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Expression of Mouse Carbonic Anhydrase VII in *E. coli* and Demonstration of Its CO_2 Hydrase Activity

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The α-carbonic anhydrase (α-CA) gene family in mammals encodes 10 CA or CA-like proteins (CA I-CA X). Although the gene for human CA VII has been cloned and characterized, the corresponding protein has not previously been purified, and hence, the CO_2 hydrase activity of its product has not as yet been demonstrated. In this study, we have cloned the mouse CA VII cDNA in an *E. coli*, glutathione-S-transferase (GST) expression vector. The CO_2 hydrase activity of the expressed protein is about 4% that of the high-activity CAII isozyme, demonstrating that this evolutionarily highly conserved protein is a catalytically active member of this CA gene family.

In mammals (and probably most amniotes), 10 independent autosomal α-carbonic anhydrase (α-CA) genes, *CA1-CA10,* (referred to as *Car-1-Car-10* in the mouse), have been identified to date that code for 10 CA or CA-related proteins, CA I-CA X, [1]. Basically, these genes code for a family of zinc metalloenzymes that catalyze the reversible hydration of CO_2 at different rates ranging from $\approx 1 \times 10^4$ sec^{-1} in CA III isozymes to extremely high rate of $\approx 1 \times 10^6$ sec^{-1} in CA II isozymes. However, two CA isoforms, CA VIII and CA X, do not appear to have any such activity due to changes in some of their critical active site residues [1].

The human CA VII gene (*CA 7*) was originally isolated and characterized from a human genomic library [2]. The gene is 10 kb long, and contains seven exons and six introns at positions identical to those characteristic of the previously-identified mammalian α-CA genes, *CA I, CA 2, CA 3* and *CA 5* [1]. CA VII mRNA has been detected at seemingly low levels in baboon salivary gland [2], rat and mouse lung [3] and more recently in the mouse brain (M. Lakkis, unpublished). The cDNA of the mouse *Car-7* was sequenced to 90% completion [4] and the deduced amino acid sequence determined. Comparison of the mouse and human CA VII sequences revealed a high percent of sequence identity (94.6%) indicating that if it was an active CA, not only would it be the most highly conserved of the active CA isozymes, but also suggests the possibility of an important biological function.

Since amino acid residues, critical for CO_2 hydrase activity, are present at the same positions in CA VII as the active CA isozymes, it was logical to assume that it also possessed such activity. The problem of the low natural availability and the difficulty of the purification of CA VII protein directly from animal tissues, was overcome by cloning this gene in a GST-expression vector and expressing it in *E. coli.* Here, we report on a study of the potential catalytic activity of the expressed CA VII protein.

MATERIALS AND METHODS

Isolation and cloning of CA VII cDNA. Total RNA was isolated from adult mouse (C57/BL6) brain using TRIzol reagent (BRL). Then mRNA was isolated using mRNA Purification Kit (Pharmacia). This mRNA was reverse

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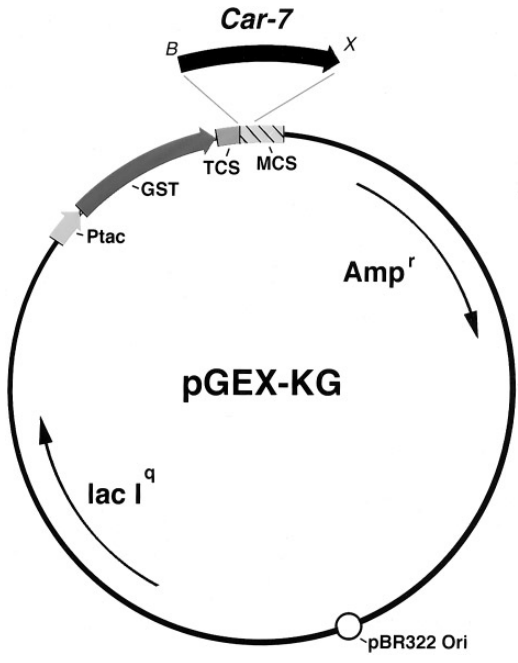

FIG. 1. Construction of the expression plasmid illustrating the insertion of *Car-7* in the pGEX-KG vector. *B: Bam*HI; *X: Xba*I; GST: Glutathione-S-transferase gene; pTac: Tac promoter; TCS: Thrombin cleavage site; MCS: Multiple cloning site.

transcribed into single stranded cDNA using Suprscript II reverse transcriptase (BRL) according to the manufacturer's instructions. The cDNA was used as a template for polymerase chain reaction (PCR) with primers derived from the extreme 5′- and 3′-ends that were tailed with *Bam*HI and *Xba*I restriction sequences respectively. The PCR was performed by using Expand High Fidelity enzyme (Boehringer Mannheim) that gives a high yield with high fidelity of DNA replication. The PCR product, a distinct band of the expected size (˜800 bp) was isolated from agarose gel using QIAEX Gel Extraction Kit (Qiagen). The PCR-amplified mouse CA VII cDNA and the pGEX-KG vector were digested with Bam H1 and Xba1 and ligated (fig. 1) such that it inserts the *Car-7* coding sequence in the correct orientation and reading frame with the ATG codon of GST to produce a fusion protein (glutathione S-transferase fusion protein). The ligation reaction was transformed into DH5α E. coli competent cells (BRL). The ampicillin resistant colonies were mass-screened by PCR using CA VII-specific primers. Four positive clones were isolated, miniplasmid preparations were made from these clones and screened again with restriction digestion. The authenticity of the clones and the correct reading frame of insertion were confirmed by DNA sequencing.

Expression and isolation of CA VII protein. Expression and purification of the protein was achieved using a modification of a previously described procedure [5], [6]. DH5α cells containing the correct clone were cultured in 30 ml of 2 $\times$ YT medium containing 50 μg/ml ampicillin with vigorous shaking overnight. Then 2 $\times$ 5 ml of this culture were inoculated into 2 $\times$ 500 ml of 2 $\times$ YT medium (with ampicillin) and grown at 37°C with vigorous shaking until an absorption value $A_{600} = 1$. At this point, IPTG (isopropyl-β-D-thiogalactoside) was added to a final concentration of 1mM and the culture continued for 4 additional hr. The cells were then harvested by centrifugation and resuspended in 10 ml of PBST (150 mM NaCl, 16m M Na_2HPO_4, 4 mM NaH_2PO_4, pH 7.3 and 1 % Triton X-100), containing 5mM EDTA, 0.1% β-mercaptoethanol, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM benzamidine and 1 mg/ml lysozyme. This cell suspension was then subjected to 3 cycles of repeated sonication on ice for about 30 sec each. The cell homogenate was centrifuged at 10,000 g for 20 min at 4°C. The clear supernatant containing the fusion protein was saved.

The fusion protein was isolated by affinity chromatography on glutathione-agarose (Sigma Chemical Company) column. The isolated supernatant (10 ml) was mixed with 5 ml of swollen glutathione-S-agarose beads that has been equilibrated with PBST and packed in a column. The column, containing the agarose beads and the isolated supernatant was incubated at 4°C for 1 hr with gentle shaking to allow the binding of the fusion protein. The column was washed with 5 $\times$ 10 ml of very cold PBST, followed by 2 $\times$ 10 ml of thrombin cleavage buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 2.5 mM $CaCl_2$, 0.1 % β mercaptoethanol), then incubated with 5 ml of the later buffer containing 10

Units of thrombin, at room temperature for 20 min. The eluate, containing CA VII protein (liberated from the fusion form) was collected, dialyzed with 50 mM Tris (pH 7.5) and concentrated in Centricon-10 concentrators (Amicon) to a final volume of about 500 μl.

Characterization and quantification of isolated CA VII. The purity of the isolated protein was analyzed by electrophoresis (15 μl) on SDS-polyacrylamide (15%) gel (PAGE) along with a standardized serial amounts of CAII protein (5, 10 and 15 μg), and a molecular weight marker (BRL). The gel was fixed in acetic-methanol and stained with 3% Coomassie blue. The relative concentration of the expressed protein was measured by densitometric scanning analysis against the CA II standards. This value was confirmed by spectrophotometry, using a calculated extinction coefficient of 44,680 M^{-1} cm^{-1} at 280 nm [7].

To confirm the identity of the expressed protein, a sample (20 μl) was electrophoresed by SDS-PAGE and electroblotted to a PVDF (polyvinylidine flouride) membrane in CAPS buffer. The protein band was detected by Coomassie staining and subjected to automated amino acid sequencing. Fifteen amino acid residues from the NH_2-terminus were sequenced in the Protein Core Facility at University of Michigan.

CO_2-hydrase activity assay. The CO_2 hydrase activity was estimated by following a modification of the CO_2-Veronal-bromothymol blue method [8], [9]. This method is based on the pH drop caused by the hydration of CO_2 to produce carbonic acid according to the equation: $CO_2 + H_2O \leftrightarrow H^+ + HCO_3^-$. This reaction occurs spontaneously; however, carbonic anhydrase greatly accelerates this reaction [9], [10]. The CO_2 hydrase activity (i.e., the specific activity of the hydration of CO_2) is measured as the difference between the velocities of the spontaneous reaction and that which occurs in the presence of the enzyme. The details of the reaction were as follows: Using all ice-cold solutions, 2 ml of CO_2-saturated dH_2O is added to 2 ml of a Veronal buffer (25 mM, pH 8.2) containing bromothymol blue (0.2%) in the presence of different amounts of enzyme dissolved in 1 ml of dH_2O or only dH_2O (blank). For the reference, 2 ml of phosphate buffer (0.2 M, pH 6.5) was used instead of CO_2-saturated dH_2O. The bromothymol blue is a pH indicator that has a blue color at alkaline pH and turns into yellowish green at acidic pH. The time required for the change in color due to the **increasing** acidity is monitored with a timer until the color of the reaction is comparable to that of the reference, i.e., until reaction reaches pH 6.5.

The enzyme activity is calculated from the time difference between the velocity of the blank reaction (t_b) and that catalyzed by the carbonic anhydrase (t_c) as follows:

$$A.U. = \frac{(t_b - t_c)}{t_c} \cdot \left(\frac{1000}{v}\right) \cdot \frac{5}{c}$$

A.U. **= Activity units/μg of enzyme**
t_b = time required by the blank reaction to reach pH 6.2
t_c = time required by the catalyzed reaction to reach pH 6.2
v = volume (μl) of CA VII sample used
c = concentration of the enzyme solution in μg/ml

The enzyme activity was measured over a range of enzyme concentrations. Another reaction was run in parallel with high activity bovine CA II isozyme for comparison. Each point measurement was repeated at least 5 times.

RESULTS AND DISCUSSION

Expression and Purification of Mouse CA VII Protein

The mouse *Car-7* cDNA was cloned in the expression vector, pGEX-KG. This expression vector is engineered to direct the expression of the cloned gene in *E. coli* as a fusion protein at the carboxy-terminal of GST, under the control of IPTG-inducible Tac promoter. Another important feature of this vector is the presence of a coding sequence for a thrombin-cleavage site between the GST and the cloned gene sequences that permits the release of the targeted protein from the GST carrier.

The induction of the Tac promoter with IPTG in the *E. coli* transformed with the cloning vector resulted in the synthesis of a 56 KDa soluble fusion protein (fig. 2, lane 5), that upon digestion with thrombin yielded two fragments, one polypeptide fragment of apparent molecular weight (M_r) of 30 KDa fragment corresponding to the expected CA VII polypeptide and a 26 KDa fragment corresponding to the GST polypeptide (fig. 2,Lane 6). Binding of this fusion protein to the glutathione agarose beads, followed by digestion with thrombin, resulted in the production of the CA VII protein (fig. 2,lane 4) as the GST remained bound to the glutathione of the column.

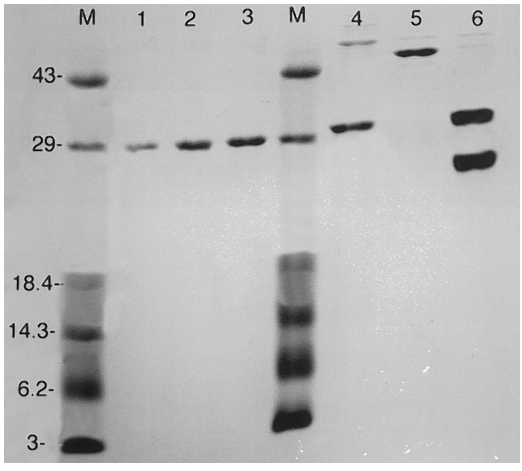

FIG. 2. SDS-PAGE of the CA polypeptides. Lanes 1, 2 & 3 : 5, 10 and 15 μg, respectively, of bovine CA II standard; Lane 4: Expressed CA VII protein, (the sequence of 15 residues at the amino-terminus of this polypeptide was determined to be, G S M T G H H G W G Y G Q D D); Lane 5: GST-CA VII fusion protein; Lane 6: Fusion protein digested with thrombin. M represents marker (BRL low range protein molecular weight standard) lanes with the numbers indicating the corresponding molecular weight in KDa.

Characterization and Quantitation of the Expressed CA VII Protein

In addition to analyzing the nucleotide sequence of the construct, we also analyzed the sequence of 15 amino acid residues from the NH_2-terminus of the expressed protein by automated Edman degradation. The resulting sequence (G S M T G H H G W G Y G Q D D), agreed fully with the sequence expected for the CA VII protein [2] with the addition of two residues from the thrombin cleavage site, before the first methionine of the expressed protein.

The quantitation of the expressed protein by densitometric scanning analysis indicated a similar value of protein concentration to that obtained by spectrophotometry, adding up to a total amount of expressed protein of about 5 mg per liter of induced bacterial culture.

CO_2-Hydrase Activity of the Expressed CA VII Protein

Due to its low levels of expression in the animal tissues, it had been difficult to isolate the CA VII protein and study its enzymatic properties. Based on its inferred amino acid sequence, it is expected to have CO_2-hydrase activity, as the active site amino acid residues remained strictly conserved in both the human and the mouse isozymes; however, it is difficult to speculate on the magnitude of this activity from the amino acid sequence. In this study, the expressed CA VII protein has been shown to have a low CO_2-hydrase activity of $\approx$17 Activity Units per μg, which represents about 4% of that of bovine CA II.

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