A Plant-Type (β-Class) Carbonic Anhydrase in the Thermophilic Methanoarchaeon Methanobacterium thermoautotrophicum

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Received 21 April 1999/Accepted 30 July 1999

Carbonic anhydrase, a zinc enzyme catalyzing the interconversion of carbon dioxide and bicarbonate, is nearly ubiquitous in the tissues of highly evolved eukaryotes. Here we report on the first known plant-type (β-class) carbonic anhydrase in the archaea. The Methanobacterium thermoautotrophicum ΔH cab gene was hyperexpressed in Escherichia coli, and the heterologously produced protein was purified 13-fold to apparent homogeneity. The enzyme, designated Cab, is thermostable at temperatures up to 75°C. No esterase activity was detected with p-phenylacetate as the substrate. The enzyme is an apparent tetramer containing approximately one zinc per subunit, as determined by plasma emission spectroscopy. Cab has a CO2 hydration activity with a kcat of 1.7 × 106 s−1 and Km for CO2 of 2.9 mM at pH 8.5 and 25°C. Western blot analysis indicates that Cab (β-class) is expressed in M. thermoautotrophicum; moreover, a protein cross-reacting to antiserum raised against the γ carbonic anhydrase from Methanosarcina thermophila was detected. These results show that β-class carbonic anhydrases extend not only into the Archaea domain but also into the thermophilic prokaryotes.

Carbonic anhydrase is a zinc-containing enzyme that catalyzes the reversible hydration of CO2 (CO2 + H2O ⇌ HCO3− + H+). In eukaryotes, the enzyme participates in various physiological functions, which include the interconversion of CO2 and HCO3− during photosynthesis and intermediary metabolism, facilitated diffusion of CO2−3, pH homeostasis, and ion transport (5, 45). Carbonic anhydrase is nearly ubiquitous in highly evolved eukaryotes, but until recently the enzyme was thought to play a minor role in prokaryotic physiology (48). All carbonic anhydrases are divided into three distinct classes (α, β, and γ) that have no sequence homology and evolved independently (22). Carbonic anhydrases from mammals (including the 10 active human isoforms) (22, 39) together with the two periplasmic enzymes from the unicellular green alga Chlamydomonas reinhardtii (16, 17) belong to the α class. The β class is comprised of enzymes from the chloroplasts of both monocotyledonous and dicotyledonous plants (22). Four prokaryotic carbonic anhydrases from species in the Bacteria domain, two belonging to the α class and two belonging to the β class, have been described (13, 20, 49, 50). The only carbonic anhydrase purified from an archaeon, Methanosarcina thermophila, is decidedly distinct from the α and β classes and is the prototype of a novel class (γ class) (2).

Crystal structures have been determined for five isozymes (CA I to CA V) of the monomeric human carbonic anhydrase (9, 14, 21, 28, 51) and the Neisseria gonorrhoeae enzyme (25) belonging to the α class. The overall folds of these monomeric enzymes are highly similar, with a 10-stranded, mainly antiparallel, β-sheet as the dominating structure. The catalytically active zinc is ligated to three histidines residues, with a water molecule acting as a fourth ligand. The structure of the prototype γ carbonic anhydrase from M. thermophila has recently been solved and found to be entirely different from those of the α-class enzymes (31). The enzyme is a trimer with three zinc-containing active sites, each located at the interface between two monomers. The zinc is coordinated in a tetrahedral geometry with three histidines and two to three putative water molecules serving as ligands (1). The main secondary structures are several parallel β-sheets forming a left-handed β-helix. Although a three-dimensional structure has yet to be determined for a β carbonic anhydrase, extended X-ray absorption fine structure and circular dichroism of the plant enzyme indicate that the zinc coordination and overall structure are quite different from those for either the α- or γ-class enzymes (10, 42).

Methanoarchaeon, the largest group within the Archaea domain, obtain energy for growth by either the production of methane from the reduction of CO2 utilizing H2 or formate as electron donors, or the conversion of the methyl groups of acetate, methanol, or methylamines to methane (8, 15). To date, the only characterized carbonic anhydrase from the Archaea domain is the γ carbonic anhydrase (Cam) from the methanoarchaeon M. thermophila, which can convert the methyl group of acetate, methanol, or methylated amines to methane. Western blot analysis indicates that Cam is expressed predominantly during growth on acetate, suggesting that this carbonic anhydrase is important for acetotrophic growth (3). Cam appears to be located outside the cell, and it has been proposed that it might be required for a CH3CO2−/H+ symport system or for efficient removal of cytoplasmically produced CO2 during growth on acetate (2).

Here we report on the initial characterization of a β carbonic anhydrase from Methanobacterium thermoautotrophicum ΔH, the first carbonic anhydrase from a CO2-reducing methanoarchaeon in the Archaea domain. Our results show that β carbonic anhydrases occur not only in the Archaea domain but also in thermophilic chemolithoautotrophs, species that represent some of the deepest branches of the universal tree of life (53).

MATERIALS AND METHODS

Cloning and hyperexpression of the cab gene in Escherichia coli. Two oligonucleotides (primers MBTCA1 [5'-GGTTTCTACAGTTATTAAG-3'] and MBTCA2 [5'-CGT AGAGGGTCTCAG-3']; partially corresponding to the amino terminus of Cab] and MBTCA2 [5'-CGT AGAGGGTCTCAG-3'; partially corresponding to the carboxy terminus of Cab]), 100 ng of M. thermoautotrophicum ΔH genomic DNA, and the GeneAmp

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DNA amplification kit (Perkin-Elmer Cetus) were used to amplify the genomic region containing the cab gene. The amplification generated Neo and BamHI restriction sites on the ends of the amplified product. The product was digested with Neo and BamHI and cloned in the appropriately digested pUC18 (Novagen) to generate plasmid pMBTCA13. E. coli BL21(DE3) competent cells were transformed with pMBTCA13, grown at 37°C in Luria-Bertani broth containing 100 μg of ampicillin per ml, and induced with 27.6 mM lactose and 0.5 mM zinc sulfate (final concentration) at an ice bath temperature of 6°C. After an induction of 3 h at 37°C, the cells were harvested by centrifugation and stored at −70°C.

**Purification of the heterologously produced Cab.** Carbonic anhydrase activity was measured at room temperature, using a modification of the electrometric method of Fairbrothers et al. (5). With the modification described by the Bradford method (58), protein concentrations were determined by the Bradford method (11), using Bio-Rad dye reagent and bovine serum albumin (Sigma) as the standard. Thawed cell paste (10 g [wet weight]) was suspended in 20 ml of buffer A (50 mM potassium phosphate, pH 6.8) and passed twice through a chilled French pressure cell at 138 MPa. The cell lysate was centrifuged at 20,000 × g for 20 min to remove cell debris. The supernatant was recentrifuged at 100,000 × g for 2 h. The cell extract was then heated at 65°C for 20 min and centrifuged at 20,000 × g for 15 min. The supernatant was loaded onto a Mono Q 10/10 anion-exchange column (Pharmacia) equilibrated with buffer A. After a 30-ml wash, the column was developed with a flow rate of 0.4 ml/min.

**Steady-state kinetics.** Initial rates of CO₂ hydration were determined by a semilogarithmic plot. The uncatalyzed rate of the reaction was determined by adding 0.15 ml of 100 mM potassium phosphate (pH 7.0) and recording the change in A₄₅₀ per min (ΔA₄₅₀ = 5000 M⁻¹ cm⁻¹). After 2 min, 15 μl of enzyme solution was added, and the catalyzed reaction was monitored for an additional 3 min.

**Molecular mass determination.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described previously (33), using 12% gels. The native molecular mass was determined by gel filtration chromatography, using a Superose 12 gel filtration column (Pharmacia) calibrated with bovine milk α-lactalbumin (14.2 kDa), chicken egg albumin (45 kDa), bovine serum albumin (66 kDa), dimer, 132 kDa), and urease (trimer, 272 kDa; hexamer, 545 kDa). Protein samples (0.5 ml) were loaded onto the column pre-equilibrated with buffer A containing 150 mM KCl, and the column was developed with a flow rate of 0.4 ml/min.

**RESULTS AND DISCUSSION**

**Heterologous production and purification of Cab.** Recently, genome sequencing (41, 46) of the thermophilic, obligately chemolithoautotrophic methanooarchaeon *M. thermoautotrophicum* ∆H revealed an open reading frame (ORF) with a deduced sequence 34.3% identical to that of CynT, the β carbonic anhydrase of *E. coli* (20). The gene, designated *cab* (carbonic anhydrase beta), was PCR amplified and cloned into the pET16b vector to produce plasmid pMBTCA13 and then expressed in *E. coli* by using the T7 promoter/polymerase expression system (54, 55). Greater than 95% of the carbonic anhydrase activity was recovered in the soluble fraction after ultracentrifugation of the *E. coli* cell extract for 2 h at 100,000 × g. By taking advantage of the thermal stability of Cab, a major purification step was the incubation of the high-speed (ultracentrifuge) soluble supernatant at 65°C for 15 min followed by centrifugation to remove the denatured *E. coli* proteins. The heterologously produced enzyme was purified 13-fold to apparent homogeneity (Table 1), as indicated by a single polypeptide band after SDS-PAGE (Fig. 1).

Most dicotyledonous plant carbonic anhydrases that have been purified and characterized are reported to be dependent on a reducing agent to retain catalytic activity. The oxidized, inactive pea enzyme could be reactivated to only 60% of the original activity by the addition of a reducing agent (26, 27). Purification in the presence of reducing agents or the addition of reducing agents to purified Cab had no affect on the catalytic activity (47). Thus, Cab joins the other two prokaryotic β carbonic anhydrases that are insensitive to oxidation (20, 49).

**Biocatalytic characterization.** (i) Thermostability. The activity of Cab was stable when the enzyme was incubated for 15 min at temperatures up to 75°C (Fig. 2). This is not surprising since the optimal growth temperature for *M. thermoautotrophicum* ∆H is between 65 and 75°C (8). Little activity was recovered when the enzyme was incubated at temperatures of 90°C or higher. Thus, Cab is the most thermostable carbonic anhydrase yet characterized.

(ii) **Inhibition.** Iodide, nitrate, and azide are potent inhibitors of Cab (IC₅₀ concentrations of inhibitor resulting in 50% inhibition of enzyme activity, determined by a semilogarithmic

<table>
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<tr>
<th>Step</th>
<th>Total activity (U/mg)</th>
<th>Protein (mg)</th>
<th>Sp act (U/mg)</th>
<th>Recovery (%)</th>
<th>Purification fold</th>
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<tr>
<td>Mono Q</td>
<td>624.0</td>
<td>16.0</td>
<td>39.0</td>
<td>70</td>
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* *Measured by the electrometric method (58). One unit = (ΔA₄₅₀/t) × 100, where t is the time for the uncatalyzed reaction and t is the time of the catalyzed reaction. *D* Determined by the biuret assay. *After ultracentrifugation at 100,000 × g for 2 h. *After incubation at 65°C for 15 min followed by centrifugation at 20,000 × g for 15 min. *After the second Mono Q step.

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**Table 1. Purification of Cab heterologously produced in *E. coli***
plot of percentage of inhibition versus logarithmic concentration of inhibitor] of 1.0, 1.5, and 2.1 mM, respectively); however, chloride and sulfate, which inhibit plant β carbonic anhydrases, had no effect on the activity of Cab (47). The insensitivity to chloride and sulfate suggests a fundamental difference in the active sites of Cab and the plant enzymes. The effect of these anions on the two other known prokaryotic β carbonic anhydrases has not been reported. The insensitivity to chloride and sulfate is also observed for Cam from *M. ther- mophila*, the only other known methanooarchaeal carbonic anhydrase (2, 3).

Although all three classes of carbonic anhydrase are inhibited by the same types of compounds, inhibition constants vary considerably between individual enzymes. Detailed structural information for enzyme-inhibitor complexes exists only for the α class carbonic anhydrases, primarily human isozymes CA I and CA II (37). Anions prevent formation of the coordinated hydroxide ion, which is essential in the catalytic hydration of CO₂ (35, 36). Nitrate belongs to a group of anions that appear to bind close to the metal ion without displacing the critical zinc-bound water molecule necessary for activity (38). Conversely, another group of anions including chloride, iodide, and azide directly coordinate to the metal ion and displace the zinc-bound water molecule (32, 40). The α class carbonic anhydrases are only weakly inhibited or not inhibited by divalent anions such as sulfate (43); thus, sulfate is only an inhibitor for plant enzymes belonging to the β class.

Cab is also susceptible to inhibition by the sulfonamides acetazolamide and ethozolamide (IC₅₀ of 0.06 and 0.007 mM, respectively). Sulfonamides inhibit the activity of carbonic anhydrases by binding to the active site zinc via the nitrogen atom of the sulfonamide group (37). Differences in the affinity of the sulfonamides have been explained by subtle differences in the active sites of the human isozymes; therefore, it is not surprising to see differences between the different classes of carbonic anhydrases.

(iii) Esterase activity. Some carbonic anhydrases belonging to the α family catalyze the reversible hydrolysis of esters. With *p*-nitrophenylacetate as a substrate, commercially available human CA II showed an esterase activity of 38.2 mol of *p*-nitrophenol per min per mol of enzyme. In contrast, Cab showed no detectable esterase activity (<0.01 mol of *p*-nitrophenol per min per mol of enzyme); thus, Cab joins other carbonic anhydrases from the β class in lacking any detectable esterase activity (20, 30). Cam, the only characterized carbonic anhydrase of the γ class, also lacks an esterase activity (3).

(iv) Subunit composition. A subunit molecular mass of 21 kDa was estimated by SDS-PAGE (Fig. 1). A subunit molecular mass of 18.9 kDa was calculated on the basis of the amino acid composition deduced from the nucleotide sequence. Native gel filtration chromatography of Cab gave an estimated molecular mass of 90 kDa. Given a calculated subunit molecular mass of 18.9 kDa, these results suggest that the native enzyme is a tetramer.

According to quaternary structure, the β carbonic anhydrases are divided into three groups represented by the dicotyledon, monocotyledon, and nonplant enzymes (7). The native molecular masses of the enzymes from dicotyledenous plants have been reported to vary between 140 and 250 kDa, with a subunit mass of 24 to 34 kDa. The oligomeric state has been recently shown to be an octamer, consisting of two covalently linked tetramers (7). Carbonic anhydrases from monocotyledenous plants have been suggested to be dimers (19). CynT from *E. coli* has been reported to be either a tetramer or a dimer, depending on the experimental conditions (20), and the β carbonic anhydrase identified in the unicellular green alga *Coccomyxa* sp. has also been shown to be a tetramer (23). Thus, the quaternary structure of Cab is like that of the enzymes from *E. coli* and *Coccomyxa* sp.

(v) Metals analysis. The carbonic anhydrases from the α, β, and γ classes contain one zinc per enzyme subunit and are thought to have a common zinc hydroxide mechanism for catalysis. A comprehensive metals analysis (20 elements) of Cab was performed by plasma emission spectroscopy using two independent enzyme preparations with similar specific activities. Since the Bradford assay was found to underestimate the concentration of Cab by a factor of 2, the biuret assay was used to determine protein concentrations for the metals analysis. The analysis revealed 1.01 and 0.97 Zn per subunit of Cab for preparations I and II, respectively, suggesting Cab contains one zinc per subunit.

Kinetic properties of Cab. Kinetic parameters for the CO₂ hydration activity were measured at 25°C in a stopped-flow spectrophotometer. The values for Cab are presented in Table 2 along with the values for the kinetically characterized β-class
carbonic anhydrases as well as those for the only two kinetically characterized prokaryotic enzymes besides Cab, the prototypical γ-class methanooarchaeal enzyme and the α-class neisserial enzyme. The $K_m$ value for CO$_2$ is similar to those of the characterized enzymes of the β-class; however, the $k_{cat}$ for Cab is lower than those for the carbonic anhydrases isolated from higher plants (6, 34) and the unicellular green alga Coccomyxa sp. (23) (Table 2). One possible reason for the greater than 10-fold difference in $k_{cat}$ and in the catalytic efficiency ($k_{cat}/K_m$) may be 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.

The values determined for Cab are the first kinetic parameters for a prokaryotic β carbonic anhydrase. Among the prokaryotic carbonic anhydrases that have been kinetically characterized, the N. gonorrhoeae α-class enzyme (13) has the highest $k_{cat}$ value and is as catalytically efficient ($k_{cat}/K_m$) as the high-activity human isozyme, CA II (29, 52). The $K_m$ values for CO$_2$ for the α and γ prokaryotic carbonic anhydrases (1, 13) are over fivefold greater than that of Cab, suggesting that Cab may have a physiological role distinct from those of the prokaryotic α- and γ-class enzymes.

**Alignment of β-class carbonic anhydrases.** On the basis of amino acid sequence comparisons, carbonic anhydrases belong to three genetically distinct classes (α, β, and γ) that evolved independently (22). We had previously identified 51 ORFs with deduced sequences having significant identity and similarity to the sequences of Cab from M. thermoautotrophicum by Blastp and tBLASTn searches of the nonredundant sequence database at the National Center for Biotechnology Information and the finished and unfinished microbial genome sequences (48). Of the 26 ORFs identified from species of the Bacteria and Archaea domains, only 2 are known to encode documented carbonic anhydrases (20, 49). Distinct from all other β carbonic anhydrase sequences, the plant sequences form two monophyletic groups representing monocotyledons and dicotyledons. The remaining sequences form four clades, and Cab is found in one of the two clades composed exclusively of prokaryotic sequences.

An alignment of the sequences that form a clade with Cab is shown in Fig. 3. Indicated in this alignment are six amino acid residues that are 100% conserved among the 62 sequences of putative β carbonic anhydrases identified in the search. Extended X-ray absorption fine structure studies of the spinach enzyme suggest that the active-site zinc is coordinated by two cysteine residues and one histidine residue (10, 42). Cysteine-32, histidine-87, and cysteine-90 of Cab are conserved among all of the sequences and would be expected to be the ligands for the active-site zinc in Cab; however, a structure is needed for definitive proof. Three other residues in Cab that are conserved among these sequences are asparagine-34, arginine-36, and glycine-50, suggesting an important structural or catalytic role. The alignment shown in Fig. 3 reveals additional residues that are 100% conserved among the members of this clade.

**Expression of Cab in M. thermoautotrophicum.** We have previously shown that carbonic anhydrase activity (0.8 U/mg) is present in M. thermoautotrophicum cell extract (48). Western blot analysis was performed to determine if this activity is at least in part due to expression of Cab in M. thermoautotrophicum. In addition to an antisera raised against Cab, antiserum was directed against the Anabaena strain PCC7120 α-carbonic anhydrase and M. thermoautotrophica γ carbonic anhydrase (Cam) were used. A cross-reacting protein of the correct size for Cab was detected by Western blot analysis using the antiserum raised against Cab (Fig. 4). No proteins cross-reacting to the antisera raised against the α carbonic anhydrase were detected; however, a protein cross-reacting to the antisera raised against the γ carbonic anhydrase from M. thermophila was detected (Fig. 4). Analysis of the genome sequence of M. thermoautotrophicum revealed an ORF encoding a protein with 30.7% identity to Cab; however, it is not yet known if the protein encoded by this ORF has 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.

Cab is the first documented carbonic anhydrase from a CO$_2$-reducing chemolithoautotrophic methanooarchaeon. These microbes have a high demand for CO$_2$ in both catabolic and anabolic reactions, suggesting that carbonic anhydrase may be essential to deliver CO$_2$ to the cell and concentrate it in the vicinity of CO$_2$-utilizing enzymes. This is analogous to the role of carbonic anhydrase in photosynthetic organisms in which the enzyme is essential for efficient CO$_2$ transport into the cell and elevation of the CO$_2$ concentration near the active site of the CO$_2$-fixing enzyme ribulose bisphosphate carboxylase (5). For example, Cab could convert bicarbonate to CO$_2$, the substrate for the formylmethanofuran dehydrogenase (57) catalyzing the first committed step of methanogenesis. In addition to utilizing CO$_2$ in its energy metabolism, M. thermoauto...
**phicum** is a chemolithoautotroph, synthesizing cell carbon from CO\(_2\). The central anabolic pathways for *M. thermoautotrophicum* are the autotrophic pathway for acetyl coenzyme A biosynthesis and the incomplete reductive tricarboxylic acid cycle (44). Some of the CO\(_2\) fixation enzymes in these pathways utilize HCO\(_3^-\); thus, interconversion between CO\(_2\) and HCO\(_3^-\) is another potential role for Cab. Similar mechanisms requiring carbonic anhydrase may also facilitate the growth of anaerobes which obtain energy by reducing carbon dioxide to either acetate (*Acetobacterium woodii* and *Clostridium thermocellum*) or methane (*Methanobacterium formicicum* and *Methanospirillum hungatei*). In fact, carbonic anhydrase activity has been detected in these anaerobes, and Western blot analysis has identified proteins that cross-react to antisera raised against Cab (12, 48).

**Conclusions.** The results presented here are the first demonstration of plant-type (β-class) carbonic anhydrases in the Archaea. The heterologously produced carbonic anhydrase from *M. thermoautotrophicum*, designated Cab, is the first documented β carbonic anhydrase from a thermophile and is thermostable at temperatures up to 75°C. It is anticipated that the thermophilic nature of Cab will facilitate the determination of a crystal structure, the first for any enzyme of the β class. The presence of β carbonic anhydrase in a thermophilic archaeon suggests that this enzyme may play a more widespread role in prokaryotic physiology than previously thought.

**ACKNOWLEDGMENTS**

We thank John Coleman and Birgit Alber for the generous gifts of antisera. We also thank Cheryl Ingram-Smith for critical reading of the manuscript.
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