A cDNA encoding the mouse carbonic anhydrase V gene was isolated by reverse transcription and polymerase chain reaction from BALB/c mouse liver mRNA. Vectors containing the full coding sequence as well as two different N-terminal truncated genes expressed enzymatically active protein in Escherichia coli. The carbonic anhydrase V produced by a vector containing the full coding sequence, which includes a possible N-terminal mitochondrial targeting signal, was proteolytically processed by E. coli and contained several amino-terminal ends. The two N-terminal-truncated vectors deleted, respectively, 1) the 29-amino acid putative targeting sequence and 2) 51 amino acids, yielding a protein equivalent to a carbonic anhydrase (CA) V isolated from mouse liver mitochondria; and both vectors produced homogeneous protein fractions. These latter two forms of CA V had identical steady-state constants for the hydration of CO₂ with maximal values of $k_\text{cat} / K_m$ at $3 \times 10^8 \text{ s}^{-1} \cdot \text{M}^{-1}$ and $k_{\text{cat}}$ at $3 \times 10^8 \text{ s}^{-1}$ with an apparent $pK_a$ for catalysis of 7.4 determined from $k_{\text{cat}} / K_m$. In catalytic properties, mouse CA V is closest to CA I; however, in inhibition by acetazolamide, ethoxzolamide, and cyanate, CA V is very similar to CA II. Mouse CA V has a tyrosine at position 64, where the highly active isozyme I has histidine serving as a proton shuttle in the catalytic site. Without Tyro-9-M showed $\sim 20\%$ as active as CA I. This is in contrast to histidine 64 in CA I, which is reported to be only a minor extent by the tyrosine at position 64.

Carbonic anhydrase (CA) catalyzes the reversible hydration of CO₂ to form bicarbonate and a proton. A carbonic anhydrase activity associated with mitochondria was noted in early reports (Datta and Shepard, 1959; Maren and Ellison, 1967). Many subsequent studies (reviewed by Dodgson (1991)) have suggested that a mitochondrial carbonic anhydrase activity is used in the metabolic pathways of gluconeogenesis and ureagenesis, both of which require bicarbonate as substrate for enzymes compartmentalized within the mitochondria. Although many of these studies postulated a unique mitochondrial form of carbonic anhydrase, the first evidence for a specific mitochondrial isozyme came from characterization of a protein purified from guinea pig liver mitochondria by Hewett-Emmett et al. (1986). They reported an amino acid sequence of 24 residues with distinct similarities to other carbonic anhydrases (this isoform was subsequently termed CA V). More recently, Amor-Gueret and Levy-Strauss (1990), while screening a mouse liver cDNA library for mRNA clones containing a mouse B2 repeat, isolated a cDNA that encoded a protein with strong sequence similarities to a carbonic anhydrase. Messenger RNA was detectable only in the liver when seven tissues were screened by Northern blotting and hybridization (Amor-Gueret and Levy-Strauss, 1990). Examination of this cDNA sequence and comparison with the partial sequence of Hewett-Emmett et al. (1986) suggested that the cDNA encodes the mouse homolog of the guinea pig mitochondrial carbonic anhydrase (CA V) with the addition of a potential mitochondrial leader sequence.

We used the cDNA sequence of Amor-Gueret and Levi-Strauss (1990) and reverse transcription-PCR to isolate and clone a full-length CA V sequence from mouse liver RNA. Naga et al. (1993) have similarly cloned a mouse CA V cDNA and used it to isolate a human CA V cDNA. We have expressed the entire mouse CA V coding sequence in a procaryotic system (Tanhauser et al., 1992). We also expressed two shorter forms of the enzyme truncated at the amino terminus, one corresponding to a protein lacking the putative mitochondrial leader sequence and the other corresponding to the amino terminus reported for guinea pig CA V (Hewett-Emmett et al., 1986). All of these expressed proteins were catalytically active in the hydration of CO₂ with identical steady-state constants and CO₂ hydration activity; it was determined to be very similar to carbonic anhydrase I in terms of the maximal values of $k_\text{cat}$ and $k_{\text{cat}} / K_m$ and ~20% as active as CA II. CA V, however, is nearly identical to CA II in inhibition by acetazolamide, ethoxzolamide, and cyanate. Using a site-specific mutant, we found that the kinetic properties of CA V are influenced to only a minor extent by the tyrosine at position 64. This is in contrast to histidine 64 in CA II, which has been shown to play a major role in proton transfer during catalysis (Tu et al., 1989).

**MATERIALS AND METHODS**

Cloning and Expression of Mouse CA V—The mouse CA V coding sequence was reverse-transcribed from BALB/c mouse liver RNA using an antisense 3'-end primer (nucleotides 1030–1049; a BamHI site was added to the 3'-end) based upon the cDNA sequence of Amor-Gueret and Levy-Strauss (1990). The products of first-strand synthesis were amplified using the polymerase chain reaction and a 5'-sense primer (nucleotides 105–123; an EcoRI site was added to the 5'-end). A major PCR product of 959 base pairs was obtained. It was inserted into a Bluescribe vector (Stratagene) that had been cut with SmaI and T-tailed (Marchuk et al., 1991). The cloned products were identified as mouse CA V by restriction site analysis and partial DNA sequencing. A full-length coding sequence, CA Vα, as well as two deletion mutants, mouse CA Vb and mouse CA Vc, were synthesized by PCR from the initial PCR-derived clones using the original 3'-oligonucleotide and 5'-deletion mutants.
three new 5'-primers, which added a new NdeI restriction site and Met start codon at the positions shown in Fig. 1. The NdeI and BamHI restriction sites allowed the constructs to be inserted into the pET31 T7 expression vector (Tanhauser et al., 1992). These clones were transformed into Escherichia coli BL21 (DE3) pLYsS, a strain optimized for T7 RNA polymerase expression, and the active carbonic anhydrases were expressed. The mutant Y64H CA V was constructed using a mutagenic oligonucleotide (Kunkel, 1985) and verified by DNA sequencing.

The substrates and the wavelength observed were as follows: Mes (pK, 7.1), 577 nm; Taps (pK, 8.3), 577 nm; Hepes (pK, 7.5), 400 nm; Tris (pK, 8.4), 400 nm; m-cresol purple (pK, 8.3), 577 nm; and phenol red (pK, 9.6), 590 nm. Solutions were maintained at a constant total ionic strength of 0.2 M by addition of NaCl.

RESULTS

Cloning and Expression of Mouse CA V—Reverse transcription of BALB/c liver mRNA followed by PCR amplification yielded several independent clones very similar to that reported by Amor-Gueret and Levi-Strauss (1990); the BALB/c sequence is incomplete, but contains several silent nucleotide changes from the B10.HH sequence. Sequences coding for three forms of mouse CA V were inserted into the T7 expression vector pET31 (Tanhauser et al., 1992); their amino termini are shown in Fig. 1. CA Va is a full-length cDNA sequence including the 29 amino acids at the NH2 terminus, which could represent the mitochondrial targeting sequence. This amino-terminal sequence has no counterpart in CA I, II, and III. CA Vb and Vc are shorter isoforms of CA V; in addition, we synthesized a mutant of CA Vc, replacing Tyr44 with His (Y64H CA Vc). Levels of CA production from these vectors ranged from 1 to 4 mg/liter of culture. Culture of the purified enzymes on SDS-polyacrylamide gels showed that mouse CA Vb and Vc and Y64H CA Vc were >95% pure and had the expected molecular masses of 31 and 28 kDa (Fig. 2). The expressed enzymes CA Va, Vb, and Vc were amino-terminally sequenced. Analysis of several preparations of CA Va indicated that it was a mixed fraction with 18–51 residues of the expected NH2-terminal sequence removed by E. coli proteases, even in the presence of several protease inhibitors during lysis and purification. CA Vb had the expected amino-terminal residues; CA Vc had the initial methionine removed (Fig. 1).

The concentrations of CA Va and Vc were determined by titration of the catalyzed 18O exchange activity with the tight-binding inhibitor ethoxzolamide. The molar absorbances of CA Va and Vc were determined to be 5.4 × 104 and 4.8 × 104 M−1 cm−1, respectively, at 280 nm; the concentrations of Y64H CA Vc were determined from 18O exchange by mass spectrometry. Over the range of pH 5–9, kcat/Km determined from 18O exchange could be

\[ \text{HCO}_3^- + \text{EtNH}_2 + \text{H}_2\text{O} \rightarrow \text{EtNH}_{2+} + \text{CO}_2 + \text{H}_2\text{O} \]

(Eq. 1)

The substrate dependence of kcat is given by R / (kcat[S]/Km + [S]), where kcat is a rate constant for maximal interconversion of CO2 and HCO3−, Km is an apparent substrate binding constant, and [S] is the concentration of CO2 or HCO3− or both (Simonsson et al., 1979). Values of kcat/Km for the enzymes were determined by nonlinear least-squares fit of the above expression for kcat to the data for varying substrate concentration or by measurement of kcat at values of [S] much smaller than Km. In theory and in practice, kcat/Km is equal to kcat/Km for the hydration of CO2 obtained by steady-state methods (Simonsson et al., 1979; Silverman, 1983).

Values of K, were obtained from 18O exchange experiments and were determined from the least-squares fit of kcat to various inhibitor concentrations to the expression for mutual depletion of free enzyme and inhibitor (Segel, 1978). Since the total substrate concentration ([CO2] + [HCO3−] = 25 mM) is much smaller than K, (>100 mM) for these experiments, we cannot differentiate between competitive and noncompetitive modes of inhibition.

Mouse Carbonic Anhydrase V

The numbering is based upon the sequence for CA II. The sequence for CA Va is the deduced amino acid sequence from the nucleotide sequence of Amor-Gueret and Levi-Strauss (1990). CA Vb is a truncated protein with the putative mitochondrial leader removed; it incidentally has the same approximate length as the nonmitochondrial isozymes CA I, II, and III. CA Vc is a truncated protein with the active site as carbonic anhydrase V isolated from mouse liver mitochondria. Sequences are from Fraser and Curtis (1986) for mouse CA I, Venta et al. (1985) for mouse CA 11, and Tweedie and Edwards (1989) for mouse CA III. Conserved residues are shown in boldface type. The daggers designate the most abundant amino-terminal residue sequences in several preparations of CA Va: Arg2 and Thr4 were the major species. The asterisks designate 3 amino acids similar to a motif found in mitochondrial signal sequence cleavage sites described by Hendrick et al. (1989).
described as dependent on the unprotonated state of a single ionizable group with $pK_a = 7.4 \pm 0.1$ and a maximum of $(3.5 \pm 0.1) \times 10^7$ s$^{-1}$ (Fig. 3). The results obtained by stopped-flow spectrophotometry were $pK_a = 7.8 \pm 0.1$ and $k_{cat}/K_m = (3.2 \pm 0.3) \times 10^7$ s$^{-1}$. The values of $k_{cat}/K_m$ and apparent $pK_a$ obtained by stopped-flow spectrophotometry and $\text{H}^\text{35}$O exchange for the longer form, CA Vb (see Fig. 1), were identical to those obtained for CA Vc. The longest form, CA Va, was subject to proteolysis, and we were not able to isolate sufficient quantities in pure forms in the longer forms do not affect catalysis.

The values of $k_{cat}$ for the hydration of CO$_2$ for the two forms CA Vb and Vc showed a monotonic increase with pH. In each case, measurement of a maximal value of $k_{cat}$ was hampered by the instability of the enzyme above pH 9. For CA Vb, there was only a hint of reaching a maximum, with values of $k_{cat}$ near $3.0 \times 10^7$ s$^{-1}$ at pH 9.5. Extended work with CA Vc gave the clearest indication of the maximal value of $k_{cat}$ at $(3.2 \pm 0.3) \times 10^7$ s$^{-1}$ and an apparent $pK_a$ of 9.2 ± 0.2 (Fig. 4). The steady-state constants for CA V and comparisons with isozymes I, II, and III are given in Table I. Comparison of the inhibition of these enzymes by the sulfonamides acetazolamide and ethoxzolamide, as well as by cyanate, are given in Table II.

The results from the catalysis of the hydrolysis of 4-nitrophenyl acetate by CA Vc at $25 ^\circ$C gave $k_{cat}/K_m = 151 \pm 10$ s$^{-1}$ and an apparent $pK_a$ of 6.7 ± 0.1. This activity is very low compared with the esterase activity of CA I1 under comparable conditions ($k_{cat}/K_m$ as great as $10^9$ s$^{-1}$) (Simonsson and Linds-kog, 1982; Pocker and Stone, 1967).

**Activity of Y64H CA Vc**—The values of $k_{cat}/K_m$ for the hydration of CO$_2$ catalyzed by Y64H CA Vc were very similar to those for CA Vc (Fig. 3), with maximal values of $k_{cat}/K_m$ and apparent $pK_a$ determined by $\text{H}^\text{35}$O exchange given in the legend to Fig. 3. Values of $k_{cat}/K_m$ determined by stopped-flow spectrophotometry were lower by as much as 3-fold compared with those in Fig. 3 in the range of pH 7–8.0; this decrease may be due to inhibition by buffer. The values of $k_{cat}$ for Y64H CA Vc were identical to those for CA Vc at pH >8; at a lower range of pH, the values of $k_{cat}$ for Y64H CA Vc were enhanced compared with those for CA Vc (Fig. 4).

**DISCUSSION**

We have cloned, expressed, and purified three types of mouse carbonic anhydrase V (forms Va, Vb, and Vc; Fig. 1) that represent possible isoforms of this enzyme in the cytoplasm and mitochondria. CA Va is a potentially full-length protein product based on the complete cDNA sequence (Amor-Gueret and Levi-Strauss, 1990). Electrophoretic analysis and amino-terminal sequencing (Figs. 1 and 2) show that this protein is a mixture of at least four species, with loss of between 18 and 51 amino acids. The initial 29 amino acid residues of mouse CA Va shown in Fig. 1 are presumably part of a mitochondrial targeting sequence and would play no role in catalysis (see below). The amino-terminal region has the structural properties of a mito-
Table II
Inhibition of isozymes of carbonic anhydrase measured by $^18$O exchange at pH 7.2-7.5 and 25 °C

| CA        | $K_i$ (mM) Acetazolamide | $K_i$ (mM) Ethoxzolamide | $K_i$ (mM) Cyanate |
|-----------|-------------------------|--------------------------|-------------------|
| Human I*  | 0.2                     | 0.002                    | 0.7               |
| Human II  | 0.06                    | 0.008                    | 30                |
| Human III | 40                      | 8                        | 30                |
| Mouse Vc  | 0.06                    | 0.005                    | 30                |

* These data for HCA I are taken from Sanyal et al. (1982) and were measured at 0 °C for acetazolamide and ethoxzolamide and at 25 °C for cyanate.

V, the shortest form of CA V (Fig. 1).

The mouse CA V sequence also contains 3 residues (shown by asterisks in Fig. 1) similar to a conserved amino acid motif found in mitochondrial leader peptides that undergo a two-step cleavage during import (Hendrick et al., 1989); this predicts a cleavage that corresponds to CA Ve in Fig. 1. This motif in mouse CA V has a proline where the consensus sequence of Hendrick et al. (1989) shows a hydrophobic residue, and the glutamates at positions 5 and 6 are inconsistent with many mitochondrial leaders. Cleavages at positions analogous to CA Ve were reported in purified guinea pig, rat, and human CA V proteins (Hewett-Emmett et al., 1986; Ohliger et al., 1993; Nagao et al., 1993). The observation that many cleavages can occur within the first 50 amino acids of CA V in our bacterial system indicates that this entire amino-terminal region may be flexible to proteolysis. We have purified CA V from mouse liver mitochondria and found a predominant fraction of protein with an amino terminus identical to that of CA Ve in Fig. 1. Nagao et al. (1993) reported that a full-length human CA V cDNA expressed in COS cells produced a weakly active carbonic anhydrase. A portion of this protein was present in a mitochondrial fraction in processed form. Processed forms were also present in cytoplasmic fractions. Nagao et al. isolated the processed form from total COS cell homogenates; it had an amino terminus that began at a position homologous to position 3 in Fig. 1, a still shorter form was extracted in the absence of protease inhibitors.

We found that the two shorter forms of CA V (forms Vb and Vc; Fig. 1) have the same catalytic properties in the hydration of CO$_2$. This is consistent with the report that a deletion mutant of HCA II lacking 16 residues at the NH$_2$ terminus is nearly fully active. This segment of CA II (as well as of CA I and III) contains Tyr$^1$, the side chain of which extends into the active-site cavity and is a component of the hydrogen-bonded array of side chains and water molecules found in the active-site cavity of the crystal structure (Eriksson et al., 1988). Mouse and human CA V contain a histidine or threonine at position 7, respectively. It seems clear that the absence of residue 7 and the NH$_2$-terminal segment in which it is found do not contribute significantly to the catalytic pathway. This is further emphasized by the result from kinetic analysis that mixtures of the longer forms of CA V, obtained from proteolytic products of CA Vc such as shown in Fig. 2, had catalytic activity equivalent to that of CA Vb and Ve.

The maximal values of the kinetic constant $k_{cat}/K_m$ for the hydration of CO$_2$ for CA Vb and Ve were $3 \times 10^7$ M$^{-1}$ s$^{-1}$. The pH profile of $k_{cat}/K_m$ appeared to be dependent on the basic form of a single ionizable group with apparent pK$_a$ near 7.4 (Fig. 3). This pK$_a$ has been shown to represent the ionization of the zinc-bound water (Simonsson and Lindskog, 1982), which in isozyme II is close to 7, although it is dependent on experimental conditions and the ionization state of the nearby His$^{64}$ (Simonsson and Lindskog, 1982). Thus, isozyme V has an apparent pK$_a$ for this zinc-bound water close to that of isozyme II. A comparison of maximal values of $k_{cat}/K_m$ for the hydration of CO$_2$ catalyzed by several isozymes is shown in Table I.

A significant difference in catalysis by isozymes I and II compared with isozyme V was found in $k_{cat}$ for hydration. For CA I and II, $k_{cat}$ is described by a titration curve with a pK$_a$ near 7 reaching a maximum at high pH (Khalifah, 1971; Steiner et al., 1975). In CA II, this behavior is a reflection of the pK$_a$ of histidine 64, the proton acceptor in the intramolecular proton shuttle that transfers a proton to solution and regenerates the zinc-bound hydroxide (Steiner et al., 1975; Silverman and Lindskog, 1988). In mouse CA Vb and Ve, we observed an increase in $k_{cat}$ for hydration with an increase in pH and an apparent pK$_a$ of 9.2 (Fig. 4). However, the maximal values of $k_{cat}$ for the hydration of CO$_2$ by mouse CA Vb and Vc are near $3 \times 10^7$ s$^{-1}$, a very sizable value. Together, these data suggest the presence of a proton acceptor in the active-site cavity with a pK$_a$ near 9. There could be more than one proton acceptor; of course, but there does not appear to be a predominant proton acceptor in the initial 51 residues of CA Vc (Fig. 1) since the deletion of this segment leaves $k_{cat}$ relatively unchanged.

One prominent difference in the active-site cavities of CA V and II is at position 64. The histidine at this position in human CA II has been shown to act as a proton shuttle, accepting protons from the zinc-bound water and transferring them to solution (Tu et al., 1989). The presence of His$^{64}$ in CA II is responsible for the apparent pK$_a$ near 7 in $k_{cat}$ for the hydration of CO$_2$ (Tu et al., 1989; Steiner et al., 1975). CA V has a tyrosine at position 64 that is a poor proton shuttle at pH 7, but is a more efficient shuttle at pH near the pK$_a$ of the phenolic hydroxyl. To assess the effects of these residues at position 64, we replaced Tyr$^{64}$ with His in CA Vc (Y64H CA Vc).

The mutant Y64H CA Vc shows very similar $k_{cat}/K_m$ values compared with CA Ve (Fig. 3). This constant, $k_{cat}/K_m$, contains rate constants for the steps of the interconversion of CO$_2$ and HCO$_3^-$ (Equation 2).

$$ CO_2 + EZnOH ~\rightleftharpoons~ EZnHCO_3^- ~\rightleftharpoons~ EZnH_2O + HCO_3^- $$

Thus, the replacement Tyr$^{64}$ → His has only small effects on the conversion of CO$_2$ to HCO$_3^-$ at the zinc; one noticeable difference is the small deviation from a single ionization of $k_{cat}/K_m$ for Y64H CA Vc at pH <7, which may be due to ionization of His$^{64}$ in this pH range.

The steady-state constant $k_{cat}$ in catalysis by carbonic anhydrase is determined by the transfer of protons from the zinc-bound water to buffers in solution, as in Equation 3 (Silverman and Lindskog, 1988).

$$ X-EZnH_2O + B \rightleftharpoons XH^+-EZnOH^- + B \rightleftharpoons X-EZnOH^- + BH^+ $$

Here, X is a residue of the enzyme capable of accepting a proton.
from the zinc-bound water and transferring it to buffer B in solution. The most prominent acceptor in Y64H CA Vc has a $p_K$ near 9.2; in fact, $k_{cat}$ values for this mutant and CA Vc are identical at pH > 8 (Fig. 4). Thus, it can be concluded that this proton acceptor is not Tyr$^{64}$, but another residue near the zinc. At pH < 7, the values of $k_{cat}$ for this mutant exceed those for CA Vc by as much as 5-fold and show a second $p_K$ near 6.2 with a maximum near $10^{-6}$ s$^{-1}$ (Fig. 4). This suggests a possible proton acceptor role of His$^{64}$ in Y64H CA Vc; His$^{64}$ is a proton acceptor in HCA II (Tu et al., 1989) and in K64H HCA III (Jewell et al., 1991). However, in HCA II, the presence of His$^{64}$ supports proton transfer to give a maximal value of $k_{cat}$ near $10^{-6}$ s$^{-1}$; in Y64H CA Vc, this maximal value appears to be closer to $10^{-4}$ s$^{-1}$. Thus, the replacement Tyr$^{64}$ → His in CA Vc does not result in $k_{cat}$ values similar to those for CA II, and the presence of Tyr$^{64}$ is not a major factor in the unique kinetic properties of CA V.

A comparison of the maximal values of the steady-state kinetic constants for the hydration of CO$_2$ appears in Table I. The maximal steady-state parameters for CA V resemble most closely those for CA I. It is useful to note that previous reports of human CA V as a low activity enzyme (Nagao et al., 1988) for CA V and Cheah et al., 1991; in K64H CA III, His$^{64}$ is a proton acceptor (Tu et al., 1989) and in K64H HCA III (Jewell et al., 1991). However, in HCA II, the presence of His$^{64}$ supports proton transfer to give a maximal value of $k_{cat}$ near $10^{-6}$ s$^{-1}$; in Y64H CA Vc, this maximal value appears to be closer to $10^{-4}$ s$^{-1}$. Thus, the replacement Tyr$^{64}$ → His in CA V does not result in $k_{cat}$ values similar to those for CA II, and the presence of Tyr$^{64}$ is not a major factor in the unique kinetic properties of CA V.

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