Catalytic Properties of Murine Carbonic Anhydrase IV*

(Received for publication, November 18, 1996, and in revised form, March 18, 1997)

Jonathan D. Hurt‡, Chingkuang Tu§, Philip J. Laipis‡, and David N. Silverman§

From the ‡Department of Pharmacology and Therapeutics and the §Department of Biochemistry and Molecular Biology, University of Florida College of Medicine, Gainesville, Florida 32610-0267

A cDNA encoding the murine carbonic anhydrase IV (mCA IV) gene, modified to resemble a form of mature human carbonic anhydrase IV (Okuyama, T., Waheed, A., Kusumoto, W., Zhu, X. L., and Sly, W. S. (1995) Arch. Biochem. Biophys. 320, 315–322), was expressed in *Escherichia coli*. Inactive inclusion bodies were collected and refolded, and active enzyme was purified; the resulting mCA IV was used to characterize the catalysis of CO2 hydration using stopped flow spectrophotometry and 18O exchange between CO2 and water. Unlike previously studied isozymes in this class of carbonic anhydrase, the pH profile for $k_{cat}$ for hydration of CO2 catalyzed by mCA IV could not be described by a single ionization, suggesting multiple proton transfer pathways between the zinc-bound water molecule and solution. A role for His64 in transferring protons between the zinc-bound water and solution was confirmed by the 100-fold lower activity of the mutant of mCA IV containing the replacement His64→Ala. The remaining activity in this mutant at pH levels near 9 suggested a second proton shuttle mechanism. The maximal turnover number $k_{cat}$ for hydration of CO2 catalyzed by mCA IV was 1.1 × 107 s⁻¹ at pH > 9. A $pK_a$ of 6.6 was estimated for the zinc-bound water molecule in mCA IV.

The mammalian carbonic anhydrases (CAs) constitute a gene family of at least seven distinct isozymes (1) that catalyze the hydration of CO2 to form bicarbonate and a proton: CO2 + H2O ⇌ HCO3⁻ + H⁺. Although these isozymes are characterized by a high degree of amino acid identity (28–59%) (2), they are quite diverse in their cellular distribution, catalytic activity, and physiological function (reviewed in Ref. 3). Among these isozymes, CA IV is the only known membrane-associated form. It was first identified and purified from bovine lung (4), although the presence of a membrane-bound carbonic anhydrase activity had been observed earlier (reviewed in Refs. 5 and 6). Subsequent purifications from human kidney (7, 8), human lung (8), and lung microsomal membranes from a variety of mammals (9) identified CA IV as a 35–52-kDa protein anchored to the membrane by a glycosyl phosphatidylinositol linkage to its C terminus. The distribution of membrane-assoc- iated carbonic anhydrases is widespread; they have been found in many secretory tissues, where they play a prominent role in, for example, the formation of ocular fluid, cerebrospinal fluid, and other secretions (reviewed in Ref. 10). Moreover, CA IV is the luminal CA in the proximal tubule of the kidney and is estimated to mediate 85% of renal bicarbonate reabsorption (10). Membrane-associated carbonic anhydrases have been found in many other tissues including the capillaries of skeletal and cardiac muscle, the colon, and the reproductive tract.

The crystal structure of human CA IV shows considerable backbone similarity to that of CA II, especially in the region of the active site (11). Two disulfide bridges appear in the structure of human CA IV (11) that are not present in CA II; these are likely responsible for the enhanced stability of CA IV against heat and SDS. Whitney and Briggle (4) reported that CA IV, unlike the other isozymes, was stable for several hours in 1 to 5% SDS, an observation that has been used to facilitate its purification.

Measurements near physiological pH have identified human (7) and bovine CA IV (12) as a fast isozyme, with a catalytic turnover $k_{cat}$ of approximately 2 × 10⁷ s⁻¹ at 0 to 1 °C, close in magnitude to that of CA II, the most efficient of the carbonic anhydrase isozymes. Early studies showed that the membrane-bound carbonic anhydrase from the brush border of the dog kidney had catalytic constants nearly identical to those of carbonic anhydrase II, suggesting that adherence to the membrane did not significantly diminish the activity to below that of CA II (13). Sulfonamides inhibit CA IV, but the average inhibition constant is 17-fold less than for CA II (12).

cDNA clones for human (14), rat (15), and mouse (16) CA IV have been isolated. Until recently, obtaining sufficient amounts of CA IV for kinetic and other studies was limited to the enzyme produced from tissue isolation or overexpression in a mammalian cell line (17). Refolding of CA IV inclusion bodies obtained from expression in *Escherichia coli* have produced sufficient quantities of active, SDS-resistant, murine carbonic anhydrase IV (mCA IV) to characterize its kinetic constants in the hydration of CO2 over a range of 4–5 pH units, allowing an examination of the mechanism of catalysis including the $pK_a$ of the zinc-bound water molecule. In this study we present the full pH profiles for catalysis of the hydration of CO2 by mCA IV using both initial velocities measured by stopped flow and 18O exchange between CO2 and water measured by mass spectrometry. The data show a very efficient enzyme but with features that are unique among the studied isozymes of the human and animal carbonic anhydrases; the maximal turnover appears to be dependent on at least two ionizations, suggesting that more than one proton shuttle group is functioning in the catalytic pathway. One of these is a shuttle group with a $pK_a$ near 7, which is identified as His64 by site-specific mutagenesis. These data are discussed in the context of a number of recent cases.

* This work was supported by Grant GM25154 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C, Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Box 100267 Health Center, University of Florida College of Medicine, Gainesville, FL 32610–0267. Tel.: 352-392-3556; Fax: 352-392-9696.

‡ University of Florida College of Medicine, Gainesville, Florida 32610–0267.

§ University of Florida College of Medicine, Gainesville, Florida 32610–0267.
suggesting the role of multiple proton transfers in catalysis and proton-translocating proteins.

MATERIALS AND METHODS

Expression and Isolation of Murine CA IV—A murine Balb/c lung carbonic anhydrase IV cDNA was isolated, and a portion of the coding sequence (see legend to Fig. 1) was expressed in E. coli strain BL21(DE3)pLysS using the pET31 T7 expression vector described by Tombaugh et al. (18). The mutant H84A mCA IV was constructed using a mutating oligonucleotide (18, 19) and verified by DNA sequencing. A truncated form of the enzyme was obtained by extending a mutating oligonucleotide (18, 19) and verified by DNA sequencing. Expression and purification of the truncated form of human CA IV isolated from lung have been shown (14). Occurring in a loop region of human CA IV, Glu12 is a glutamate (Fig. 1). Residue 12 is a glutamate in human CA IV corresponding to a serine in the human sequence (17). One additional change occurring during construction of the murine CA IV expression clone; this converted a lysine at position 12 to a glutamate (Fig. 1). Residue 12 is a glutamate in human CA IV (14). Occurring in a loop region of human CA IV, Glu12 is a surface residue that extends into solvent and has no interactions with other residues in the crystal form of the enzyme (11). We anticipate that the presence of glutamate at position 12 has no effect on the catalysis by murine CA IV.

Catalytic Activity—The rate constant $R_{Cat}/[E]$ for the interconversion of CO$_2$ and HCO$_3^-$ at chemical equilibrium catalyzed by mCA IV was measured as a function of total concentration of all

$\text{HCO}_3^-$ + E$\text{ZnH}_2$O $\rightleftharpoons$ E$\text{ZnO}_2$H + CO$_2$ + H$_2$O (Eq. 1)

$\text{EZnO}_2$H + BH $\rightleftharpoons$ E$\text{ZnO}_2$H$_2$ + B $\rightleftharpoons$ E$\text{ZnH}_2$O + H$_2$O (Eq. 2)

$\text{HCO}_3^-$ + CO$_2$ + H$_2$O, $\text{Ka}$ (pH 8.4) with methoxzolamide to inhibit mCA IV. The $S_\text{eff}$ were determined by nonlinear least squares methods (Kinzffiter, Elsevier-Biosoft). The values of $S_\text{eff}$ for catalysis of CO$_2$ was slow, we determined the overall rate and subtracted from it the rate in the presence of 20 $\mu$M ethoxzolamide to inhibit mCA IV. The difference was the component of the overall catalysis due to the $S_\text{eff}$ at the active site. Initial velocities were determined under the conditions given in the legend to Fig. 4. The value of $S_\text{eff}$ for catalysis was too large to measure because it exceeded the solubility of substrate.

As a result, we were limited to observing catalytic rates that were first order in the substrate from which we obtained $k_{cat}/S_\text{eff}$. 

RESULTS

Murine CA IV—A murine CA IV cDNA coding sequence closely corresponding in structure to human CA II was inserted into a pET31 vector and protein expressed in E. coli BL21(DE3)pLysS (18). An alignment of the full-length murine CA IV protein sequence with that of human CA II is shown in Fig. 1. The two arrows in Fig. 1 show the initial and terminal amino acids of the expressed mCA IV used in this study. Mature human and rat CA IV isolated from lung have been shown to be N terminally truncated by 18 and 17 amino acids, respectively, to remove the putative plasma membrane targeting sequence (15). The murine CA IV coding sequence was truncated to mimic that of the endogenous rat protein, removing the first 17 amino acids and converting the next residue into a start methionine. The aspartate in the mouse sequence that immediately follows this methionine corresponds to the second residue in the mature rat protein (9, 15). Human CA IV is C terminally cleaved during maturation immediately after serine 266 and attached to a glycosylphosphatidylinositol anchor by this residue. This membrane anchor can be enzymatically removed without altering enzyme activity, and a fully active truncated form of human CA IV can be expressed without the residues C terminal to serine 266 (corresponding to position 258 in Fig. 1) and lacking attachment to a glycosylphosphatidylinositol anchor (17). The form of murine CA IV examined here is C terminally truncated two amino acids beyond the corresponding serine in the human sequence (17). One additional change occurred during construction of the murine CA IV expression clone; this converted a lysine at position 12 to a glutamate (Fig. 1). Residue 12 is a glutamate in human CA IV (14). Occurring in a loop region of human CA IV, Glu12 is a surface residue that extends into solvent and has no interactions with other residues in the crystal form of the enzyme (11). We anticipate that the presence of glutamate at position 12 has no effect on the catalysis by murine CA IV.

Steady-state Constants—A stopped flow spectrophotometer (Applied Photochemistry Model SF.17MV) was used to measure initial velocities of the hydration of CO$_2$. Since this catalysis produces protons as well as HCO$_3^-$, we measured the initial rate of hydration by recording the absorbance change of a pH indicator (24). Saturated CO$_2$ solutions were made by bubbling CO$_2$ into water at 25 °C. Syringes with gas-tight seals were used to make CO$_2$ dilutions from 17 to 0.24 mm. The $pK_a$ of the buffer indicator pairs, and the observed wavelengths, were as follows: Mes ($pK_a$ 6.1) with chlorophenol red ($pK_b$ 6.3, 574 nm); Mops ($pK_a$ 7.2) with phenol red ($pK_b$ 7.5, 557 nm); Taps ($pK_a$ 8.4) with m-cresol purple ($pK_b$ 8.3, 578 nm); and 8-8 Amino-5-Methylphenol blue ($pK_b$ 8.9, 590 nm). The buffer concentration was 25 mM, unless indicated otherwise, and the total ionic strength for each buffer-indicator pair system was maintained at 0.1 M by the addition of the appropriate amount of Na$_2$SO$_4$. Solutions contained 4 $\mu$M EDTA. The mean of four to eight reaction traces of the first 5 to 10% of the reaction was used to determine initial rates. The uncatalyzed rates were subtracted, and the rate constants $k_{cat}$ and $k_{eff}$ were determined by nonlinear least squares methods. The values of $S_\text{eff}$ for catalysis of CO$_2$ were below 10 mM at pH < 8.5 and reached a maximal value near 20 mM at pH approaching 10; hence, we were able to achieve concentrations of CO$_2$ approaching saturation over most of the pH range tested. The S.E. values in $k_{cat}$ and $k_{eff}/S_\text{eff}$ were $\pm$5% at most.

Rate of Ester Hydrolysis—The catalysis by mCA IV of the hydrolysis of 4-nitrophenyl acetate was measured by following the increase in absorbance at 348 nm, corresponding to the isobestic point of nitrophenol and the nitrophenolate ion using the molar absorptivity 5.0 × 10$^4$ M$^{-1}$·cm$^{-1}$ (25). Because the catalysis of this hydrolysis by mCA IV was slow, we determined the overall rate and subtracted from it the rate in the presence of 20 $\mu$M ethoxzolamide to inhibit mCA IV. The difference was the component of the overall hydrolysis due to the hydrolysis at the active site. Initial velocities were determined under the conditions given in the legend to Fig. 4. The value of $S_\text{eff}$ for catalysis was too large to measure because it exceeded the solubility of substrate.

As a result, we were limited to observing catalytic rates that were first order in the substrate from which we obtained $k_{cat}/S_\text{eff}$. 

$\text{HCO}_3^-$ + CO$_2$ + H$_2$O, $\text{Ka}$ (pH 8.4) with methoxzolamide to inhibit mCA IV. The $S_\text{eff}$ were determined by nonlinear least squares methods (Kinzffiter, Elsevier-Biosoft). The values of $S_\text{eff}$ for catalysis of CO$_2$ was slow, we determined the overall rate and subtracted from it the rate in the presence of 20 $\mu$M ethoxzolamide to inhibit mCA IV. The difference was the component of the overall catalysis due to the hydrolysis at the active site. Initial velocities were determined under the conditions given in the legend to Fig. 4. The value of $S_\text{eff}$ for catalysis was too large to measure because it exceeded the solubility of substrate.

As a result, we were limited to observing catalytic rates that were first order in the substrate from which we obtained $k_{cat}/S_\text{eff}$. 

$\text{HCO}_3^-$ + CO$_2$ + H$_2$O, $\text{Ka}$ (pH 8.4) with methoxzolamide to inhibit mCA IV. The $S_\text{eff}$ were determined by nonlinear least squares methods (Kinzffiter, Elsevier-Biosoft). The values of $S_\text{eff}$ for catalysis of CO$_2$ was slow, we determined the overall rate and subtracted from it the rate in the presence of 20 $\mu$M ethoxzolamide to inhibit mCA IV. The difference was the component of the overall catalysis due to the hydrolysis at the active site. Initial velocities were determined under the conditions given in the legend to Fig. 4. The value of $S_\text{eff}$ for catalysis was too large to measure because it exceeded the solubility of substrate.
species of CO₂, with data at pH 6.7 given in Fig. 2. The dependence of $R_\text{cat}/[E]$ on the sum of the concentrations [CO₂] + [HCO₃⁻] was nearly linear with very little evidence of saturation. For example, the very slight curvature in the data of Fig. 2 is consistent with a value of $k_\text{cat}^{\text{eff}}$ of 270 ± 30 ms⁻¹, where $S$ represents all CO₂ species, both CO₂ and HCO₃⁻. Because this large value of $k_\text{cat}^{\text{eff}}$ greatly exceeded the solubility of CO₂ and HCO₃⁻, we were not able to obtain a value of $k_\text{cat}^{\text{ex}}$ in Equation 3. However, we were able to obtain $k_\text{cat}^{\text{ex}}/K_\text{cat}^{\text{eff}}$ from the slope of plots such as the one in Fig. 2 or from studies with ([CO₂] + [HCO₃⁻]) < $K_\text{cat}^{\text{eff}}$.

The ratio $k_\text{cat}^{\text{ex}}/K_\text{cat}^{\text{eff}}$ for the hydration of CO₂ was determined by two methods, measurement by mass spectrometry of the exchange of $^{18}$O between CO₂ and water and measurement by stopped flow of the initial velocity of CO₂ hydration. When $[S]$ < $K_\text{m}$ there is an equilibrium distribution of enzyme forms also in steady state; hence, the ratio $k_\text{cat}^{\text{ex}}/K_\text{cat}^{\text{eff}}$ (for S = CO₂) obtained by $^{18}$O exchange in theory and in practice equivalent to $k_\text{cat}^{\text{ex}}/K_\text{m}$ for hydration of steady-state measurements (22). The $^{18}$O method was carried out at a total concentration of 25 mM of all CO₂ species under conditions (given in the legend to Fig. 3) for which ([CO₂] + [HCO₃⁻]) < $K_\text{cat}^{\text{eff}}$.

This approach for catalysis by mCA IV yielded a maximal value of $k_\text{cat}^{\text{eff}}/K_\text{m}$ of 3.2 ± 0.1 × 10⁷ M⁻¹ s⁻¹ with an apparent $pK_a$ for the catalysis of 6.6 ± 0.1 (Fig. 3). The presence of up to 200 mM imidazole in solution had no effect on $k_\text{cat}^{\text{eff}}/K_\text{m}$ measured by $^{18}$O exchange (shown for 100 mM in Fig. 3). The measurement of $k_\text{cat}^{\text{ex}}/K_\text{m}$ for hydration of CO₂ by stopped flow gave data with less precision (data not shown); the analysis of these results yielded a maximal value of $k_\text{cat}^{\text{ex}}/K_\text{m}$ of 5.0 ± 0.2 × 10⁷ M⁻¹ s⁻¹ with an apparent $pK_a$ of 7.3 ± 0.2. The solvent hydrogen isotope effect determined for mCA IV by $^{18}$O exchange was $P_\text{(H₂O/\text{H}_{2}^{18}O)}$ = 0.83 ± 0.11, measured at pH 6.8 and 25 °C in solutions containing no buffers. The $^{18}$O exchange studies extended to catalysis by the mutant H64A mCA IV yielded a maximal value of $k_\text{cat}^{\text{ex}}/K_\text{m}$ of 6.3 ± 0.2 × 10⁷ M⁻¹ s⁻¹ with an apparent $pK_a$ of 7.3 ± 0.1 (Fig. 3).

The value of the apparent $pK_a$ for catalysis by mCA IV was confirmed by measurement of the catalytic hydrolysis of 4-nitrophenyl acetate. The inherent efficiency of mCA IV in this catalysis was very low. To ensure that we were measuring catalysis at the same active site as is involved in hydration of CO₂, we determined that component of the overall rate of catalytic hydrolysis of 4-nitrophenyl acetate that was inhibited in the presence of 20 μM ethoxzolamide, a specific and potent ($K_i$ = 16 nM) inhibitor of mCA IV. The maximal value of $k_\text{cat}^{\text{ex}}/K_\text{m}$ for this hydrolysis was 20 ± 1 M⁻¹ s⁻¹ with an apparent $pK_a$ of 6.5 ± 0.2 (Fig. 4).

The rate constant $R_\text{cat}/[E]$ for the proton transfer-dependent release of $^{18}$O-labeled water from the active site (Equation 2) was measured by mass spectrometry in the absence of buff-
The kinetic constant $k_{cat}/K_m$ for hydration of CO$_2$ catalyzed by murine CA IV in the absence of buffers (●), murine CA IV in the presence of 100 mM imidazole (■), and murine H64A CA IV in the absence of buffers (△). Data were obtained at 25 °C using $^{18}$O exchange. The total concentration of all CO$_2$ species ([CO$_2$] + [HCO$_3^-$/CO$_3^{2-}$]) was 25 mM, and the total ionic strength of solution was maintained at 0.2 M by addition of Na$_2$SO$_4$. Solutions also contained 5 mM EDTA. The solid line is a least squares fit to a single ionization for murine CA IV in the absence of buffers with $pK_a$ of 6.6 ± 0.1 and a maximal value of $k_{cat}/K_m$ of 3.2 ± 0.1 × 10$^7$ M$^{-1}$ s$^{-1}$; the dashed line is a fit to H64A CA IV and has $pK_a$ of 7.3 ± 0.1 and a maximal value of $k_{cat}/K_m$ of 6.3 ± 0.2 × 10$^7$ M$^{-1}$ s$^{-1}$.

The catalytic turnover number for the hydration of CO$_2$ catalyzed by mCA IV and measured at steady state had a maximal value of 80 ± 20 s$^{-1}$ at pH 7.2 (data not shown). In contrast, the value of $R_{H2O}/[E]$ catalyzed by wild-type mCA IV was enhanced 50% by addition of imidazole at the level of saturation (50–100 mM imidazole) under the same conditions.

The catalytic turnover number for the hydration of CO$_2$ catalyzed by mCA IV and measured at steady state had a maximal value of 80 ± 20 s$^{-1}$ at pH 7.2 (data not shown). In contrast, the value of $R_{H2O}/[E]$ catalyzed by wild-type mCA IV was enhanced 50% by addition of imidazole at the level of saturation (50–100 mM imidazole) under the same conditions.

The kinetic constant $k_{cat}/K_m$ for the hydrolysis of 4-nitrophenyl acetate catalyzed by murine CA IV at 25 °C in solutions containing 5 mM EDTA and 25 mM of one of the following buffers: pH 5.2–6.2, Mes; pH 6.8–7.4, Mops; pH 7.8–8.0, Heps. The data represent that component of the catalytic velocity that was abolished by the addition of 20 μM of the carbonic anhydrase inhibitor ethoxzolamide. The solid line is a least squares fit of the data to a single ionization with $pK_a$ of 6.5 ± 0.2 and a maximal value of $k_{cat}/K_m$ of 20 ± 1 M$^{-1}$ s$^{-1}$.

The data represent that component of the catalytic velocity that was abolished by the addition of 20 μM of the carbonic anhydrase inhibitor ethoxzolamide. The solid line is a least squares fit of the data to a single ionization with $pK_a$ of 6.5 ± 0.2 and a maximal value of $k_{cat}/K_m$ of 20 ± 1 M$^{-1}$ s$^{-1}$.
His64 to the zinc and in hydrogen bonding to the zinc-murine CA IV with that of human CA II is shown in Fig. 1. The isozyme of carbonic anhydrase. An amino acid alignment of this form of CA IV and cytosolic CA II, a well studied and efficient extension, an enzyme designed to resemble both the mature CA IV lacking an N-terminal signal peptide and C-terminal cupric ion Cu$_{2}$$^{+}$ listed above. The solid line least squares fit and yielded two values of pK$_{a}$ 6.3 ± 0.4 and 9.1 ± 0.1.

FIG. 6. The pH dependence of the steady-state turnover number $k_{cat}$ for the hydration of CO$_{2}$ catalyzed by murine CA IV (●) and by murine H64A CA IV (□) measured at 25 °C in the presence of 4 μM EDTA and 25 mM of one of the following buffers: pH 5.6–6.9, Mes; pH 6.6–7.3, Mops; pH 7.0–7.9, Hepes; pH 8.0–8.7, Taps; pH 8.7–10, Ches. The experiments identified by the open square were identical to those for murine CA IV with the exception that they also contained 25 mM imidazole in addition to one each of the buffers listed above. The solid line for wild-type murine CA IV was obtained by least squares fit and yielded two values of pK$_{a}$ 6.3 ± 0.4 and 9.1 ± 0.1.

DISCUSSION

In this study we describe the catalytic properties of murine CA IV lacking an N-terminal signal peptide and C-terminal extension, an enzyme designed to resemble both the mature form of CA IV and cytosolic CA II, a well studied and efficient isozyme of carbonic anhydrase. An amino acid alignment of this murine CA IV with that of human CA II is shown in Fig. 1. The overall sequence identity of 37% includes residues involved in coordination to the zinc and in hydrogen bonding to the zinc-bound water molecule (Thr$^{199}$, Glu$^{106}$) that are conserved in all of the animal carbonic anhydrases. Moreover, the crystal structures of human CA IV and human CA II in the region of their active sites are very similar (11). Through these similarities, the general catalytic pathway of murine CA IV can be rationalized by analogies to isozyme II as described in Equations 5 and 6.

$$\text{CO}_2 + \text{EZnOH}^- + \text{H}_2\text{O} \rightleftharpoons \text{HCO}_3^- + \text{EZnH}_2\text{O} \quad (\text{Eq. 5})$$

$$\text{His}^{64-}\text{EZnH}_2\text{O} + \text{B} \rightleftharpoons \text{H} \text{His}^{64-}\text{EZnOH}^- + \text{B} \rightleftharpoons \text{His}^{64-}\text{EZnOH}^- + \text{BH} \quad (\text{Eq. 6})$$

Here BH represents buffer in solution and/or a possible proton shuttle of the enzyme. This scheme of Equations 5 and 6 has been supported by considerable evidence for the mechanism of CA II (26), and its use for mCA IV is consistent with a number of features of this work: a solvent hydrogen isotope effect near unity, $D(k_{cat}/K_m) = 0.83 \pm 0.11$, indicating the lack of proton transfer in Equation 5; a solvent hydrogen isotope effect on $R_{cat}/[E]$ of 1.9 ± 0.4 consistent with primary proton transfer in Equation 6; and an effect of buffer imidazole on $k_{cat}$ but not on $K_m$.

However, despite these similarities between CA II and CA IV, catalysis by murine CA IV appears unique among the isozymes of carbonic anhydrase based on the evidence of Fig. 6 that its catalysis involves multiple proton transfer pathways. This evidence as well as the role of His$^{64}$ in the proton shuttle is discussed below.

Role of His$^{64}$—The maximal turnover for CO$_{2}$ hydration catalyzed by CA II has been carefully considered in previous work to be determined in rate almost entirely by the intramolecular proton transfer from the zinc-bound water molecule to His$^{64}$ (27, 28). The similar values of $k_{cat}$ for isozymes II and IV as well as the presence and similar conformation of His$^{64}$ in both of these isozymes (11) strongly suggest that the same intramolecular proton transfer contributes to the maximal velocity of CA IV. The maximal turnover number $k_{cat}$ for the hydration of CO$_{2}$ catalyzed by mCA IV at 25 °C was observed to be $1.1 \times 10^6$ s$^{-1}$ (Fig. 6), quite close in magnitude to the $k_{cat}$ value of $1.4 \times 10^6$ s$^{-1}$ measured under comparable conditions for human CA II (24).

Further support for the role of His$^{64}$ in CA IV comes from $^{18}$O-exchange studies. The rate constant for the release of $^{18}$O-labeled water from mCA IV, $R_{cat}/[E]$ shown in Fig. 5, reveals a bell-shaped dependence on pH very similar to that of CA II (29). This curve is fit well by the superposition of two ionizations (Equation 4) describing the presence of the zinc-bound hydroxide and the presence of a second group that donates a proton to the zinc-bound hydroxide, such as the imidazolium side chain of His$^{64}$. These $^{18}$O-exchange data confirm the pK$_{a}$ of 6.6 for the ionization of the zinc-bound water and also provide the pK$_{a}$ of 6.9 ± 0.1 for the second ionization (Fig. 5). Again this value is close to that determined by the titration of the proton magnetic resonance of the C-2 proton of His$^{64}$ in human CA II, which yields a value of pK$_{a}$ 7.1 (30). These same $^{18}$O-exchange data of Fig. 5 give a maximal rate constant for intramolecular proton transfer from His$^{64}$ to the zinc-bound hydroxide...
molecule of \( k_B = 1.4 \pm 0.2 \times 10^6 \text{ s}^{-1} \), consistent with the maximal value of \( k_{\text{cat}} \), which is also determined by this intramolecular proton transfer. That these values should be nearly identical for proton transfer-dependent processes in the hydration and dehydration directions is the result of the values of the \( pK_a \) for donor and acceptor being nearly identical at \( pK_a = 7 \).

The role of His\(^{64} \) as proton donor in CA IV is further supported by the inhibition of catalysis by cupric ions and by the much reduced values of \( k_{\text{cat}} \) and \( R_{\text{H2O}} \) for the mutant H64A mCA IV. The data for the unmodified mCA IV showed inhibition of \( R_{\text{H2O}} \) by cupric ions (IC\(_{50} = 6 \times 10^{-7} \text{ M} \), with no effect on the rate of interconversion of CO\(_2\) and HCO\(_3^-\) (\( R_1/\text{[E]} \)) up to a concentration of 40 \( \mu \text{M} \) (Fig. 7). These data are consistent with an inhibition of the proton-transfer pathway with no effect on the function of the zinc-bound hydroxide in the first stage of catalysis that produces HCO\(_3^-\). An analogous result was obtained with CA II, in which \( R_{\text{H2O}} \) was inhibited by Cu\(^{2+} \) with an IC\(_{50} \) of 1.0 \( \times 10^{-7} \text{ M} \) and by Hg\(^{2+} \) with an IC\(_{50} \) of 1.6 \( \times 10^{-7} \text{ M} \) with no effect on \( R_1 \) (31). This was interpreted as an inhibition of the function of His\(^{64} \) as a proton shuttle and was confirmed by the crystal structure of the Hg\(^{2+}\)-CA II complex showing a superposition of structures, about half with mercuic ion bound to N\( \delta \) and about half bound to N\( \epsilon \) of the imidazole side chain of His\(^{64} \) (32). The similar inhibition of CA IV and CA II by cupric ions is strong evidence for the role of His\(^{64} \) in proton transfer by CA IV. Moreover, the inhibited value of \( R_{\text{H2O}}/\text{[E]} \) near 2 \( \times 10^{-3} \text{ s}^{-1} \) for CA IV in the presence of excess Cu\(^{2+} \) (Fig. 7) is close to the value of \( R_{\text{H2O}}/\text{[E]} \) for the mutant H64A CA IV in which His\(^{64} \) is replaced by alanine (Fig. 5) and is close to the value of \( R_{\text{H2O}}/\text{[E]} \) for H64A CA II (29), suggesting that this binding of Cu\(^{2+} \) has nearly completely inhibited the proton transfer role of a shuttle group. The remaining proton-transfer activity is due to other less efficient proton-transfer residues of the protein or to water in the active site.

These inhibition data suggesting a proton shuttle role for His\(^{64} \) were supported by the much lower values of \( k_{\text{cat}} \) for hydration and \( R_{\text{H2O}}/\text{[E]} \) catalyzed by the mutant H64A mCA IV (Figs. 5 and 6). Both of these rate constants are determined largely by proton transfer from or to the zinc-bound water or hydroxide (27, 29) in CA II. This is further confirmed by the significant enhancement of \( R_{\text{H2O}} \) and \( k_{\text{cat}} \) for H64A mCA IV by addition of imidazole, achieving a chemical rescue by providing the proton-transfer group from solution (Fig. 6).

### Multiple Proton-Transfer Pathways

Although the maximal values of \( k_{\text{cat}} \) for hydration are similar for mCA IV and CA II, there is a significant difference: the pH dependence of CA II can be described by a single ionization (24), that of the proton acceptor His\(^{64} \), but the pH dependence of \( k_{\text{cat}} \) for mCA IV is more complex, indicating the influence of at least two ionizable groups (Fig. 6).\(^4\) One of these groups is His\(^{64} \) acting as a proton shuttle, as described above, and the second is at least one other proton shuttle of pK\(_a \) near 9 that has not yet been identified but that is similar to that observed in CA V (33). The activation of \( k_{\text{cat}} \) through chemical rescue caused by the presence of 25 mm imidazole (Fig. 6) suggests that the depression in the pH profile of \( k_{\text{cat}} \) for mCA IV near pH 7 is due to reduced capacity of the proton shuttle. That is, mCA IV has the capacity to attain higher values of \( k_{\text{cat}} \) with an external proton acceptor, a result that suggests that the function of the internal proton shuttle His\(^{64} \) in mCA IV is partially blocked. One possible explanation for the more complex pH profile of mCA IV may be the presence of threonine at position 65, whereas human CA II has an alanine at this position (Fig. 1). This Thr\(^{65} \) may block the proton shuttle function of His\(^{64} \) in a manner similar to that of Phe\(^{65} \) in CA V (33). However, in CA II Jackman et al. (34) found no effect of the replacement Ala\(^{65} \rightarrow \) Thr on the function of the proton-shuttle mechanism involving His\(^{64} \). It is noteworthy that murine CA IV has glutamine at residue 63, whereas human CA II has glycine at this position; hence, in this position also murine CA IV has a bulkier side chain that could hinder the role of His\(^{64} \) as a proton shuttle. Tamai et al. (41) have reported evidence consistent with the influence of residue 63 in the overall catalysis by CA IV; the replacement Glu\(^{63} \rightarrow \) Gln in bovine CA IV decreases catalytic activity measured in a colorimetric assay near neutral pH, and the replacement Gln\(^{63} \rightarrow \) Gly in murine CA IV increases activity. This role of residue 63 can also explain the differences in the pH profile for \( k_{\text{cat}} \) obtained for murine CA IV in this work and that of Baird et al. (42) for human CA IV. Baird et al. observed a pH profile for \( k_{\text{cat}} \) catalyzed by human CA IV that is consistent with a single ionization; at physiological pH this value of \( k_{\text{cat}} \) is greater by about 40-fold than that of murine CA IV. These observations represent one of the largest kinetic differences both qualitatively and quantitatively between a single isozyme of carbonic anhydrase from different animal species.

The data for \( k_{\text{cat}} \) describing CO\(_2\) hydration catalyzed by H64A mCA IV, although lacking the proton shuttle His\(^{64} \), clearly show the emergence of a second proton shuttle in the enhanced activity at the higher pH range of Fig. 6. Hence, murine CA IV demonstrates at least two pathways for intramolecular proton transfer. Its further study may clarify the properties of multiple proton transfers and clarify the role of such processes in more complex cases of multiple proton pathways recently reported in enzymes (glycaminide ribonucleotide transformylase (35)) and in proton-translocation proteins (cytochrome oxidase (36) and photosynthetic reaction center (37)). Each of these cases has a similarity to our results in that mutation of a single proton-transfer residue leaves considerable activity, indicating that these residues are nonessential when mutated individually and suggesting multiple proton-transfer pathways.

### Interconversion of CO\(_2\) and HCO\(_3^-\)

The ratio \( k_{\text{cat}}/K_m \) contains rate constants for the catalysis up to the first irreversible step in the pathway, which in the hydration direction is the release of HCO\(_3^-\) in Equation 5. Thus, \( k_{\text{cat}}/K_m \) contains information on the rate of interconversion of CO\(_2\) and HCO\(_3^-\) but not on the proton-transfer steps by which the zinc-bound hydroxide is regenerated (Equation 6). The maximal value of \( k_{\text{cat}}/K_m \) for hydration of CO\(_2\) catalyzed by mCA IV was similar when measured at 25 °C by \( ^{18} \text{O} \) exchange (3.2 \( \times 10^5 \text{ M}^{-1} \text{s}^{-1} \); Fig. 3) and by stopped flow spectrophotometry (5.0 \( \times 10^5 \text{ M}^{-1} \text{s}^{-1} \); data not shown). The apparent pK\(_a \) for this activity was 6.6 (Fig. 3). Hence, when Wistrand and Knuttila (7) and Maren et al. (12) made measurements of \( k_{\text{cat}}/K_m \) for hydration at pH near 7.0–7.8 they were close to maximal values; their measurements were made at 0 to 1 °C and yielded \( k_{\text{cat}}/K_m \) near 2 \( \times 10^7 \text{ M}^{-1} \text{s}^{-1} \) for human and bovine CA IV. These values are to
be compared with the maximal value of $k_{cat}/K_m$ for human CA II of $1.5 \times 10^8 \text{M}^{-1}\text{s}^{-1}$ at 25 °C (24).

The pH dependence of $k_{cat}/K_m$ for hydration of CO$_2$ catalyzed by mCA IV gave an apparent $pK_a$ of 6.6 ± 0.1 measured by $^{18}$O exchange at chemical equilibrium (Fig. 3); this is a direct indication of the $pK_a$ of the zinc-bound water molecule (38). A consistent result is the $pK_a$ of 6.5 ± 0.2 for the hydrolysis of 4-nitrophenyl acetate catalyzed by mCA IV (Fig. 4). However, for reasons that are unclear, the apparent $pK_a$ measured from $k_{cat}/K_m$ at steady state gave a value somewhat larger, 7.3 ± 0.2. The value of the corresponding $pK_a$ for CA II is 6.9 but depends significantly on ionic strength and sulfate concentration (38). In view of these similarities, it is interesting that the maximal value of $k_{cat}/K_m$ for the hydrolysis of 4-nitrophenyl acetate catalyzed by mCA IV at 20 ± 1 $\text{M}^{-1}\text{s}^{-1}$ is less by a factor of at least 100 than that measured for CA II, which is as great as 3 $\times$ $10^6$ $\text{M}^{-1}\text{s}^{-1}$ (27, 39). The replacement His$^{64}$ → Ala in mCA IV had an effect on $k_{cat}/K_m$ for hydration of CO$_2$ measured by $^{18}$O exchange; the maximal value is enhanced somewhat and the apparent $K_m$ of 7.3 ± 0.1 is also increased compared with that of the unmodified mCA IV (Fig. 3). These are small effects relative to those observed for $k_{cat}$ and $R_{H_2O}$ but reflect the influence of the side chain of residue 64 on the ionization of the zinc-bound water and the related effect on the rate of interconversion of CO$_2$ and HCO$_3^-$.

Acknowledgments—We acknowledge the technical assistance of Yanping Zhang and Yang Wang.

REFERENCES
1. Hewett-Emmett, D., and Tashian, R. E. (1996) Mol. Phylogenet. Evol. 5, 50–77
2. Tashian, R. E. (1989) BioEssays 10, 186–192
3. Dodgson, S. J. (1991) in The Carbonic Anhydrases (Dodgson, S. J., Tashian, R. E., Gros, G., and Carter, N. D., eds) pp. 297–306, Plenum Publishing Corp., New York
4. Whitney, P. L., and Briggle, T. V. (1982) J. Biol. Chem. 257, 12056–12059
5. Maren, T. H. (1980) Ann. N. Y. Acad. Sci. 341, 246–258
6. Wistrand, P. J. (1984) Ann. N. Y. Acad. Sci. 429, 195–206
7. Wistrand, P. J., and Kovaltzipa, K.-G. (1989) Kidney Int. 35, 851–859
8. Zhu, X. L., and Sly, W. S. (1996) J. Biol. Chem. 271, 8795–8801
9. Waheed, A., Zhu, X. L., and Sly, W. S. (1992) J. Biol. Chem. 267, 5308–3311
10. Sly, W. S., and Hu, P. Y. (1995) Annu. Rev. Biochem. 64, 375–401
11. Stamat, N., Nair, S. K., Okuyama, T., Waheed, A., Sly, W. S., and Christiansen, D. W., (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 13589–13594
12. Maren, T. H., Wynns, G. C., and Wistrand, P. J. (1993) Mol. Pharmacol. 44, 901–905
13. Vincent, S. H., and Silverman, D. N. (1980) Arch. Biochem. Biophys. 205, 51–56
14. Okuyama, T., Sato, S., Zhu, X. L., Waheed, A., and Sly, W. S. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 1315–1319
15. Fleming, R. E., Crouch, E. C., Ruzicka, C. A., and Sly, W. S. (1993) Am. J. Physiol. 265, L627–L635
16. Tamai, S., Cody, L. B., and Sly, W. S. (1996) Biochem. Genet. 34, 31–43
17. Okuyama, T., Waheed, A., Kusumoto, W., Zhu, X. L., and Sly, W. S. (1995) Arch. Biochem. Biophys. 320, 315–322
18. Tanhauser, S. M., Jewell, D. A., Tu, C. K., Silverman, D. N., and Laiups, P. J. (1992) Gene 117, 113–117
19. Kunkel, T. A., Begene, K., and McClary, J. (1991) Methods Enzymol. 204, 125–139
20. Tu, C. K., Thomas, H. G., Wynns, G. C., and Silverman, D. N. (1986) J. Biol. Chem. 261, 10100–10103
21. Silverman, D. N. (1982) Methods Enzymol. 87, 732–752
22. Simonson, I., Jonsson, B.-H., and Lindsuego, S. (1979) Eur. J. Biochem. 93, 409–417
23. Silverman, D. N., Tu, C. K., Chen, X., Tanhauser, S. M., Kresge, A. J., and Laiups, P. J. (1996) Biochemistry 35, 10757–10762
24. Khalifah, R. G. (1971) J. Biol. Chem. 246, 2561–2573
25. Verpoorte, J. A., Mehta, S., and Edsall, J. T. (1967) J. Biol. Chem. 242, 4221–4229
26. Silverman, D. N., and Lindsuego, S. (1988) Acc. Chem. Res. 21, 30–36
27. Steiner, H., Jonsson, B.-H., and Lindsuego, S. (1975) Eur. J. Biochem. 59, 253–259
28. Bowlett, R. S. (1984) J. Protein Chem. 3, 369–393
29. Tu, C. K., Silverman, D. N., Forsman, C., Jonsson, B. H., and Lindsuego, S. (1989) Biochemistry 28, 7913–7918
30. Campbell, I. D., Lindsuego, S., White, A. I. (1975) J. Mol. Biol. 96, 597–614
31. Tu, C. K., Wynns, G. C., and Silverman, D. N. (1981) J. Biol. Chem. 256, 9466–9470
32. Eriksen, A. E., Jones, T. A., and Liljas, A. (1986) in Zinc Enzymes (Bertini, I., Luchinat, C., Maret, W., Zeppezauer, M., eds) pp. 317–326, Birkhauser, Boston
33. Heck, R. W., Boriack-Sjodin, P. A., Qian, M. Z., Tu, C. K., Christiansen, D. W., Laiups, P. J., and Silverman, D. N. (1996) Biochemistry 35, 11605–11611
34. Jackman, J. E., Merz, K. M., and Fierke, C. A. (1996) Biochemistry 35, 16421–16428
35. Warren, M. S., Marolewski, A. E., and Benkovic, S. J. (1996) Biochemistry 35, 8855–8862
36. Mitchell, D. M., Fettet, J. R., Milis, D. A., Adelroth, P., Pressler, M. A., Kim, Y., Aasa, R., Brezinski, P., Malmström, B. G., Alben, J. O., Babcock, G. T., Ferguson-Miller, S., and Gennis, R. B. (1996) Biochemistry 35, 13089–13093
37. Aciou, L., and Michel, H. (1995) Biochemistry 34, 7967–7972
38. Simonson, I., and Lindsuego, S. (1982) Eur. J. Biochem. 123, 29–36
39. Pocker, Y., and Stone, T. (1967) J. Biol. Chem. 242, 4174–4180
40. Dayhoff, M. O., Barber, W. C., and Hunt, L. T. (1983) Methods Enzymol. 91, 524–545
41. Tamai, S., Waheed, A., Cody, L. B., and Sly, W. S. (1997) Proc. Natl. Acad. Sci. U. S. A. 93, 13647–13652
42. Baird, T. T., Waheed, A., Okuyama, T., Sly, W. S., and Fierke, C. A. (1997) Biochemistry 36, 2669–2678
