Characterization of Heterologously Produced Carbonic Anhydrase from *Methanosarcina thermophila*

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The gene encoding carbonic anhydrase from *Methanosarcina thermophila* was hyperexpressed in *Escherichia coli*, and the heterologously produced enzyme was purified 14-fold to apparent homogeneity. The enzyme purified from *E. coli* has properties (specific activity, inhibitor sensitivity, and thermostability) similar to those of the authentic enzyme isolated from *M. thermophila*; however, a discrepancy in molecular mass suggests that the carbonic anhydrase is posttranslationally modified in either *E. coli* or *M. thermophila*. Both the authentic and heterologously produced enzymes were stable to heating at 55°C for 15 min but were inactivated at higher temperatures. No esterase activity was detected with *p*-nitrophenylacetate as the substrate. Plasma emission spectroscopy revealed approximately 0.6 Zn per subunit. As judged from the estimated native molecular mass, the enzyme is either a trimer or a tetramer. Western blot (immunoblot) analysis of cell extract proteins from *M. thermophila* indicates that the levels of carbonic anhydrase are regulated in response to the growth substrate, with protein levels higher in acetate than in methanol- or trimethylamine-grown cells.

Carbonic anhydrase catalyzes the reversible hydration of CO_2 and is widely distributed in nature. The enzyme participates in various physiological functions, which include interconversion of CO_2 and HCO_3^- during photosynthesis and intermediary metabolism, facilitated diffusion of CO_2, pH homeostasis, and ion transport (4, 36). Three classes of carbonic anhydrases have been proposed on the basis of amino acid sequence comparisons (1, 13). The mammalian class (α) includes seven isozymes from higher vertebrates and two isozymes from the microalga *Chlamydomonas reinhardtii*. These are by far the best-studied carbonic anhydrases. The three-dimensional structures for human isozymes I and II, bovine isozyme III, and murine isozyme V have been resolved by X-ray crystallography and refined (7, 11, 17, 21). The catalytic mechanism has been studied in detail and involves a Zn-hydroxide anion as the catalytic group (for reviews, see references 27, 33, and 42). The chloroplast carbonic anhydrases from higher plants and two bacterial enzymes make up the plant class (β) which contains a Zn binding motif different from that of the mammalian class (8, 32). The mammalian and plant classes are believed to have a common mechanism for CO_2 hydration (8).

The recently discovered carbonic anhydrase from *Methanosarcina thermophila* (Cam) represents the prototype of the third (γ) class of carbonic anhydrases (1). Methanogenic anaerobes are the largest group within the *Archaea*, a domain which is phylogenetically closer to the *Eucarya* than to the *Bacteria* (40). *M. thermophila* is one of the metabolically more diverse methanogens in that it obtains energy for growth by converting the methyl groups of acetate, methanol, or methylamines to methane (6). During methanogenesis from acetate, oxidation of the carbonyl group provides electrons for reduction of the methyl group, leaving CO_2 as the second product (12). A metabolic switch from methanol to acetate elevates carbonic anhydrase activity (20, 22), suggesting that this enzyme is important for growth on acetate. It has been proposed that carbonic anhydrase might be required for a $CH_3CO_2^-/H^+$ symport system or for efficient removal of cytoplasmically produced CO_2 (1).

Although carbonic anhydrase activity in *M. thermophila* reaches the highest levels during growth on acetate, very little Cam is produced (1). The difficulties encountered by acetotrophic growth of this strict anaerobe, and the low yields of purified Cam, have precluded the determination of several biochemical features of the enzyme. Here we report the heterologous hyperproduction of Cam with high specific activity and an initial biochemical characterization of the first representative of the γ class of carbonic anhydrases. We also show that the amount of Cam is regulated in response to the growth substrate.

MATERIALS AND METHODS

Hyperexpression of the *cam* gene encoding Cam and production of the enzyme in *Escherichia coli*. Two 25-mer oligonucleotides (primer I, 5′-AGCCTCATATG CAGGAAATAACCGT-3′, partially corresponding to nucleotides encoding amino acids 35 to 39 of Cam; primer II, 5′-TGCGCGGATCCAAGATAGATA TCTA-3′, partially corresponding to nucleotides 380 to 395 downstream of the *cam* gene), 100 ng of chromosomal *M. thermophila* DNA (1), and the GeneAmp DNA amplification reagent kit (Perkin-Elmer Cetus) were used to amplify a 1,054-bp genomic region of *M. thermophila* containing the *cam* gene. This amplification generated *Nde*I and *Bam*HI sites on either end of the amplified fragment and excluded the coding region for the putative signal sequence (1). The PCR product was blunt ended with T4 DNA polymerase, digested with *Nde*I and *Bam*HI, and cloned into the appropriately restriction enzyme digested pT7/7 vector (35) to yield pBA1416NB. Competent *E. coli* BL21(DE3) cells (34) were transformed with pBA1416NB, grown at 37°C in Luria-Bertani broth containing 100 μg of ampicillin per ml, and induced at an A_{600} of 0.6 to 1.0 with 0.4 mM isopropylthiogalactopyranoside (IPTG) and 0.5 mM zinc sulfate (final concentrations). After additional growth for 2.5 h at 37°C, the cells were harvested and stored in liquid nitrogen until use.

Purification of heterologously produced Cam. Cam activity was measured at room temperature by using a modification of the electrometric method of Wilbur and Anderson (39) as described previously (1). Protein concentrations were determined by the method of Bradford (9), using Bio-Rad dye reagents and bovine serum albumin (Pierce) as a standard. Thawed cell paste (15 g [wet weight]) was suspended in 20 ml of buffer A (50 mM potassium phosphate [pH 6.8] containing 1 μM zinc sulfate) and passed twice through a chilled French

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TABLE 1. Purification of Cam heterologously produced in *E. coli*

Step	Total activity (U)[a]	Protein (mg)[b]	Sp act (U/mg)	Recovery (%)	Purification (fold)
Cell extract[c]	300,000	630	470	100	1
Q-Sepharose	260,000	72	3,700	88	7.8
Phenyl-Sepharose	240,000	36	6,600	79	14

[a] One unit = $(t_0 - t)/t$, where t_0 is the time for the uncatalyzed reaction and t is the time for the enzyme-catalyzed reaction.

[b] Determined by the Bradford protein assay.

[c] After ultracentrifugation at $100,000 \times g$ for 2 h.

pressure cell at 20,000 lb/in^2 (1 lb/in^2 = 6.9 kPa). After the first passage, 0.25 mg of DNase I and 0.1 mg of RNase A were added, and the mixture was incubated for 10 min at 4°C. The cell lysate was centrifuged at $20,000 \times g$ for 15 min. The supernatant solution was recentrifuged at $100,000 \times g$ for 2 h. The cell extract was applied to a Q-Sepharose column (Pharmacia HiLoad 26/10) equilibrated with buffer A. The column was washed with 150 ml of buffer A, and then 1 liter of a linear gradient increasing from 0 to 1 M NaCl was applied at 2.0 ml/min. The peak of carbonic anhydrase activity which eluted between 0.44 and 0.5 M NaCl was pooled, solid ammonium sulfate was added to 1.5 M, and the sample was equilibrated overnight at 4°C. After centrifugation of the mixture at $20,000 \times g$ for 15 min, the supernatant was loaded onto a phenyl-Sepharose column (Pharmacia HiLoad 26/10) equilibrated with buffer B (100 mM potassium phosphate [pH 7.0] containing 1 μM zinc sulfate and 1.5 M ammonium sulfate). After a 150-ml wash, the column was developed with a 500-ml linear gradient decreasing from 1.5 to 0 M ammonium sulfate at 2.0 ml/min. The peak of activity eluted between 0.75 and 0.6 M ammonium sulfate, and the purified enzyme was stored at −20°C.

Molecular mass determination. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed as described previously (25), using 12% gels. The native molecular mass was determined by fast protein liquid chromatography, using a Superose 12 (Pharmacia) gel filtration column calibrated with RNase A (13.7 kDa), chymotrypsinogen (25 kDa), ovalbumin (43 kDa), and bovine serum albumin (67 kDa; dimer, 134 kDa). Protein samples (0.2 ml) were injected onto the column and equilibrated with buffer C (50 mM potassium phosphate [pH 7.1] containing 150 mM NaCl and 1 μM zinc sulfate), and the column was developed at a flow rate of 0.4 ml/min.

N-terminal analysis. The N-terminal sequence of the purified Cam heterologously produced in *E. coli* was determined with a model 470 gas peptide sequencer (Applied Biosystems). The phenylthiohydantoin derivatives were identified with an on-line Applied Biosystems liquid chromatograph.

Metals analysis. A comprehensive metals analysis (20 elements) was carried out by inductively coupled plasma atomic emission spectroscopy, using a Jarrel Ash Plasma Comp 750 instrument at the University of Georgia, Athens. All solutions were prepared in plasticware, using deionized water (18 MΩ). Dialysis tubing was treated as described previously (3), and buffers were made metal free by passage over a Chelex 100 (Bio-Rad) column (18). Samples of two independent enzyme preparations were concentrated in dialysis tubing (cutoff, 3.5 kDa) embedded in dry polyethylene glycol (M_r = 8,000; Sigma) before dialysis against a total of 1 liter of metal-free buffer (20 mM potassium phosphate [pH 6.8]) for 20 to 24 h. A sample of each enzyme preparation was analyzed for metals content. A third sample was prepared for metals analysis by further dialysis of one of the above-described preparations against a total of 1 liter of 20 mM potassium phosphate (pH 7.0) containing 100 mM dipicolinic acid for 24 h and then chromatography on a PD-10 gel filtration column (Bio-Rad) equilibrated with metal-free buffer. Protein concentrations were determined by the biuret method (15), using bovine serum albumin and chicken egg white lysozyme (Sigma) as standards. Protein concentrations were also estimated by using the A_{280} and the extinction coefficient (ε = 15,990 M^{-1} cm^{-1}) calculated from the deduced amino acid sequence of the *cam* gene (excluding the putative signal sequence). Results obtained by the two methods agreed well but indicated that the Bradford method (used during purification of the enzyme) underestimated the carbonic anhydrase concentration 6.8-fold.

Esterase activity. Activity for *p*-nitrophenylacetate hydrolysis was determined at 21 to 25°C, using a modification of the method of Armstrong et al. (2). The reaction mixture (1.35 ml) contained 0.5 ml of freshly prepared 3 mM *p*-nitrophenylacetate in aqueous 3% (vol/vol) acetone and 0.85 ml of H$_2$O. The uncatalyzed rate of the reaction was determined by adding 0.15 ml of 100 mM potassium phosphate (pH 7.0) containing 1 μM zinc sulfate and recording the change in A_{348} per minute (Δε = 5,000 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.

Western blotting (immunoblotting). *M. thermophila* TM-1 was grown in 160-ml serum vials in 100 ml of medium (28) containing either 100 mM acetate, methanol, or trimethylamine as the substrate. Cell extracts were prepared as previously described (1). Polyclonal antibodies directed against the purified het-

erologously produced Cam were raised in New Zealand White rabbits (Cocalico Biological Corp., Reamstown, Pa.). *M. thermophila* cell extract proteins were separated by SDS-PAGE (25) on 12% gels and electrotransferred to a polyvinylidene difluoride membrane (Immobilon PVDF; Millipore) as described previously (38). Additional protein binding sites were blocked by incubating the membrane in buffer D (10 mM Tris-HCl [pH 8.0] containing 150 mM NaCl and 0.05% Tween 20) with the addition of 0.1% casein and 0.1% gelatin. A 1:20,000 dilution of anti-Cam antiserum and 1:7,500 dilution of anti-rabbit immunoglobulin G-alkaline phosphatase conjugate in buffer D were used. The antibody-antigen complex was detected with 5-bromo-4-chloro-3-indolylphosphate and 4-nitroblue tetrazolium chloride as recommended for the ProtBlot Western Blot AP system (Promega).

Materials. All chemicals were of reagent grade and purchased from Sigma or Fisher. T4 DNA polymerase, T4 DNA ligase, and restriction endonucleases were from New England Biolabs, United States Biochemical, or Bethesda Research Laboratories. Plasmid T7-7 and *E. coli* BL21(DE3) were gifts from S. Tabor and W. Studier, respectively. Specific oligonucleotides were from Research Genetics, Huntsville, Ala. Molecular mass standards were from Bio-Rad (SDS-PAGE) or Pharmacia (gel filtration). Highly purified human and bovine carbonic anhydrase isozymes II were obtained from Sigma. Western blotting reagents were from Boehringer Mannheim.

RESULTS AND DISCUSSION

Purification of Cam heterologously produced in *E. coli*. The amino acid sequence deduced from the gene encoding Cam identified a 34-amino-acid N-terminal sequence with properties characteristic of signal peptides in secretory proteins (1). Cam was produced in *E. coli* by using a T7 promoter/polymerase expression system (35) and plasmid pBA1416NB containing the *cam* gene excluding the coding region for the putative signal sequence. About 90% of carbonic anhydrase activity was recovered in the soluble fraction after ultracentrifugation of *E. coli* cell extract for 2 h at $100,000 \times g$. The heterologously produced enzyme was purified 14-fold (Table 1) to apparent homogeneity, as indicated by a single protein band after SDS-PAGE (Fig. 1). The subunit molecular mass, as estimated by SDS-PAGE, was 37 kDa. The N-terminal sequence (Met-Gln-Glu-Ile-Thr) of the heterologously produced Cam was identical to that of the authentic enzyme (1) except for the N-terminal methionine, which is a consequence of the pBA1416NB construct to ensure efficient expression of the *cam* gene. The specific activity (6,600 U/mg [Bradford protein assay]) is higher than that previously reported (1) for authentic Cam purified from *M. thermophila* (4,900 U/mg [Bradford protein assay]). The higher specific activity is likely the result of less inactivation when fewer steps are used compared with purification of the authentic enzyme.
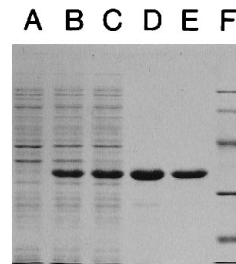

FIG. 1. SDS-PAGE of heterologously produced Cam at various steps during purification from *E. coli*. Lane A, 9.5 μg of cell extract protein from *E. coli* carrying pT7-7 without an insert; lane B, 10 μg of cell extract protein from *E. coli* carrying the *cam* gene on a T7-7 plasmid (pBA1416NB); lane C, 10 μg of cell extract protein after centrifugation at $100,000 \times g$ for 2 h; lane D, 2 μg of protein from the Q-Sepharose column step; lane E, 1 μg of protein from the phenyl-Sepharose column step; lane F, molecular mass markers (rabbit muscle phosphorylase *b*, 97.4 kDa; bovine serum albumin, 66.2 kDa; hen egg white ovalbumin, 45 kDa; bovine carbonic anhydrase, 31 kDa; soybean trypsin inhibitor, 21.5 kDa; hen egg white lysozyme, 14.4 kDa). The gel was stained with Coomassie brilliant blue R-250.
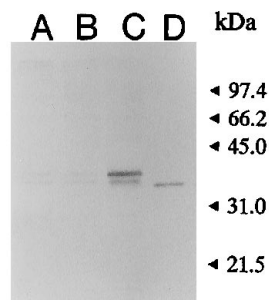
FIG. 2. Western blot analysis of cell extract protein from *M. thermophila* grown on different substrates. Lane A, 20 μg of cell extract protein from methanol-grown cells; lane B, 20 μg of cell extract protein from trimethylamine-grown cells; lane C, 20 μg of cell extract protein from acetate-grown cells; lane D, 1 ng of Cam heterologously produced in and purified from *E. coli*. The blot was probed with anti-Cam antiserum and detected with anti-rabbit IgG-alkaline phosphatase conjugate. Molecular mass markers are shown at the right.

Regulation of Cam synthesis in response to the growth substrate. Cam activity is higher in acetate- than in methanol-grown cells (20). Western blot analysis (Fig. 2) using antibodies raised against the heterologously produced Cam detected two protein bands (40 and 38 kDa) in cell extracts of methanol-, trimethylamine-, and acetate-grown cells. The major (40-kDa) band had the molecular mass reported for authentic Cam purified from *M. thermophila* (1). Migration of heterologously produced Cam (37 kDa) was consistent with that on a Coomassie blue-stained SDS-polyacrylamide gel of the purified enzyme (Fig. 1), and the relative mobility was unaffected by mixing it with cell extract (data not shown). The apparent discrepancy in subunit molecular masses is unexplained; however, Cam may be posttranscriptionally modified in *M. thermophila*, perhaps by glycosylation as reported for other secreted archaeal proteins (26). The 38-kDa band detected in Western blots of cell extracts may represent an incompletely modified form of Cam, a degradation product of the completely modified 40-kDa form, or a second carbonic anhydrase from *M. thermophila*. Furthermore, the results cannot rule out the possibility that the 40-kDa Cam is posttranslationally modified in *E. coli* by C-terminal cleavage yielding the 37-kDa enzyme.

The amounts of Cam detected in cell extracts of *M. thermophila* (Fig. 2) were considerably less in cells grown on methanol or trimethylamine than in acetate-grown cells, corresponding to a decrease in specific activities of Cam in cell extracts of acetate-, methanol-, and trimethylamine-grown cells (0.26, <0.02, and 0.03 U of cell extract protein per ml, respectively). These results indicate that the amount of Cam is regulated. In addition to *M. thermophila* (20), carbonic anhydrase activity is higher in acetate- than in methanol-grown *Methanosarcina barkeri* (22). The up-regulation of Cam protein levels in acetate-grown *M. thermophila* suggests that this enzyme is important for acetotrophic growth and that the increase in Cam activity associated with growth on acetate results, at least in part, from increased levels of Cam. Assuming that Cam is located outside the cell membrane, it is proposed that the enzyme may be required for a $CH_3CO_2^-/H^+$ symport system or for efficient removal of cytoplasmically produced CO_2 (1). The availability of specific polyclonal antibodies will allow the determination of the subcellular location of the enzyme in *M. thermophila* by electron microscopic immunochemistry. The localization is crucial for elucidating the physiological role of carbonic anhydrase in acetotrophic anaerobes.

Biochemical characterization of heterologously produced purified Cam. (i) Subunit composition. A subunit molecular mass of 37 kDa was estimated by SDS-PAGE (Fig. 1). A subunit molecular mass of 22.9 kDa was calculated on the basis of the amino acid composition deduced from the gene, suggesting that SDS-PAGE overestimates the size of this highly acidic protein with a calculated pI of 4.0. Abnormally slow SDS-PAGE migration has been reported for other highly acidic proteins, including a 56.7-kDa transducer protein from *Halobacterium salinarium* with a calculated pI of 3.9, for which a molecular mass of 97 kDa is reported on the basis of SDS-PAGE (41). Native gel filtration chromatography of heterologously produced Cam estimated a molecular mass of 74 kDa, compared with 84 kDa reported for the authentic enzyme (1). Given a calculated subunit molecular mass of 22.9 kDa, these results suggest that the native enzyme is either a trimer or a tetramer; indeed, analysis of the crystal structure (24) indicates that the heterologously produced enzyme forms a trimer.

(ii) Inhibition. The results in Table 2 indicate that authentic Cam and the heterologously produced enzyme are equally susceptible to inhibition by sulfonamides and anions. The previously reported iodide inhibitor constant for authentic Cam (1) was overestimated because the effect of high concentrations of iodide (>10 mM) on the uncatalyzed rate of CO_2 hydration was not considered; however, this was accounted for in determining an inhibitor constant (0.24 M) for heterologously produced Cam. With an inhibitor constant of 0.24 M, iodide joins other monovalent anions (Table 2) which are about 10-fold less effective as an inhibitor of Cam compared with the human isozyme II (1).

(iii) Esterase activity. Some carbonic anhydrases from mammalian sources catalyze the reversible hydrolysis of esters (29). With *p*-nitrophenylacetate as a substrate, commercially available bovine carbonic anhydrase showed an esterase activity of 36.8 mol of *p*-nitrophenylacetate per min per mol of enzyme, in contrast to no detectable activity (<0.03 mol of *p*-nitrophenylacetate per min per mol of enzyme [biuret protein assay]) for Cam. Thus, Cam is one of several carbonic anhydrases (14, 16, 23, 37) for which esterase activity appears to be absent.

(iv) Thermostability. The activity of authentic Cam was stable when the enzyme was incubated for 15 min at temperatures up to 55°C (Fig. 3), the optimal temperature for growth of the organism. Little activity was recovered when the enzyme was incubated above 75°C. The thermostability profile of heterologously produced Cam was not significantly different (Fig. 2). The results are consistent with similar folding structures for the two enzymes.

(v) Metals analysis. On the basis of sequence comparisons, three classes of carbonic anhydrases have been proposed (1,

TABLE 2. Comparison of inhibition constants for heterologously produced and authentic Cam

Inhibitor	IC_{50} (M)[a]	
	Heterologously produced in *E. coli*	Authentic[b]
Acetazolamide	5.7×10^{-4}	4.0×10^{-4}
Sulfanilamide	$>6 \times 10^{-3}$	$>5 \times 10^{-3}$
Ethoxyzolamide	2.6×10^{-5}	ND
Azide	3.1×10^{-3}	3.4×10^{-3}
Cyanate	1.7×10^{-4}	2.8×10^{-4}
Cyanide	2.7×10^{-4}	3.1×10^{-4}

[a] IC_{50} is the concentration of inhibitor resulting in 50% inhibition of the enzyme and was determined by a semilogarithmic plot of percentage of inhibition versus logarithmic concentration of inhibitor (30). The sodium salts of azide and cyanate and the potassium salts of iodide and cyanide were used.
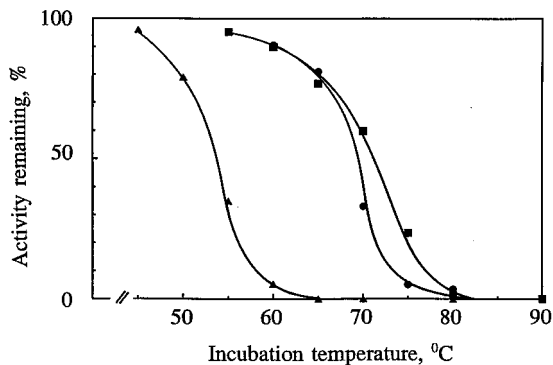[b] Previously published values (1). ND, not determined.

FIG. 3. Thermostability of carbonic anhydrases. The enzymes were incubated for 15 min at the indicated temperatures. The enzyme solutions were cooled to 4°C before determination of the activities relative to those of samples kept at 4°C throughout the experiment. Symbols: ●, authentic Cam (4,100 U/mg [Bradford protein assay]); ■, heterologously produced Cam purified from *E. coli* (5,600 U/mg [Bradford protein assay]); ▲, human carbonic anhydrase isozyme II (5,700 U/mg; Sigma [Bradford protein assay]).

13). The mammalian class (α), and more recently the plant class (β), have been studied in detail. The carbonic anhydrases of both classes contain 1 Zn per enzyme subunit and likely have a common Zn-hydroxide mechanism for catalysis (8). Cam is the only member of the third (γ) class, even though several putative gene products have significant amino acid sequence identity (Fig. 4), particularly with respect to conserved

histidine residues, which is consistent with metal ligation; however, it is not known if any of these putative gene products contain Zn or exhibit carbonic anhydrase activity. A comprehensive metals analysis (20 elements) of heterologously produced Cam was done by plasma emission spectroscopy using two independent enzyme preparations. Preparation I had specific activities of 5,100 U/mg as determined by the Bradford protein assay and 740 U/mg, using either the biuret method or the A_{280} to determine protein concentrations. Most likely, the Bradford protein assay (also used during enzyme purification) underestimates the amount of Cam; thus, the biuret assay was used to determine protein concentrations for metals analysis. The analysis revealed 0.51 and 0.63 Zn per 22.9-kDa subunit for enzyme preparations I and II (740 and 780 U/mg [biuret protein assay]) that were extensively dialyzed against metal-free buffer. There was no significant loss in specific activity following dialysis. A sample from preparation II that was further treated with dipicolinic acid (19) was fully active (770 U/mg [biuret protein assay]) and contained 0.57 Zn per enzyme subunit. Identical treatment of commercially available bovine carbonic anhydrase removed Zn from the enzyme (data not shown), as demonstrated by introducing Co(II) into the vacant Zn binding site, which produced a change in the visible spectrum of the enzyme (5). With the cobalt replacement method, additional attempts to remove Zn from the *M. thermophila* enzyme by dialysis against dipicolinic acid in the presence of 4 M urea or by dialysis against 1,10 phenanthroline were also unsuccessful. The results suggest that Cam contains tightly

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Cam    QEITVDEFSNIRENPVTPWNPEPSAPVIDPTAY-IDPQASVIGEVTIGANV
CcmM          MPSPTTVPVAT-AGRLAEPYIDPAAQ-VHAIASIIGDVRIAAGV
CaiE          MERTLTTVSYYAFEGLIPVVHPTA-FVHPSAVLIGDVIVGAGV
Fbp                   MKYRLGDARVEAHPDSW-IAPSAAVIGKVRLDAGA
Cox    MLCLRLKWANWFGRKFGINFGLQTIEQFQMLYAFEDREPKLLGDNYFIADSADVIGSVIIHNNV
Eco                (+ 78)   PYRDLFPQIGQRV-MIDDSSVVIGDVRLADDV
Ara           DKAPIVDKDA-FVAPSASVIGDVHIGRGS

                                                *
Cam    MVSPMASIRSDEGMPIFVGDRSNVQDGVVLHALETINEEGEPIEDNIVEVDGKEYAVYIGNNVS
CcmM   RVAAGVSIRADEGAPFQVGKESILQEGAVIHGLEYGRVLGD---------DQADYSVWIGQRVA
CaiE   YIGPLASLRGDYGR-LIVQTGANIQDGCIMHG------------------YCDTDTIVGENGH
Fbp    SVWFGAVLRGDNEL-IHIGEHSNVQDGSVMHT------------------DMGYPLTLGKGVT
Cox    SILPHAVIRADNEV-IEIGEGSNVQDGALLHT------------------DPGIPMRVGKGVT
Eco    GIWPLVVIRGDVHY-VQIGARTNIQDGSMLHVTHKSS-----------YNPDGNPLTIGEDVT
Ara    SIWYGCVLRGDVNT-VS-GSGTNIQDNSLVHVAKSNLS-----------GKVHPYIIGDNVT

        *     *
Cam    LAHQSQVHGPAAVGDDTFIGMQAFV-FKSKVGNNCVLEPRSAAIGVTIPDGRYIPAG-MVVTSQ
CcmM   ITHKALIHGPAYLGDDCFVGFRSTV-FNARVGAGSVIMMHALVQDVEIPPGRYVPSG-AIITTQ
CaiE   IGHGAILHG-CVIGRDALVGMNSVIMDGAVIGEESIVAAMSF-----VKAGFHGEKRQLLMGTP
Fbp    VGHNAMLHG-CSVGDYSLVGINAVILNGAKIGKYCIIGANAL-----IPEGKEIPDGSLVMGSP
Cox    IAHRAMLHG-CTIGDHSVIAIGAIVMNNAIIGKNCIIGANAL-----ILENQKIPDGSLVIGSP
Eco    VGHKVMLHG-CTIGNRVLVGMGSILLDGAIVEDDVMIGAGSL-----VPQNKRLESGYLYLGSP
Ara    IGHSAVLHG-CTVEDETFIGMGATLLDGVVVEKHGMVAAGAL------CT?KHQNSFWRG?G

Cam    AEADKLPEV---TDDYAYSHTNEAVYYVNVHLAEGYKETS
CcmM   QQADRLPEVRPEDREFARHIIGSPPVIVRSTPAATADFHSTPTPSPLRPSSSEATTVS   (+ 320)
CaiE   ARAVRSVSD---DELHWKQLNTKEYQDLVGRCHASLHETQPLRQMEENRPRLQGTTDVTPKR
Fbp    GKVVRELSE---PQKKMLEASAAHYVHNARRYARDLVEDPA
Cox    GKVKSQLSQ---KQIEEMQGYAKHYIEKIDRFKHELKVFQRVK
Eco    VKQIRPLSD---EEKAGLRYSANNYVKWKDEYLDQGNQTQP
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FIG. 4. Comparison of the deduced amino acid sequence of Cam with the deduced sequences of a putative CO_2-concentrating-mechanism protein (CcmM) from *Synechococcus* sp. (31), the putative CaiE protein from *E. coli* (10), the ferripyochelin-binding protein (Fbp) from *Pseudomonas aeruginosa*, a putative protein from *Coxiella burnetii* (Cox), a putative protein from *E. coli* (Eco), and a partial protein sequence from *Arabidopsis thaliana* (Ara) (GenBank database accession numbers M82832, S38220, and U18997 and EST database accession number T04294). Question marks indicate ambiguity in the deduced amino acid sequence of Ara. Identical amino acids are shaded, and the asterisks indicate histidines conserved among all six proteins. Inserts, shown as dashes, were included to obtain maximum manual alignment. The C terminus of the CcmM protein and the N terminus of the Eco protein extend 320 and 78 residues beyond the Cam sequence as indicated.

bound Zn; indeed, analysis of the crystal structure (24) indicates the presence of 1 Zn per subunit. It is not yet known if the Zn has a catalytic role, as reported for the other two classes of carbonic anhydrases, or only a structural function.

ACKNOWLEDGMENTS

REFERENCES

1. **Alber, B. E., and J. G. Ferry.** 1994. A carbonic anhydrase from the archaeon *Methanosarcina thermophila*. Proc. Natl. Acad. Sci. USA **91:**6909–6913.
2. **Armstrong, J. M., D. V. Myers, J. A. Verpoorte, and J. T. Edsall.** 1966. Purification and properties of human erythrocyte carbonic anhydrases. J. Biol. Chem. **241:**5137–5149.
3. **Auld, D. S.** 1988. Metal-free dialysis tubing. Methods Enzymol. **158:**13–14.
4. **Badger, M. R., and G. D. Price.** 1994. The role of carbonic anhydrase in photosynthesis. Annu. Rev. Plant Physiol. Plant Mol. Biol. **45:**369–392.
5. **Bertini, I., and C. Luchinat.** 1983. Cobalt(II) as a probe of the structure and function of carbonic anhydrase. Acc. Chem. Soc. **16:**272–279.
6. **Boone, D. R., W. B. Whitman, and P. Rouvière.** 1993. Diversity and taxonomy of methanogens, p. 35–80. *In* J. G. Ferry (ed.), Methanogenesis: ecology, physiology, biochemistry & genetics. Chapman & Hall, London.
7. **BoriackSjodin, P. A., R. W. Heck, P. J. Laipis, D. N. Silverman, and D. W. Christianson.** 1995. Structure determination of murine mitochondrial carbonic anhydrase V at 2.45-angstrom resolution: implication for catalytic proton transfer and inhibitor design. Proc. Natl. Acad. Sci. USA **92:**10949–10953.
8. **Bracey, M. H., J. Christiansen, P. Tovar, S. P. Cramer, and S. G. Bartlett.** 1994. Spinach carbonic anhydrase: investigation of the zinc-binding ligands by site-directed mutagenesis, elemental analysis, and EXAFS. Biochemistry **33:**13126–13131.
9. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
10. **Eichler, K., F. Bourgis, A. Buchet, H.-P. Kleber, and M.-A. Mandrand-Berthelot.** 1994. Molecular characterization of the *cai* operon necessary for carnitine metabolism in *Escherichia coli*. Mol. Microbiol. **13:**775–786.
11. **Eriksson, A. E., and A. Liljas.** 1993. Refined structure of bovine carbonic anhydrase III at 2.0 Å resolution. Proteins **16:**29–42.
12. **Ferry, J. G.** 1992. Methane from acetate. J. Bacteriol. **174:**5489–5495.
13. **Fukuzawa, H., E. Suzuki, Y. Komukai, and S. Miyachi.** 1992. A gene homologous to chloroplast carbonic anhydrase (*icfA*) is essential to photosynthetic carbon dioxide fixation by *Synechococcus* PCC7942. Proc. Natl. Acad. Sci. USA **89:**4437–4441.
14. **Gill, S. R., P. J. Fedorka-Cray, R. K. Tweten, and B. P. Sleeper.** 1984. Purification and properties of the carbonic anhydrase of *Rhodospirillum rubrum*. Arch. Microbiol. **138:**113–118.
15. **Gornall, A. G., C. J. Bardawill, and M. M. David.** 1948. Determination of serum proteins by means of the Biuret reaction. J. Biol. Chem. **177:**751–766.
16. **Guilloton, M. B., J. J. Korte, A. F. Lamblin, J. A. Fuchs, and P. M. Anderson.** 1992. Carbonic anhydrase in *Escherichia coli*. A product of the *cyn* operon. J. Biol. Chem. **267:**3731–3734.
17. **Håkansson, K., M. Carlson, L. A. Swensson, and A. Liljas.** 1992. Structure of the native and apocarbonic anhydrase II and structure of some of its anion-ligand complexes. J. Mol. Biol. **227:**1192–1204.
18. **Holmquist, B.** 1988. Elimination of adventitious metals. Methods Enzymol. **158:**6–12.
19. **Hunt, J. B., M.-J. Rhee, and C. B. Storm.** 1977. A rapid and convenient preparation of apocarbonic anhydrase. Anal. Biochem. **79:**614–617.
20. **Jablonski, P. E., A. A. DiMarco, T. A. Bobik, M. C. Cabell, and J. G. Ferry.** 1990. Protein content and enzyme activities in methanol- and acetate-grown *Methanosarcina thermophila*. J. Bacteriol. **172:**1271–1275.
21. **Kannan, K. K., M. Ramanadham, and T. A. Jones.** 1984. Structure, refinement, and function of carbonic anhydrase isozymes: refinement of human carbonic anhydrase I. Ann. N. Y. Acad. Sci. **429:**49–60.
22. **Karrasch, M., M. Bott, and R. K. Thauer.** 1989. Carbonic anhydrase activity in acetate grown *Methanosarcina barkeri*. Arch. Microbiol. **151:**137–142.
23. **Kisiel, W., and G. Graf.** 1972. Purification and characterization of carbonic anhydrase from *Pisum sativum*. Phytochemistry **11:**113–117.
24. **Kisker, C., H. Schindelin, B. E. Alber, J. G. Ferry, and D. C. Rees.** A left-handed β-helix revealed by the crystal structure of a carbonic anhydrase from the archaeon *Methanosarcina thermophila*. EMBO J., in press.
25. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
26. **Lechner, J., and F. Wieland.** 1989. Structure and biosynthesis of procaryotic glycoproteins. Annu. Rev. Biochem. **58:**173–194.
27. **Liljas, A., K. Håkansson, B. H. Jonsson, and Y. Xue.** 1994. Inhibition catalysis of carbonic anhydrase. Recent crystallographic analyses. Eur. J. Biochem. **219:**1–10.
28. **Lovley, D. R., and J. G. Ferry.** 1985. Production and consumption of H_2 during growth of *Methanosarcina* spp. on acetate. Appl. Environ. Microbiol. **49:**247–249.
29. **Pocker, Y., and S. Sarkanen.** 1978. Carbonic anhydrase: structure, catalytic versatility, and inhibition. Adv. Enzymol. **47:**149–274.
30. **Ponticello, G. S., M. B. Freedman, C. N. Habecker, P. A. Lyle, H. Schwam, S. L. Varga, M. E. Christy, W. C. Randall, and J. J. Baldwin.** 1987. Thieno-thiopyran-2-sulfonamide: a novel class of water-soluble carbonic anhydrase inhibitors. J. Med. Chem. **30:**591–597.
31. **Price, G. D., S. M. Howitt, K. Harrison, and M. R. Badger.** 1993. Analysis of a genomic DNA region from the cyanobacterium *Synechococcus* sp. strain PCC7942 involved in carboxysome assembly and function. J. Bacteriol. **175:** 2871–2879.
32. **Rowlett, R. S., M. R. Change, M. D. Wirt, D. E. Sidelinger, J. R. Royal, M. Woodroffe, Y.-F. A. Wang, R. P. Saha, and M. G. Lam.** 1994. Kinetic and structural characterization of spinach carbonic anhydrase. Biochemistry **33:** 13967–13976.
33. **Silverman, D. N., and S. Lindskog.** 1988. The catalytic mechanism of carbonic anhydrase: implications of a rate-limiting proteolysis of water. Acc. Chem. Res. **21:**30–36.
34. **Studier, F. W., and B. A. Moffatt.** 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. J. Mol. Biol. **189:**113–130.
35. **Tabor, S., and C. C. Richardson.** 1985. A bacteriophage T7 RNA polymerase/promoter system for controlled expression of specific genes. Proc. Natl. Acad. Sci. USA **82:**1074–1078.
36. **Tashian, R. E.** 1990. The carbonic anhydrases: widening perspectives on their evolution, expression and function. Bioessays **10:**186–192.
37. **Tobin, A. J.** 1970. Carbonic anhydrase from parsley leaves. J. Biol. Chem. **245:**2656–2666.
38. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76:**4350–4354.
39. **Wilbur, K. M., and N. G. Anderson.** 1948. Electrometric and colorimetric determination of carbonic anhydrase. J. Biol. Chem. **176:**147–154.
40. **Woese, C. R., O. Kandler, and M. L. Wheelis.** 1990. Towards a natural system of organisms: proposal for the domains Archaea, Bacteria, and Eucarya. Proc. Natl. Acad. Sci. USA **87:**4576–4579.
41. **Yao, V. J., and J. L. Spudich.** 1992. Primary structure of an archaebacterial transducer, a methyl-accepting protein associated with sensory rhodopsin I. Proc. Natl. Acad. Sci. USA **89:**11915–11919.
42. **Zheng, Y.-J., and K. M. Merz, Jr.** 1992. Mechanism of the human carbonic anhydrase II catalyzed hydration of carbon dioxide. J. Am. Chem. Soc. **114:** 10498–10507.