *phaM* gene is located upstream in an operon with *phaN* which encodes a transcriptional repressor. Disruption of the *phaM* gene, encoding a protein of 199 amino acids with a subunit molecular mass of 21.1 kDa, did not affect catabolism of phenylacetic acid. Thus, the role of PhaM is unknown; however, PhaM shares high identity to the *E. coli* CaiE suggesting it may play a similar role. Although carbonic anhydrase activity has not yet been reported for either CaiE or PhaM, it is expected that both of these are Zn-containing enzymes since the conserved histidine residues are present.

As is the case with the γ class carbonic anhydrases, some prokaryotes contain multiple sequences with identity to Cam (Fig. 10, Table 2). For example, *E. coli* and *Synechocystis* sp. strain 6803 each contain two while *P. aeruginosa* contains three. It is interesting to note that all of the putative γ class carbonic anhydrase sequences shown in Fig. 9 are from prokaryotes and none are from the Eukarya domain. Partial ESTs (expressed sequence tags) with sequence similarity to γ class carbonic anhydrases have been identified from humans and *A. thaliana*. These ESTs contain the active site histidines and the conserved Arg-59, Asp-61 and Gln-75.

Previous analysis of sequences with identity to the γ class carbonic anhydrase from *M. thermophila* estimated divergence times that place the root of the γ class approximately 4.2 billion years ago, with the major cluster of bacterial and archaeal carbonic anhydrases having a common ancestor approximately 2.2 billion years ago, suggesting that γ class carbonic anhydrases are extremely ancient enzymes existing before the divergence of the Archaea and Bacteria domains [14]. An ancient origin for β and γ class carbonic anhydrases in prokaryotes may suggest a role for these enzymes in the recent endosymbiotic theory for the origin of eukaryotic organisms [169,170]. In this theory, a symbiotic association between a H_2- and CO_2-utilizing methanoarchaeon and a H_2- and CO_2-producing fermentative species from the Bacteria domain led to engulfment of the latter which evolved into the mitochondrion. Carbonic anhydrases may have facilitated this symbiosis by assisting in CO_2 transport between the fermentative species and the methanogenic host.

7. Other potential physiological roles for prokaryotic carbonic anhydrases

As discussed in the previous sections, two general roles have been suggested for known carbonic anhydrases: (i) transport of CO_2 or HCO_3^- and (ii) to provide CO_2 or HCO_3^- for enzymatic reactions. Physiological roles have been investigated largely in photosynthetic microbes; however, carbonic anhydrases occur across the spectrum of prokaryotic metabolism in both the Archaea and Bacteria domains and many individual species contain more than one class (Table 2), indicating that carbonic anhydrases have far more extensive and diverse roles in prokaryotes than previously recognized. These results suggest novel roles for this enzyme in prokaryotic physiology. What are these potential roles? A few possibilities are discussed below.

A large number of enzymes from non-photosynthetic microbes catalyze reactions (Table 3) for which carbonic anhydrase could be expected to provide CO_2/HCO_3^- in the vicinity of the active site or to remove CO_2/HCO_3^- to improve the energetics of the reaction. This latter role would be particularly important for decarboxylation reactions coupled to energy generation. Methylmalonyl-CoA

Table 3
Prokaryotic enzymes with CO_2 or HCO_3^- as substrate or product

Substrate	EC number	Enzyme	Reaction	Cofactor
HCO_3^-/CO_2	4.2.1.1	carbonic anhydrase	$CO_2+H_2O \rightleftharpoons HCO_3^-$	
	4.3.99.1	cyanase	cyanate+$HCO_3^- \rightleftharpoons$ carbamate+CO_2	
HCO_3^-	4.1.1.31	phosphoenolpyruvate carboxylase	phosphoenolpyruvate+HCO_3^-+ATP $\rightleftharpoons$ oxaloacetate+ADP+P_i	
	6.3.4.16	carbamoyl-phosphate synthase	ammonia+ATP+HCO_3^-+$H_2O \rightleftharpoons$ carbamoyl-phosphate+ADP+P_i	
	6.4.1.1	pyruvate carboxylase	pyruvate+ATP+$HCO_3^- \rightleftharpoons$ oxaloacetate+ADP+P_i	biotin
	6.4.1.2	acetyl-CoA carboxylase	acetyl-CoA+ATP+$HCO_3^- \rightleftharpoons$ malonyl-CoA+ADP+P_i	biotin
	6.4.1.3	propionyl-CoA carboxylase	propanoyl-CoA+ATP+$HCO_3^- \rightleftharpoons$ methylmalonyl-CoA+ADP+P_i	biotin
	6.4.1.4	methylcrotonoyl-CoA carboxylase	3-methylcrotonoyl-CoA+ATP+$HCO_3^- \rightleftharpoons$ 3-methylglutaconyl-CoA+ADP+P_i	biotin
	6.4.1.5	geranyl-CoA carboxylase	geranyl-CoA+ATP+$HCO_3^- \rightleftharpoons$ 3(4-methylpent-3-en-1-yl)-pent-2-enedioyl-CoA+ADP+P_i	biotin
CO_2	1.1.1.38	malate dehydrogenase	malate $\rightleftharpoons$ pyruvate+CO_2	NAD^+
	1.1.1.42	isocitrate dehydrogenase	isocitrate $\rightleftharpoons$ 2-oxoglutarate+CO_2	$NADP^+$
	1.1.1.44	phosphogluconate dehydrognease	6-phospho-D-gluconate $\rightleftharpoons$ D-ribulose 5-phosphate+CO_2	$NADP^+$
	1.2.1.43	formate dehydrogenase	formate $\rightleftharpoons$ CO_2	$NADP^+$
	1.2.4.2	oxoglutarate dehydrogenase	2-oxoglutarate+lipoamide $\rightleftharpoons$ *S*-succinyldihydrolipoamide+CO_2	thiamine diphosphate
	1.2.4.4	3-methyl-2-oxobutanoate dehydrogenase	3-methyl-2-oxobutanoate+lipoamide $\rightleftharpoons$ *S*-(2-methoxypropanoyl)-dihydrolipoamide+CO_2	thiamine diphosphate
	1.2.7.1	pyruvate synthase	pyruvate+CoA+ferredoxin$_{ox}$ $\rightleftharpoons$ acetyl-CoA+CO_2+ferredoxin$_{red}$	
	1.2.99.2	carbon monoxide dehydrogenase	CO+$H_2O \rightleftharpoons$ CO_2	
	1.2.99.5	formylmethanofuran dehydrogenase	methanofuran+$CO_2 \rightleftharpoons$ formylmethanofuran+H_2O	
	1.4.4.2	glycine dehydrogenase	glycine+lipoprotein $\rightleftharpoons$ *S*-aminomethyldihydrolipoylprotein+CO_2	pyridoxal phosphate
	1.13.12.4	lactate 2-monooxygenase	lactate+$O_2 \rightleftharpoons$ acetate+H_2O+CO_2	FMN
	2.1.3.1	methylmalonyl-CoA carboxyltransferase	2-methyl-3-oxopropanoyl-CoA+pyruvate $\rightleftharpoons$ propanoyl-CoA+oxaloacetate	biotin
	3.5.1.5	urease	urea+$H_2O \rightleftharpoons$ 2 ammonia+CO_2	
	3.5.3.21	methylenediurea deiminase	methylenediurea+$H_2O \rightleftharpoons$ N-urea+ammonia	
	4.1.1.1	pyruvate decarboxylase	2-oxo-acid $\rightleftharpoons$ aldehyde+CO_2	thiamine diphosphate
	4.1.1.3	oxaloacetate decarboxylase	oxaloacetate $\rightleftharpoons$ pyruvate+CO_2	biotin
	4.1.1.4	acetoacetate carboxylase	acetoacetate $\rightleftharpoons$ acetone+CO_2	
	4.1.1.7	benzoylformate carboxylase	benzoylformate $\rightleftharpoons$ benzaldehyde+CO_2	thiamine diphosphate
	4.1.1.8	oxalyl-CoA decarboxylase	oxalyl-CoA $\rightleftharpoons$ formyl-CoA+CO_2	thiamine diphosphate
	4.1.1.9	malonyl-CoA decarboxylase	malonyl-CoA $\rightleftharpoons$ acetyl-CoA+CO_2	
	4.1.1.11	aspartate-1 carboxylase	L-aspartate $\rightleftharpoons$ β-alanine+CO_2	pyruvoyl group
	4.1.1.14	valine decarboxylase	L-valine $\rightleftharpoons$ 2-methylpropanamine+CO_2	pyridoxal phosphate
	4.1.1.15	glutamate decarboxylase	L-glutamate $\rightleftharpoons$ 4-aminobutanoate+CO_2	pyridoxal phosphate
	4.1.1.17	ornithine decarboxylase	L-ornithine $\rightleftharpoons$ putrescine+CO_2	pyridoxal phosphate
	4.1.1.18	lysine decarboxylase	L-lysine $\rightleftharpoons$ cadaverine+CO_2	pyridoxal phosphate
	4.1.1.19	arginine decarboxylase	L-arginine $\rightleftharpoons$ agmatine+CO_2	pyridoxal phosphate
	4.1.1.20	diaminopimelate decarboxylase	*meso*-2,6-diaminoheptanedioate $\rightleftharpoons$ L-lysine+CO_2	pyridoxal phosphate
	4.1.1.21	phosphoribosylaminoimidazole carboxylase	1-(5-phosphoribosyl)-5-amino-4-imidazolecarboxylate $\rightleftharpoons$ 1-(5-phosphoribosyl)-5-aminoimidazole+CO_2	
	4.1.1.22	histidine carboxylase	L-histidine $\rightleftharpoons$ histamine+CO_2	pyridoxal phosphate
	4.1.1.23	orotidine-5′-phosphate decarboxylase	orotidine-5′-phosphate $\rightleftharpoons$ UMP+CO_2	
	4.1.1.25	tyrosine carboxylase	L-tyrosine $\rightleftharpoons$ tyramine+CO_2	pyridoxal phosphate
	4.1.1.36	phosphopantothenoylcysteine decarboxylase	4-phosphopantothenoyl-L-cysteine $\rightleftharpoons$ pantotheine-4-phosphate+CO_2	
	4.1.1.37	uroporphyrinogen decarboxylase	uroporphyrinogen III $\rightleftharpoons$ coporphyrinogen+4 CO_2	
	4.1.1.39	Rubisco	D-ribulose-1,5-biphosphate+$CO_2\rightleftharpoons$ 2 D-3-phosphoglycerate	
	4.1.1.41	methylmalonyl-CoA decarboxylase	2-methyl-3-oxo-propanoyl-CoA $\rightleftharpoons$ propanoyl-CoA+CO_2	ρ-hydroxy-mercuribenzoate
	4.1.1.47	tartronate-semialdehyde carboxylase	2 glyoxylate $\rightleftharpoons$ tartronate-semialdehyde+CO_2	thiamine pyrphosphate
	4.1.1.49	phosphoenolpyruvate carboxykinase	oxaloacetate+ATP $\rightleftharpoons$ phosphoenolpyruvate+ADP+CO_2	
	4.1.1.50	adenosylmethionine decarboxylase	*S*-adenosyl-L-methionine $\rightleftharpoons$ (5-deoxy-5-adenosyl)(3-aminopropyl) methylsulfonium salt+CO_2	pyruvate

Table 3 (continued)

Substrate	EC number	Enzyme	Reaction	Cofactor
	4.1.1.55	4,5-dihydroxyphtalate decarboxylase	4,5-dihydroxyphtalate ⇌ 3,4-dihydroxybenzoate+CO_2	
	4.1.1.57	methionine carboxylase	*L*-methionine ⇌ 3-methylthiopropanamide+CO_2	pyridoxal phosphate
	4.1.1.59	gallate carboxylase	3,4,5-trihydroxybenzoate ⇌ pyrogallol+CO_2	
	4.1.1.61	4-hydrobenzoate decarboxylase	4-hydroxybenzoate ⇌ phenol+CO_2	
	4.1.1.63	protocatechuate decarboxylase	3,4-dihydrobenzoate ⇌ catechol+CO_2	
	4.1.1.64	2,2-dialkylglycine decarboxylase	2,2-dialkylglycine+pyruvate ⇌ dialkyl ketone+L-alanine+CO_2	pyridoxal phosphate
	4.1.1.65	phosphatidylserine decarboxylase	phosphatidyl-L-serine ⇌ phosphatidylethanolamine+CO_2	
	4.1.1.70	glutaconyl-CoA carboxylase	pent-2-enoyl-CoA ⇌ but-2-enoyl-CoA+CO_2	biotin
	4.1.1.71	2-oxoglutarate decarboxylase	2-oxoglutarate ⇌ succinate semialdehyde+CO_2	thiamine diphosphate
	4.1.1.72	branched chain 2-oxoacid decarboxylase	(3*S*)-3-methyl-2-oxopentanoate ⇌ 2-methylbutanol+CO_2	thiamine diphosphate
	4.1.1.73	tartrate decarboxylase	(*R*,*R*)-tartrate ⇌ D-glycerate+CO_2	NAD^+
	6.3.4.14	biotin carboxylase	biotin-carboxyl-carrier protein+ATP+CO_2 ⇌ carboxyl-biotin-carboxyl-carrier protein+ADP+P_i	D-biotin

decarboxylase, oxaloacetate decarboxylase and glutaconyl-CoA decarboxylase form a unique family of decarboxylases [171–173] that utilize the free energy of the decarboxylation reaction as the driving force for active transport of Na^+ ions across the membrane. The decarboxylation of dicarboxylic acids (oxalate, malonate, glutarate and malate) can serve as the sole energy source for the growth of fermenting bacteria [173]. The free energy change for the decarboxylation reaction is quite small ($\Delta G^{\circ\prime} = -20$ kJ mol^{-1}), equivalent to approximately one-third the amount of energy required for ATP synthesis from ADP and phosphate under physiological conditions [173]. Thus, the energy from decarboxylation cannot be conserved by substrate-level phosphorylation. In the fermentation of malonate, succinate and glutarate, this energy is converted into an electrochemical gradient of sodium ions across the membrane by membrane-bound decarboxylase sodium ion pumps. Alternatively, in the fermentation of oxaloacetate and malate, the combined action of a soluble decarboxylase with a dicarboxylate/monocarboxylate antiporter establishes an electrochemical proton gradient. Na^+- or H^+-coupled F_1F_0 ATP synthases then utilize the electrochemical Na^+ or H^+ gradients for ATP synthesis. This new type of energy conservation, known as decarboxylation phosphorylation, is responsible for ATP synthesis in several anaerobic bacteria [173]. Carbonic anhydrase can be envisaged to remove the CO_2 product of the decarboxylation reactions by conversion to HCO_3^- to drive the decarboxylation reaction forward. This principle could also be important for *R. rubrum* which obtains energy for growth by oxidizing carbon monoxide and reducing protons [$CO+H_2O \rightleftharpoons CO_2+H_2$, $\Delta G^{\circ\prime} = -20$ kJ mol^{-1}] [174].

A significant amount of CO_2 fixation occurs in a diversity of non-photosynthetic prokaryotes where carbonic anhydrase would be expected to play important roles in the transport and concentration of CO_2 inside the cell. The enzymes that fix inorganic carbon utilize either CO_2 or HCO_3^-; thus, carbonic anhydrase may be necessary to interconvert these species to ensure an adequate concentration in the vicinity of the enzyme. The autotrophic methanoarchaeon *M. thermoautotrophicum* is an example of a non-photosynthetic species that fixes CO_2 and contains carbonic anhydrase. The synthesis of oxaloacetate is an important reaction in the CO_2-fixation pathways for methanoarchaea. Oxaloacetate is the starting point of an incomplete reductive tricarboxylic acid cycle that terminates at α-ketoglutarate and provides precursors for cell material and coenzyme biosynthesis [175]. *M. thermoautotrophicum* possesses two enzymes, pyruvate carboxylase and phosphoenolpyruvate (PEP) carboxylase, for the synthesis of oxaloacetate [176]. HCO_3^- has been shown to be the substrate for both of these enzymes. A precedent exists for carbonic anhydrase providing HCO_3^- to both pyruvate carboxylase and PEP carboxylase in eukaryotic systems. Human CA V is a mitochondrial enzyme that provides HCO_3^- for pyruvate carboxylase in the liver and kidney [11]. More recently, CA V has also been shown to provide HCO_3^- for pyruvate carboxylase in pancreatic islets [177]. Additionally, carbonic anhydrase plays a crucial role in the photosynthesis of C_4 plants by acting as the first enzyme in a pathway catalyzing the fixation of CO_2 into C_4 acids [178]. The enzyme provides HCO_3^- to PEP carboxylase, the initial carboxylation reaction of C_4 photosynthesis. To provide substrate for this carboxylation reaction, carbonic anhydrase rapidly converts CO_2 entering the mesophyll cells from the atmosphere to HCO_3^-.

Mammalian cells utilize cellular CO_2 as a tool to perform various physiological functions by the interconversion of an uncharged species (CO_2) to a charged species (HCO_3^-) catalyzed by carbonic anhydrase. For example, chloride transport is mediated by an antiport mechanism in which the chloride anion is exchanged with HCO_3^- [12]. CO_2 is ubiquitous in prokaryotic metabolism suggesting they too are likely to take advantage of this tool for var-

ious physiological functions such as solute transport. Database searches have identified three sequences (*S. coelicolor* SC2, *M. bovis* MB3 and *M. tuberculosis* MT3) that appear to encode bifunctional proteins (Fig. 8). These sequences encode putative transmembrane proteins whose N-terminal halves show similarity to low-affinity sulfate transporters and C-terminal regions show similarity to β class carbonic anhydrases. Thus, carbonic anhydrase may play a role in the transport of a compound using either a symport or antiport mechanism.

In addition to the interconversion of CO_2 and HCO_3^-, carbonic anhydrase has been shown to catalyze several other reactions, such as aldehyde hydration [179] and the hydrolysis of carboxylic esters [180] and various halogen derivatives [181,182]. Esters of phenols [179,180,183] and sulfonic and phosphoric acids [184,185] have been reported to act as substrates for this enzyme. Unfortunately, these activities were identified in studies with the α class human isozymes and it is not known whether the β or γ class enzymes can also catalyze these reactions. With the large reservoir of prokaryotic carbonic anhydrases, additional catalytic functions would not be unexpected.

8. Conclusions and outlook

Why is it important to study prokaryotic carbonic anhydrases when so much is already known about the plant and mammalian enzymes? Past reports of carbonic anhydrase in microbes from the Archaea and Bacteria domains have been infrequent implying a narrow function for this enzyme in prokaryotes. The enzyme has been purified from only five prokaryotic species and previous efforts to detect carbonic anhydrase activity in certain prokaryotes left the impression that carbonic anhydrases are not important to prokaryotic physiology. It is now known that carbonic anhydrases from prokaryotes are generally of very low abundance and strictly regulated, often making their presence difficult to detect. The recent discovery of the γ class, the availability of antisera to prokaryotic enzymes representing each of the classes and the availability of genome sequences of many prokaryotes has now allowed for a more comprehensive search. These analyses have demonstrated that carbonic anhydrases are indeed widespread and nearly ubiquitous among metabolically and phylogenetically diverse prokaryotes from both the Bacteria and Archaea domains, suggesting that this enzyme plays a major role in prokaryotic physiology.

Genes encoding carbonic anhydrases from all three classes have been identified in the prokaryotes with the β and γ classes predominating (Figs. 6, 8 and 9). Since no significant sequence similarities are observed between representatives of the three classes, carbonic anhydrase provides an excellent example of convergent evolution of catalytic function. Why three different classes have evolved and what advantage one class may provide over another is unknown. Interestingly, many prokaryotes contain putative carbonic anhydrase genes from more than one class (Table 2) and some even contain genes from all three known classes (Table 2). In addition, some prokaryotes contain multiple genes encoding putative carbonic anhydrases from the same class (see Section 4.2). Amazingly, *P. aeruginosa* contains six sequences that could encode carbonic anhydrase, three from the β class and three from the γ class. The multiplicity of carbonic anhydrases in many prokaryotes underscores their importance in prokaryotic physiology and suggests novel roles for the enzyme.

Are there any undiscovered classes of carbonic anhydrase? Analysis of the completed genome sequences of *Borrelia burgdorferi*, *Chlamydia trachomatis*, *Mycoplasma pneumoniae*, *Mycoplasma genitalium*, *Thermatoga maritima*, and *Treponema pallidum* from the Bacteria domain and *Archaeoglobus fulgidus* from the Archaea domain indicated an absence of open reading frames with deduced sequences having significant identity to α, β or γ class carbonic anhydrases [14]. Whether these organisms possess a different class of carbonic anhydrase or do not require this enzymatic activity remains to be answered. Since carbonic anhydrases seem to be ancient enzymes and there are already three known classes that were independently invented, it would not be surprising to find even more classes. One method to identify genes encoding carbonic anhydrase from these prokaryotes would be by genetic complementation of an *E. coli cynT* mutant [88].

In addition to biochemical and physiological studies, carbonic anhydrases have been exploited for biotechnological purposes such as the design of metal ion sensors and de novo catalytic sites. For example, carbonic anhydrase transducers have been created that measure picomolar concentrations of Cu(II), Zn(II), Cd(II), Co(II) and Ni(II) in complex media as determined by the changes in the fluorescence lifetime and intensity of site-specifically labeled fluorescent variants of a human CA II apoenzyme [186,187]. Other technological uses for carbonic anhydrase such as in CO_2 scrubbers can also be imagined.

Although there are many questions to be answered about the enzymology and structure of prokaryotic carbonic anhydrases, one major question still remains: what are the roles of carbonic anhydrases in prokaryotic physiology? Most of the information known about the function of this enzyme in prokaryotes relates to its role in the fixation of CO_2 by cyanobacteria; however, the research reviewed here strongly supports that prokaryotes from diverse metabolic and phylogenetic origins contain multiple carbonic anhydrase genes. Undoubtedly, future research will uncover diverse novel functions for this ubiquitous prokaryotic enzyme.

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