Catalysis and Inhibition of Human Carbonic Anhydrase IV†

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Received October 23, 1996; Revised Manuscript Received January 8, 1997⊗

ABSTRACT: Carbonic anhydrase IV (CA IV) is a membrane-bound form of carbonic anhydrase. We have characterized the catalytic activity and inhibition of recombinant human CA IV. CA IV is a high-activity isozyme in CO_2 hydration with a pH-independent k_{cat} value (1.1×10^6 s^{-1}) comparable to that of CA II (8×10^5 s^{-1}). Furthermore, CA IV is more active in HCO_3^- dehydration than is CA II as illustrated by the nearly 3-fold increase in k_{cat}/K_M to 3×10^7 M^{-1} s^{-1}. However, the esterase activity of CA IV is decreased 150-fold compared to CA II. The catalytic mechanisms of CA II and CA IV are nearly identical. Both isozymes show similar dependence on buffer concentration with the rate-limiting step at high buffer concentration being intramolecular proton transfer, although the intramolecular proton transfer for CA IV is 3 times faster than that observed with CA II. Additional positive charges in the active site of CA IV stabilize anions as indicated by a decreased pK_a for the Zn-bound water compared to CA II (6.2 vs 6.9), as well as lower inhibition constants for a variety of anions, including halides, sulfate, formate, acetate, and bicarbonate. CA IV is also activated by low concentrations (<20 mM) of chloride, bromide, and phosphate. Activation by phosphate suggests that the phospholipid anchor may be acting both as an extracellular tether and as a protein activator. Finally, the affinity of CA IV for sulfonamide inhibitors is decreased up to 65-fold compared to CA II as demonstrated by fluorescence titration. The increased bicarbonate activity and altered pH profile are consistent with the proposed physiological role of CA IV in renal bicarbonate reabsorption.

The carbonic anhydrases (EC 4.2.1.1) are a family of zinc metalloenzymes that catalyze the reversible hydration of carbon dioxide in a two-step reaction to yield bicarbonate and a proton (eq 1a,b). At least seven different mammalian

$$H_2O + E{\cdot}Zn{-}OH^- + CO_2 \leftrightarrow H_2O + E{\cdot}Zn{-}HCO_3^- \leftrightarrow E{\cdot}Zn{-}H_2O + HCO_3^- \quad (1a)$$

$$E{\cdot}Zn{-}H_2O + B^- \leftrightarrow E{\cdot}Zn{-}OH^- + BH \quad (1b)$$

isozymes with varying activity, tissue specificity, and physiological roles (CA I to CA VII)[1] have been identified (Dodgson, 1991; Hewett-Emmett & Tashian, 1996). Two isozymes, CA I and II, are expressed in high concentrations in red blood cells and in lower concentrations in the cytosol of other tissues while CA III is present mainly in the cytoplasm of muscle tissue cells. CA IV is the only membrane-bound form of carbonic anhydrase and has been observed in lung, kidney, brain, and eye tissues. CA V is expressed in mitochondria while isozymes CA VI and VII are found in saliva and salivary glands, respectively (Sly & Hu, 1995).

CA II is the most active of the cytosolic isozymes with a turnover number of $>10^6$ s^{-1} for catalysis of CO_2 hydration, and a second-order rate constant that approaches that of the diffusion control limit at 10^8 M^{-1} s^{-1} (Silverman & Lindskog, 1988). In comparison, mouse CA V (Heck et al., 1994), the mitochondrial carbonic anhydrase, is 5-fold less active than CA II while CA I (Khalifah, 1971) is 3-fold less active than isozyme II even though it is the predominant CA constituent in erythrocytes. CA III is the least active soluble isozyme, with a k_{cat}/K_M 500-fold less than that of CA II (Jewell et al., 1991).

The physiological importance of CA II is demonstrated by a CA II deficiency syndrome in humans that is characterized by renal tubular acidosis, osteopetrosis, and, in some cases, mental retardation (Sly & Hu, 1995). This is a genetic disease where CA II activity is absent in both kidneys and erythrocytes while CA IV is expressed and functional (Sato et al., 1990). Therefore, CA II and CA IV play important but different physiological roles, and the functions of these isozymes are related to their different cellular locations. In the kidney, CA II is localized to the cytoplasm of certain

† This work was supported by the National Institutes of Health (GM40602). Additionally, C.A.F. gratefully acknowledges the receipt of an American Heart Association Established Investigator Award and a David and Lucile Packard Foundation Fellowship in Science and Engineering. T.T.B. is supported in part by a Ford Foundation Predoctoral Fellowship.

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⊗ Abstract published in *Advance ACS Abstracts,* February 15, 1997.

[1] Abbreviations: AZA, acetazolamide (5-acetamido-1,3,4-thiadiazole-2-sulfonamide); BZA, benzenesulfonamide; CA I, II, etc., human carbonic anhydrase isozyme I, II, etc.; CHES, 2-(*N*-cyclohexylamino)-ethanesulfonic acid; DNSA, dansylamide (5-dimethylamino-1-naphthalenesulfonamide); EDTA, (ethylenedinitrilo)tetraacetic acid; EZA, ethoxzolamide (6-ethoxy-2-benzothiazolsulfonamide); GPI, glycophosphatidylinositol; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PNPA, *p*-nitrophenyl acetate; SDS, sodium dodecyl sulfate; SHIE, solvent hydrogen isotope effect; SNA, sulfanilamide; TAPS, 3-Tris[(hydroxymethyl)methyl]aminopropanesulfonic acid; Tris, Tris(hydroxymethyl)aminomethane; Val-121, valine at position 121 using CA I numbering; other residues are described similarly using the three-letter amino acid code.

S0006-2960(96)02663-3 CCC: $14.00

cells of renal tubules and collecting ducts where it is important for acidification of urine (Liljas et al., 1972; Sato et al., 1990; Sly et al., 1983; 1985a,b) while membrane-bound CA IV is located in the apical plasma membranes of the brush border in the proximal convoluted tubule and the thick ascending limb of Henle (Brown et al., 1990). CA IV plays a major role in bicarbonate reabsorption in the kidney (Maren et al., 1993) as well as modulating the pH in the lumen (Brechue et al., 1991; Lucci et al., 1983). CA IV is also localized in pulmonary endothelial cells where it catalyzes the dehydration of serum bicarbonate to CO_2 (Whitney & Briggle, 1982). Furthermore, CA IV is found in the endothelial cells of an ocular capillary bed (Hageman et al., 1991; Maren et al., 1993), suggesting that this isozyme may also be a target for carbonic anhydrase inhibitors that are used in the treatment of glaucoma (Maren, 1987).

CA IV is posttranslationally cleaved and modified by addition of a glycophosphatidylinositol (GPI) tail that anchors the enzyme to the membrane (Zhu & Sly, 1990). Furthermore, this enzyme contains two disulfide linkages that contribute to its stability in the presence of 5% sodium dodecyl sulfate (SDS), a concentration that inactivates CA II. The specific activity for CO_2 hydration catalyzed by CA IV in membrane fractions of human lung, human kidney, or calf kidney is comparable to that of CA II (Wistrand, 1984; Zhu & Sly, 1990). However, CA II is antigenically and genetically distinct from CA II (Hageman et al., 1991). The gene for human CA IV has been isolated from a λgt10 kidney cDNA library and expressed in COS cells (Okuyama et al., 1992). Furthermore, a variant with 45 amino acids deleted from the C-terminal end, including a signal sequence and the signal for posttranslational cleavage and transfer to the GPI anchor, encodes a fully active, soluble form of CA IV in COS cells (Okuyama et al., 1995). We have used this recombinant CA IV to determine steady-state kinetic parameters of this isozyme for comparison with other CA isozymes.

To further delineate the functional properties and physiological role(s) of human carbonic anhydrase IV, we have measured the pH dependence of the steady-state kinetic parameters for CO_2 hydration, bicarbonate dehydration, and ester hydrolysis catalyzed by CA IV. These data indicate that the catalytic and kinetic mechanism of CA IV is comparable to that of CA II, except that the k_{cat}/K_M for bicarbonate dehydration increases 3-fold, and the pK_a of the zinc-bound water molecule is significantly decreased in CA IV (pK_a = 6.2 vs 6.9). At physiological pH, CA IV has greater dehydrase activity than CA II, consistent with its physiological role in bicarbonate dehydration. Our studies also show that compared to CA II, isozyme IV is more sensitive to inhibition by sulfate and other anions and less sensitive to inhibition by some sulfonamides. These different properties can be rationalized in light of the recently determined crystal structure of CA IV (Stams et al., 1996). This detailed kinetic characterization of human carbonic anhydrase IV provides both clues to the physiological importance of this isozyme and valuable information about structure−function relationships in carbonic anhydrase.

MATERIALS AND METHODS

Catalytic Activity. Recombinant human kidney CA IV was expressed and purified from either *Escherichia coli* or CHO cells as described (Okuyama et al., 1992; Waheed et al., 1996). This CA IV is 266 amino acids in length with N-terminal and C-terminal truncations to delete the leader signal peptide and the sequence required for the attachment of the GPI anchor, respectively. The molecular weight of the enzyme (M_r = 29 800) was calculated from the amino acid sequence deduced from the cDNA sequence. The protein concentration (in milligrams per milliliter) was initially determined using the Lowry method with bovine serum albumin as a standard (Lowry et al., 1951).

The second-order rate constant, k_{cat}/K_M, for ester hydrolysis was determined at 25 °C using an assay mixture containing 0.1−1.0 μM enzyme and 0.5 mM *p*-nitrophenyl acetate (PNPA). The reaction was carried out in 50 mM buffer, ionic strength maintained at 0.1 M with Na_2SO_4, using either Tris−SO_4 (pH 7.5, 8.5) or CHES (pH 9.5). The initial rate of hydrolysis was monitored at 348 nm [ϵ_{348} = 5000 M^{-1} cm^{-1} (Armstrong et al., 1966)]. Background rates were determined by measuring the initial rate of PNPA hydrolysis in the presence of 20 μM acetazolamide.

Initial rates of CO_2 hydration and HCO_3^- dehydration catalyzed by CA IV (40 nM) were determined at 25 °C using the changing pH indicator method of Khalifah (1971) and either a KinTek or an Applied Photophysics stopped-flow apparatus. The buffer concentration was 50 mM and contained 0.1 mM EDTA with the ionic strength maintained at 0.1 M by addition of Na_2SO_4. Carbon dioxide hydrase activity was measured at varied pH (6.1−9.0) and CO_2 concentration (6−24 mM). Bicarbonate dehydrase activity was measured at varied pH (5.5−7.8) and concentrations of $NaHCO_3$ (5−75 mM). The buffer/indicator pairs and pH values employed were as follows: MES/chlorophenol red (pH 5.5−6.5), MOPS/*p*-nitrophenol (pH 6.8−7.5), and TAPS/*m*-cresol purple (pH 7.8−9.0). The rate constants, k_{cat} and k_{cat}/K_M, were determined by fitting the observed initial rates to the Michaelis−Menten equation. The standard errors and pH-independent values of k_{cat} and k_{cat}/K_M for CO_2 hydration and HCO_3^- dehydration were determined by fitting the pH-dependent values to eqs 2 and 3, respectively, using Kaleidagraph (Synergy Software). Solvent hydrogen isotope effects on CO_2 hydration and HCO_3^- dehydration were measured using solutions with a final concentration of 94−98% D_2O (Cambridge Isotopes). The pD value was calculated by adding 0.4 to the pH measured using a pH electrode (Schowen & Schowen, 1982).

$$k_{obs} = \frac{k}{1 + 10^{(pK_a - pH)}} \quad (2)$$

$$k_{obs} = \frac{k}{1 + 10^{(pH - pK_a)}} \quad (3)$$

The buffer dependence of CO_2 hydration catalyzed by HCA IV was measured by separately varying the concentration of CO_2 (6−24 mM) and buffer (1−50 mM) using the buffer/indicator combinations described previously. The buffer-dependent rate constants were determined by fitting the data to the Michaelis−Menten equation.

Inhibitor Binding. Inhibition of CA IV-catalyzed bicarbonate dehydration by sulfate was measured from pH 5.5 to 7.0 using the pH-indicator assay (Khalifah, 1971). Assay solutions consisted of 50 mM buffer, 0.1 mM EDTA, 5−75 mM $NaHCO_3$, 0.010−1.0 M Na_2SO_4, and 40 nM CA IV.

Inhibition by other anions (chloride, bromide, iodide, formate, acetate, or phosphate) was measured similarly in 50 mM MES, pH 5.5, with addition of 2−100 mM sodium salt of the anion. The k_{cat}/K_M values were determined by fitting the v_{init} versus [HCO_3^-] data to the Michaelis−Menten equation at various concentrations of anion. For sulfate, iodide, formate, and acetate, K_I was determined by fitting these data to eq 4a assuming competitive inhibition. For chloride, bromide, and phosphate, K_I was determined using equation 4b where K_{Act} is the activation equilibrium constant and A is the activation factor. The pH-independent K_I for sulfate was calculated using eq 5.

$$\left(\frac{k_{cat}}{K_M}\right)_{obs} = \frac{k_{cat}/K_M}{1 + [I]/K_I} \quad (4a)$$

$$\left(\frac{k_{cat}}{K_M}\right)_{obs} = \frac{k_{cat}/K_M\{1 + A([I]/K_{Act})\}}{1 + [I]/K_{Act} + [I]/K_I + [I]^2/K_{Act}K_I} \quad (4b)$$

$$K_{I_{obs}} = K_I(1 + 10^{(pH-pK_a)}) \quad (5)$$

The dansylamide (DNSA) dissociation constant was determined by measuring the increase in fluorescence upon binding of DNSA to CA IV (λ_{ex} = 280 nm, λ_{em} = 470 nm) in 10 mM Tris−SO_4, pH 8 at 25 °C, and 20−100 nM CA IV (Chen & Kernohan, 1967). The fluorescence signal was measured 30−60 s after addition of an aliquot of DNSA to CA IV in an Aminco-Bowman Series 2 Luminescence Spectrometer. The binding constant was determined by fitting the results to a binding isotherm (eq 6) where F_{total} is

$$\text{fraction } F_{total} = \frac{F_{obs} - F_{ini}}{F_{end} - F_{ini}} = \frac{1}{1 + K_{DNSA}/[DNSA]} \quad (6)$$

the total fluorescence, F_{obs} is the observed fluorescence, F_{ini} is the initial fluorescence of protein in the absence of DNSA, and F_{end} is the end point fluorescence. The binding constants of other sulfonamide inhibitors were determined by competition with DNSA; 20 nM CA IV was incubated with 20 μM DNSA to form E·DNSA, and then the inhibitor of interest (I) was added to compete for the binding site to form E·I, decreasing the observed fluorescence. The dissociation constants were determined by fitting the results to eq 7 where F_{ini} is the initial fluorescence and I is either acetazolamide, sulfanilamide, benzenesulfonamide, or ethoxzolamide.

$$\text{fraction } F_{total} = \frac{F_{obs} - F_{end}}{F_{ini} - F_{end}} = \frac{1}{1 + (K_{DNSA}/[DNSA])(1 + [I]/K_I)} \quad (7)$$

RESULTS

CO_2 Hydration and HCO_3^- Dehydration. The pH dependence of CO_2 hydration and HCO_3^- dehydration catalyzed by recombinant CA IV was measured using the changing pH-indicator method (Khalifah, 1971) as described under Materials and Methods. For catalysis of CO_2 hydration by CA IV, the pH-independent steady-state values of $k_{cat}^{CO_2}$ and $K_M^{CO_2}$ are both slightly larger than those of CA II (Figure 1; Table 1). However, the pH-independent second-order rate constant, $k_{cat}/K_M^{CO_2}$, for CA IV is almost 2-fold less than that of CA II (Figure 1; Table 1). The differences observed for the HCO_3^- dehydrase activities of CA IV and CA II are more pronounced (Figure 2, Table 1); both the pH-independent $k_{cat}^{HCO_3^-}$ and $k_{cat}/K_M^{HCO_3^-}$ for dehydration of HCO_3^- catalyzed by CA IV are nearly 2-fold larger than those observed for CA II. These data are consistent with literature reports illustrating that CA IV is a high-activity isozyme, as determined by a bubbling CO_2 assay using membrane fractions of CA IV (Maren et al., 1993; Okuyama et al., 1992), and are the first kinetic data describing the physiologically important dehydrase activity of CA IV. These data demonstrate that bicarbonate is a better substrate for CA IV than it is for CA II.

The pH dependence of k_{cat} for CO_2 hydration and bicarbonate dehydration catalyzed by CA II mainly reflects the dependence of the activity on the ionization state of His-64 (Silverman & Lindskog, 1988; Steiner et al., 1975). This histidine serves as an intramolecular proton shuttle in the catalytic mechanism of CA II (Steiner et al., 1975; Tu et

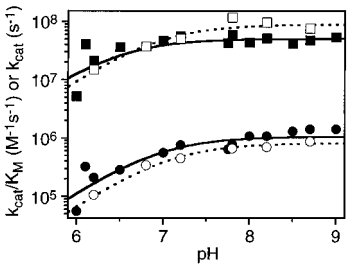

FIGURE 1: pH dependence of CO_2 hydrase activity. CO_2 hydrase activity catalyzed by either 40 nM CA II (○, □) or CA IV (●, ■) was measured using the pH-indicator assay as a function of the concentration of CO_2 (6−24 mM) in 50 mM buffer (pH 6.0−9.0), 25 °C, ionic strength = 0.1 M, with sodium sulfate as described under Materials and Methods. The steady-state kinetic parameters k_{cat}/K_M (□, ■) and k_{cat} (○, ●) were derived from fitting these data to the Michaelis−Menten equation. The pH-independent rate constants and pK_a values were determined by fitting the observed rate constants to eq 2. Results are listed in Table 1.

Table 1: pK_as and pH-Independent Steady-State Kinetic Parameters of CA II and CA IV

	HCA II	HCA IV
CO_2 hydration[a]		
k_{cat} (μs^{-1})	0.79 ± 0.08	1.1 ± 0.1
pK_a	7.0 ± 0.1	7.1 ± 0.1
$k_{cat}^{H_2O}/k_{cat}^{D_2O}$	3.9[b]	2.1 ± 0.3
K_M (mM)	8.8 ± 0.6	22 ± 2
k_{cat}/K_M (μM^{-1} s^{-1})	90 ± 6	51 ± 2
pK_a	6.9 ± 0.1	6.5 ± 0.2
k^{H_2O}/k^{D_2O}	1.0[b]	1.1 ± 0.1
HCO_3^- dehydration[c]		
k_{cat} (μs^{-1})	0.44 ± 0.05	0.72 ± 0.06
pK_a	6.8 ± 0.2	6.8 ± 0.2
$k_{cat}^{H_2O}/k_{cat}^{D_2O}$	3.8[d]	2.1 ± 0.2
K_M (mM)	37 ± 3	26 ± 4
k_{cat}/K_M (μM^{-1} s^{-1})	12 ± 0.6	28 ± 2
pK_a	6.9 ± 0.1	6.2 ± 0.1
k^{H_2O}/k^{D_2O}	1.2[d]	1.2 ± 0.1

[a] pH-independent kinetic parameters for CO_2 hydration were determined in 50 mM buffer, 25 °C, using the stopped-flow pH-indicator assay, as described in the legend of Figure 2. SHIE were measured in ≥94% D_2O at pH 9.0, as described in the legend of Figure 4. [b] Data taken from Jackman et al. (1996). [c] pH-independent kinetic parameters for HCO_3^- dehydration were determined in 50 mM buffer, 25 °C, using the stopped-flow pH-indicator assay, as described in the legend of Figure 3. SHIE were measured in ≥94% D_2O at pH 5.5, as described in the legend of Figure 4. [d] Data taken from Steiner et al. (1975).

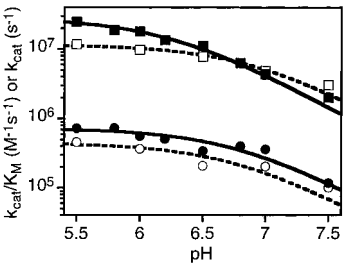

FIGURE 2: pH dependence of HCO_3^- deydrase activity. HCO_3^- dehydrase activity was measured using the pH-indicator assay as described under Materials and Methods as a function of the concentration of HCO_3^- (5−75 mM) in 50 mM buffer (pH 5.5−8.0), 25 °C, ionic strength = 0.1 M, with sodium sulfate and either 40 nM CA II (○, □) or CA IV (●, ■). The data were fit to eq 3 to obtain pH-independent values of k_{cat}/K_M (□, ■), k_{cat} (○, ●), and pK_a. Results are listed in Table 1.

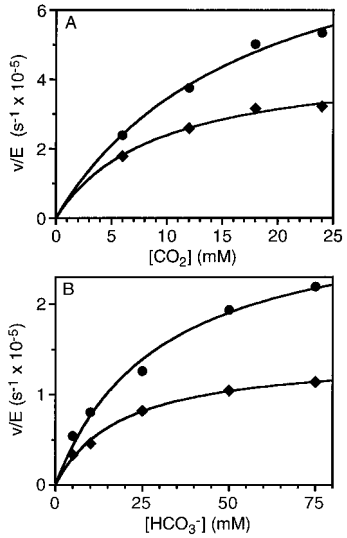

FIGURE 3: Solvent deuterium isotope effect of CO_2 hydration and HCO_3^- dehydration catalyzed by HCA IV. (A) Initial rates for CO_2 hydration catalyzed by 40 nM HCA IV were measured in 50 mM TAPS, pH 9, 25 °C, 0.1 mM EDTA, ionic strength maintained at 0.1 M with sodium sulfate, as a function of the concentration of CO_2 (6−24 mM) in either H_2O (●) or D_2O (◆) as described in the legend of Figure 1. (B) Initial rates for HCO_3^- dehydration catalyzed by 40 nM HCA IV were measured in 50 mM MES, pH 5.5, 25 °C, 0.1 mM EDTA, ionic strength maintained at 0.1 M with sodium sulfate, as a function of the concentration of HCO_3^- (5−75 mM) in either H_2O (●) or D_2O (◆) as described in the legend of Figure 2. The lines were fit to the data using the Michaelis−Menten equation. The calculated SHIE is listed in Table 1.

al., 1989) and is also conserved in CA IV (Okuyama et al., 1992). Fitting the pH dependence of k_{cat} to a single ionization reveals similar pK_as for CA II and CA IV in both CO_2 hydration and bicarbonate dehydration (Table 1), suggesting that the pK_a of His-64 in CA IV is similar to CA II. The pH dependence of k_{cat}/K_M is also consistent with the ionization of a single group (Figures 1 and 2); in CA II, this reflects mainly the dependence of the activity on the ionization state of the zinc-bound solvent molecule with a pK_a of 6.9 [Table 1 (Coleman, 1967; Lindskog, 1966)]. The observed pK_a in k_{cat}/K_M for CO_2 hydration and bicarbonate dehydration catalyzed by CA IV is decreased to 6.5 and 6.2, respectively (Figures 1 and 2, Table 1). The higher pK_a value in k_{cat}/K_M for CO_2 hydration likely reflects the difficulty of measuring CO_2 hydration at low pH since the pH dependence of anion inhibition of HCO_3^- dehydration catalyzed by CA IV (discussed later) is consistent with a pK_a of 6.2 for zinc-bound water. Therefore, the pK_a of zinc-bound water is decreased in CA IV relative to CA II.

Solvent Hydrogen Isotope Effects. A large solvent hydrogen isotope effect (SHIE) on k_{cat} at high buffer concentration indicates that an intramolecular proton transfer step is the rate-limiting step in the hydration and dehydration reactions catalyzed by CA II (Steiner et al., 1975). To ascertain whether this is also true for CA IV, we measured the hydrase activity at pD 9.0 and the dehydrase activity at pD 5.5 catalyzed by CA IV in deuterium oxide at high buffer concentrations (Figure 3) to quantify the SHIE. Little isotope effect was observed on k_{cat}/K_M (Table 1), indicating that the rate-limiting step at either low [CO_2] or low [HCO_3^-] does not involve proton transfer. However, for catalysis of CO_2 hydration and HCO_3^- dehydration, CA IV exhibited a SHIE ($k_{cat}^{H_2O}/k_{cat}^{D_2O}$) of 2.1 and 2.2, respectively, indicating that the rate-limiting step does contain proton transfer. These data suggest that intramolecular proton transfer is the rate-limiting step in k_{cat} for CA IV as well as CA II. The turnover number for CA IV is higher than CA II for both CO_2 hydration and HCO_3^- dehydration (Table 1), suggesting that the intramolecular proton transfer is faster in this isozyme. Also, assuming a common intrinsic SHIE, the decreased observed SHIE indicates that proton transfer is only partially rate-limiting in k_{cat}; therefore, these estimated increases in the intramolecular proton transfer steps in CA IV compared to CA II are lower limits.

Intermolecular Proton Transfer. At low concentrations, buffer molecules enhance the rate constant for catalysis of CO_2 hydration and HCO_3^- dehydration catalyzed by CA II (Jonsson et al., 1976; Rowlett & Silverman, 1982; Taoka et al., 1994). Intermolecular proton transfer between His-64 of CA II to a buffer molecule in solution is rate-limiting for CA II under conditions of high substrate and low buffer concentrations. For CO_2 hydration, the basic form of the buffer acts as the terminal intermolecular proton acceptor in the relay of a proton from zinc-bound water in the active site to solvent. The rate constant for intermolecular proton transfer is dependent on the pK_a of the buffer and approaches the diffusion-controlled limit observed for proton transfer between small molecules (Rowlett & Silverman, 1982). To test whether CA IV-catalyzed CO_2 hydration is similarly dependent on buffer concentration, we measured the rate constants for CO_2 hydration as a function of buffer concentration using MES (pH 6.5, 6.8), MOPS (pH 6.8, 7.8), and TAPS (pH 7.8, 9.0) buffers (Figure 4). In each buffer, the rate constant for CO_2 hydration showed a hyperbolic dependence on the concentration of the basic species of buffer in a manner similar to that observed for CA II. Additionally, at various concentrations of buffer, plots of E/v_{init} vs 1/[CO_2] yield parallel lines (Figure 4, inset), indicating that a "ping-pong" kinetic mechanism is being used where there are two separate, independent steps that do not involve the formation of a ternary complex between CA IV, substrate, and buffer (Segel, 1975). This is additional confirmation that the kinetic scheme (eq 1) derived for CA II (Silverman & Lindskog, 1988) is also valid for CA IV.

Esterase Activity. Although the primary physiological reaction catalyzed by the carbonic hydrases is the reversible hydration of carbon dioxide, CA II also catalyzes hydrolysis of small aromatic esters (Pocker & Sarkanen, 1978). To quantitate the esterase activity of CA IV, we measured

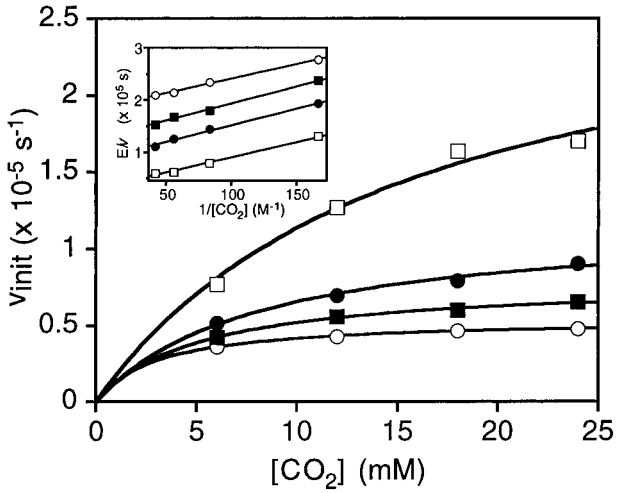

FIGURE 4: Buffer dependence of CO_2 hydration catalyzed by CA IV. CO_2 hydrase activity was measured as described in the legend of Figure 1 as a function of the concentration of CO_2 (6−24 mM) at 5 mM (○), 8 mM (■), 15 mM (●), and 50 mM (□) MES base, pH 6.5. Similar results were obtained at higher pH (pH 6.5−9.0; data not shown). Inset: A double-reciprocal plot of these data demonstrates that the slope (K_M/k_{cat}) is not dependent on the buffer concentration, indicating a "ping-pong" mechanism.

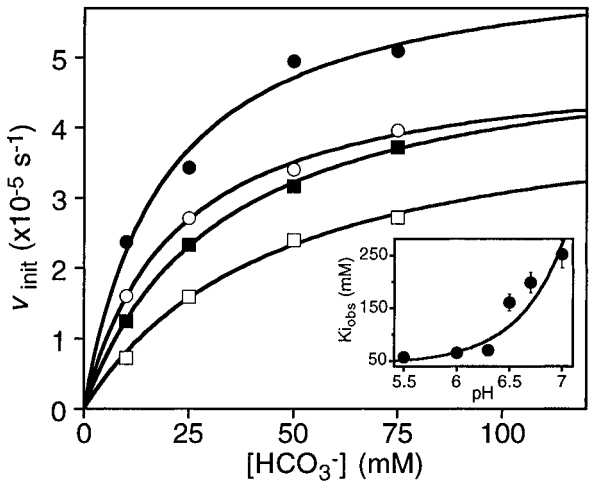

FIGURE 5: Sulfate inhibition of CA IV-catalyzed bicarbonate dehydration. Bicarbonate dehydration catalyzed by CA IV (40 nM) was measured using the pH indicator assay as a function of both the concentration of bicarbonate (5−75 mM) and sulfate [0 mM (●), 25 mM (○), 75 mM (■), or 150 mM (□)] in 50 mM MES, pH 5.5, 25 °C. The steady-state kinetic parameter $(k_{cat}/K_M)_{obs}$ was determined by fitting these data to the Michaelis−Menten equation. $K_I^{SO_4^{2-}}$ was determined using eq 4. Similar data were obtained at higher pH (pH 5.5−7; data not shown). Inset: The pH-independent K_I (44 ± 6 mM) and observed pK_a (6.2 ± 0.1) was determined from fitting the pH dependence of $(K_I^{SO_4^{2-}})_{obs}$ to eq 5.

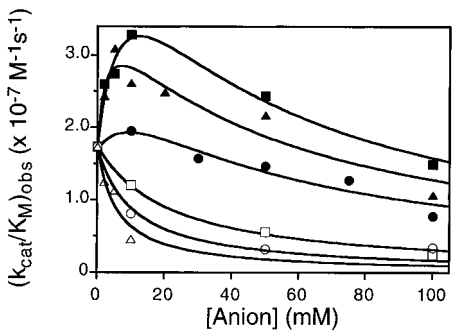

FIGURE 6: Activation and inhibition of CA IV-catalyzed bicarbonate dehydration by anions. Bicarbonate dehydrase activity was measured as a function of both bicarbonate (5−75 mM) and anion concentration (0−100 mM) as described in the legend of Figure 5. The steady-state kinetic parameter $(k_{cat}/K_M)_{obs}$ was determined at various chloride (■), bromide (▲), phosphate (●), acetate (□), iodide (○), or formate (△) concentrations by fitting these data to the Michaelis−Menten equation. K_I values for iodide, acetate, and formate were determined using eq 4a while K_I and K_{ACT} for chloride, bromide, and phosphate were determined using eq 4b. These data are listed in Table 2.

hydrolysis of *p*-nitrophenyl acetate (PNPA) catalyzed by CA IV. At high pH, k_{cat}/K_M is 16.7 M^{-1} s^{-1} (pH 9.5), 150-fold lower than the pH-independent k_{cat}/K_M of 2500 M^{-1} s^{-1} for PNPA hydrolysis catalyzed by CA II (Krebs & Fierke, 1993). Therefore, the esterase activity of CA IV is much lower than that observed for CA II and is comparable to the esterase activity of CA III (Tu et al., 1986). Furthermore, the pH dependence of the CA IV esterase activity (k_{cat}/K_M is 7.5 M^{-1} s^{-1} and 6.8 M^{-1} s^{-1} at pH 7.5 and 8.5, respectively) does not directly parallel the pH dependence of the CO_2 hydrase activity, suggesting that this reaction is not catalyzed by the active site zinc-bound hydroxide, as similarly suggested for CA III (Tu et al., 1986). Therefore, these values represent an upper limit for the active site esterase activity.

Anion Inhibition. The CO_2 hydrase activity of CA II and other isozymes, particularly CA I, is inhibited by a number of anions, uncompetitively at high pH (near 9) and noncompetitively at neutral pH (Tibell et al., 1984). Many anions inhibit CA activity by direct coordination with the catalytic zinc of carbonic anhydrase (Liljas et al., 1994), as in the case of sulfate. Sulfate binds more tightly to zinc-bound water forms of the enzyme than zinc-bound hydroxide; therefore, sulfate inhibition is competitive with bicarbonate and pH dependent with a lower K_I at lower pH (Simonsson & Lindskog, 1982). To determine if isozyme IV is similarly inhibited by sulfate, we measured the pH dependence of sulfate inhibition of CA IV-catalyzed bicarbonate dehydration (Figure 5). Our results show that the pH-independent K_I for sulfate is 44 ± 6 mM with an observed pK_a of 6.2 ± 0.1 (Figure 5, inset). This value is consistent with the ionization constant of zinc-bound water and suggests that sulfate binds to the $Zn-H_2O$ form of CA IV. These data demonstrate CA IV is inhibited by sulfate more strongly than CA II (K_I > 0.2 M; data not shown).

To determine if CA IV is inhibited by other anions as well, we measured the K_I for inhibition of bicarbonate dehydration by chloride, bromide, iodide, formate, acetate, and phosphate at pH 5.5 (Figure 6, Table 2). These pH-independent inhibition constants are significantly lower than values previously measured at neutral pH since the anions bind to the zinc−water form of the enzyme (Maren & Conroy, 1993; Segel, 1975; Zhu & Sly, 1990). In addition to inhibiting the dehydrase activity of CA IV, certain anions, including chloride, bromide, and $H_2PO_4^-$, increase $k_{cat}/K_M^{HCO_3^-}$ at concentrations less than 20 mM (Figure 6). These data can be fit using eq 4b derived for a model in which CA IV has two independent anion binding sites, one that increases k_{cat}/K_M and one that inhibits this activity. However, the constants derived from this fit (Table 2) have large errors since the maximal k_{cat}/K_M of "activated" CA IV is also unknown. Fitting the data to eq 4b (Figure 6) indicates that k_{cat}/K_M is increased 3.7-, 2.2-, and 2.9-fold, respectively, for chloride, bromide, and phosphate activation.

Sulfonamide Inhibitors. Sulfonamides effectively inhibit carbonic anhydrases by direct coordination of the anionic

Table 2: Inhibition of CA IV by Anions

anion	CA IV K_I (mM)[a]	CA IV K_{Act} (mM)[a]	CA I K_I (mM)	CA II K_I (mM)	CA III K_I (mM)
chloride	36 ± 30	9 ± 8	6[b]	200[b]	6[b]
bromide	52 ± 27	2.3 ± 2		63[c]	
iodide	11 ± 3		0.3[b]	26[b]	1.1[b]
bicarbonate	44[d]			85[d]	
formate	6 ± 2			20[c]	
acetate	22 ± 2			79[c]	
sulfate	44 ± 6[e]			≥200[e]	1[f]
phosphate	27	26			<100[g]

[a] Values of K_I and K_{Act} were determined as described in the legend of Figure 6. [b] Taken from Maren and Sanyal (1983). [c] Taken from Liljas et al. (1994). [d] Calculated from k_2/k_{-2} in Table 4. [e] pH-independent K_I was calculated as described in the legend of Figure 5. [f] Taken from Rowlett et al. (1991). [g] Taken from Paranawithana et al. (1990).

Table 3: Dissociation Constants of Sulfonamide Inhibitors[a]

sulfonamide inhibitor	dissociation constant (μM) CA II	CA IV
dansylamide	1.0 ± 0.1	1.30 ± 0.1
acetazolamide	0.011 ± 0.002	0.039 ± 0.003
ethoxzolamide	$(3.8 \pm 0.3) \times 10^{-4}$	0.025 ± 0.006
sulfanilamide	1.0 ± 0.03	4.6 ± 0.05
benzenesulfomanide	0.19 ± 0.01	6.4 ± 0.3

[a] K_D values were determined by fluorescence titration (λ_{ex} = 280 nm, λ_{em} = 470 nm) using 20 nM CA II or CA IV at 25 °C in 10 mM Tris−SO_4, pH 8.0, ionic strength = 0.1 M with sodium sulfate, as described under Materials and Methods. The values for K_{DNSA} were determined by fitting the data to eq 6 while the other dissociation constants were determined by fitting the data to eq 7 using the measured K_{DNSA}.

sulfonamido nitrogen with the catalytic zinc ion (Liljas et al., 1994). To compare the sulfonamide affinity of CA IV to CA II, we measured the dissociation constants of several known sulfonamide inhibitors (Maren, 1984). The dissociation constant for a fluorescent sulfonamide, dansylamide (DNSA), was determined by measuring the fluorescence enhancement upon binding DNSA to carbonic anhydrase (λ_{ex} = 280 nm, λ_{em} = 470 nm) (Chen & Kernohan, 1967). The dissociation constants for acetazolamide (AZA), ethoxzolamide (EZA), sulfanilamide (SNA), and benzenesulfonamide (BZA) were determined by competition with DNSA as the decrease in fluorescence due to the disappearance of E·DNSA and the concomitant formation of an E·I complex, where I is AZA, EZA, BZA, or SNA (Fierke et al., 1991). In all five cases, the data are best fit to a single dissociation constant. The affinity of CA IV for DNSA is comparable to that of CA II while CA IV binds acetazolamide, sulfanilamide, ethoxzolamide, and benzenesulfonamide 3−65-fold more weakly (Table 3). These decreases are comparable to previously determined decreases in the K_I for sulfonamide inhibition of CA IV (Conroy & Maren, 1995; Maren et al., 1993; Zhu & Sly, 1990).

DISCUSSION

The amino acid sequence of CA IV, deduced from its cDNA sequence, has a great deal of similarity with the cytosolic members of the carbonic anhydrase family; CA IV contains 43 amino acids that are common to CAs I, II, III, VI, and VII (Okuyama et al., 1992). The largest degree of sequence identity is shared with human CA II at 36%. The sequence identities with the remaining human isozymes are 31% for CA I, 30% for CA III, 33% for CA VI, and 32% for CA VII. Of the 17 highly conserved "active site" residues found in most other CAs, 16 are present in CA IV. The differing amino acid is a Pro-202→Thr substitution. In CA II, an alanine substituted for proline at position 202 retains the *cis*-configuration and destabilizes the folded state of CA II by 5 kcal/mol (Tweedy et al., 1993). Similarly, the threonine substituted for proline at position 202 in CA IV remains in the *cis*-configuration (Stams et al., 1996). However, one of the disulfide linkages in CA IV is made with Cys-203 which is directly adjacent to the *cis*-Thr peptide linkage. Consequently, the energetic cost of the Pro-202→Thr substitution is likely offset by the additional stability provided by this disulfide bond.

Crystal structures of CAs I, II, III, IV, and V have been solved, and all show a great deal of similarity in overall topology (Boriack-Sjodin et al., 1995; Eriksson & Liljas, 1993; Kannan et al., 1984; Liljas et al., 1972; Stams et al., 1996). The recent determination of the high-resolution crystal structure of CA IV has demonstrated that the zinc binding site and the hydrophobic substrate binding site are very similar to that of CA II (Stams et al., 1996). However, changes are observed in an active site loop containing a *cis*-peptide linkage between Pro-201−Thr-202, the segment between Val-131 and Asp-136, and the electropositive surface potential near the C-terminus. These differences in primary and tertiary structure likely lead to the observed functional differences between CA II and CA IV.

Catalytic Mechanism. The reaction catalyzed by CA II occurs in two separate and distinct steps [a "ping-pong" kinetic mechanism (Segel, 1975)]. The first step involves CO_2 and HCO_3^- interconversion (eq 1a), and the second involves the transfer of a proton from zinc-bound water in the active site to buffer in solution via a proton shuttle group (eq 1b). Similarly, for all of the carbonic anhydrase isozymes that have been characterized in detail (CA I, CA II, CA III, and CA V), this general catalytic mechanism has also been verified. Furthermore, all of the data presented here indicate that the kinetic scheme derived for CA II (Silverman & Lindskog, 1988) is also valid for CA IV.

On the other hand, the different isozymes have evolved divergent proton transfer pathways. The proton shuttle groups vary from His-200 in CA I (Engstrand et al., 1995; Lindskog et al., 1984) and His-64 in CA II (Steiner et al., 1975; Tu et al., 1989) to the direct transfer to bulk solvent without an intervening proton shuttle in CA III (Jewell et al., 1991). The proton shuttle group in CA V is currently proposed to be near Tyr-131 (Boriack-Sjodin et al., 1995; Heck et al., 1994, 1996). For CA IV, the similarities in the pK_a values of the proton shuttle groups of CA II and CA IV (determined from the pH dependence of k_{cat}, Table 1), in conjunction with the crystal structure of CA IV showing that His-64 is positioned to facilitate transfer of a proton from the active site to solution (Stams et al., 1996), indicate that the intramolecular proton acceptor for CA IV is His-64 as well.

Reaction Coordinate Diagram. Even though CA II and CA IV follow the same general kinetic mechanism, the data indicate that these two isozymes vary in subtle, but important, ways in individual steps. To further illustrate the differences between CA II and CA IV, we have constructed a free-energy profile comparing the two high-activity isozymes of carbonic

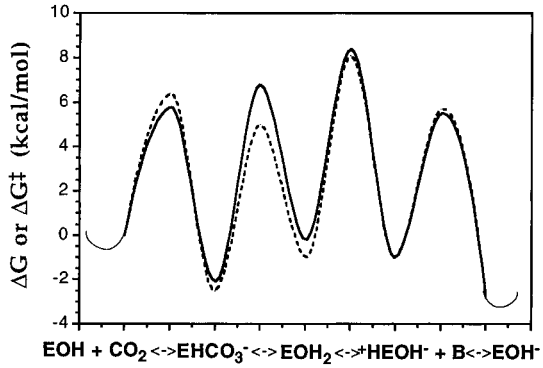

FIGURE 7: Proposed reaction coordinate diagram for CA IV at 25 °C. The reaction coordinate for CA IV is a solid line; that for CA II is a dotted line. $\Delta G^{\ddagger}$ values for CA II and CA IV were calculated from rate constants listed in Table 4 using the Arrhenius equation where A is equal to 6×10^{12} s^{-1} (Fersht, 1985). ΔG values were calculated from the equilibrium constants for each step in the reaction ($\Delta G = -RT \ln K_{eq}$). The standard state is 1 M for all reagents, and the pH is 8.2, maintained with dimethylimidazole ($pK_a = 8.2$).

Table 4: Calculated Rate Constants for CA II[a] and CA IV

rate constant	values		units
	CA II[a]	CA IV	
k_1	1.3×10^8	3.4×10^8	M^{-1} s^{-1}
k_{-1}	1.8×10^6	1.0×10^7	s^{-1}
k_2	1.7×10^7	1.8×10^6	s^{-1}
k_{-2}	2.0×10^8	4.1×10^7	M^{-1} s^{-1}
k_3	1.2×10^6	3.1×10^6	s^{-1}
k_{-3}	1.2×10^6	7.8×10^5	s^{-1}
k_4	4.0×10^8	1.0×10^8	M^{-1} s^{-1}
k_{-4}	2.0×10^7	5.0×10^6	M^{-1} s^{-1}

[a] Data taken from Behravan et al. (1990).

anhydrase (Figure 7). To construct this free-energy profile, we estimated the value of each of the eight individual rate constants in eq 8 (Table 4) from the following parameters. According to eq 8, the pH-independent steady-state parameters are described by: $k_{cat}/K_M^{CO_2} = k_1k_2/(k_{-1} + k_2)$, $k_{cat}/K_M^{HCO_3^-} = k_{-1}k_{-2}/[(k_{-1} + k_2)(1 + K_{Zn}/K_{His})]$, $k_{cat}/K_M^{Buffer} = k_4/(1 + K_{His}/K_{Zn})$, $k_{cat}^{CO_2} = k_2k_3/(k_2 + k_3)$, and $k_{cat}^{HCO_3^-} = k_{-1}k_{-3}/(k_{-1} + k_{-3})$ where K_{Zn} and K_{His} are the ionization constants of zinc-bound water and His-64, respectively. Also, the equilibrium constant for the intramolecular proton transfer, k_3/k_{-3} is equal to the ratio K_{Zn}/K_{His}. The values of the ionization constants for His-64 and zinc-bound water were taken from the pH profiles of k_{cat} and k_{cat}/K_M, respectively, for CO_2 hydration and HCO_3^- dehydration (Table 1). Furthermore, the overall equilibrium for this reaction is equal to $K_A^{H_2CO_3} = (k_{cat}/K_M^{CO_2})(K_{Zn})(k_{cat}/K_M^{HCO_3^-}) = (k_1k_2k_3k_4)/(k_{-1}k_{-2}k_{-3}k_{-4})$. Finally, assuming a similar intrinsic isotope effect, the decreased observed SHIE in the pH-independent k_{cat} for CO_2 hydration catalyzed by CA IV compared to CA II [Table 1 (Jackman et al., 1996; Steiner et al., 1975)] indicates that proton transfer is not the sole rate-contributing step. For the proposed mechanism (eq 8), the ratios of the steps that contribute to the rate-limiting step can be described by eq 9 (Schowen & Schowen, 1982) where C is the commitment factor for the forward hydration reaction, $C_f = k_3/k_2$, assuming that the isotope-sensitive step at high buffer concentration is intramolecular proton transfer (k_3, k_{-3}) with an intrinsic isotope effect of 4. Using these relationships along with the experimental data, we have estimated the values of the individual rate constants (Table 4) and used these values to construct a free-energy profile comparing isozymes II and IV (Figure 7).

$$H_2O + EZn{-}OH^- + CO_2 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}}$$

$$EZn{-}HCO_3^- + H_2O \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EZn{-}H_2O + HCO_3^-$$

$$EZn{-}H_2O \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} H{-}EZn{-}OH^- \underset{k_{-4}[BH^+]}{\overset{k_4[B]}{\rightleftharpoons}} EZn{-}OH^- \quad (8)$$

$$\left(\frac{k_{cat}^{H_2O}}{k_{cat}^{D_2O}}\right)_{obs} = \frac{C + (k_{cat}^{H_2O}/k_{cat}^{D_2O})_{intrinsic}}{C + 1} \quad (9)$$

The free-energy diagram illustrates a number of interesting differences between isozymes II and IV that cause the bicarbonate dehydrase activity of CA IV to be the highest of all the isozymes characterized to date. Although the pK_a values for ionization of the proton shuttle groups are identical, the pK_a of the zinc-bound water molecule is decreased significantly from 6.9 to 6.2. Therefore, the enzyme stabilizes the anionic zinc−hydroxide relative to zinc−water by ≈0.9 kcal/mol. In this respect, CA IV is similar to CA III, the least active isozyme of the CA family, where the pK_a of the zinc-bound solvent molecule is <5.5 (Engberg & Lindskog, 1984; Tu et al., 1986). Furthermore, the affinity of CA IV for bicarbonate is increased by ≈0.4 kcal/mol relative to CA II, closer to the values observed for CA I (Behravan et al., 1990; Kogut & Rowlett, 1987). The increased affinity of CA IV for bicarbonate causes the rate constant for bicarbonate dissociation to decrease ≈10-fold compared to CA II. Therefore, this step becomes partially rate-limiting for $k_{cat}^{CO_2}$, causing the observed decrease in the SHIE. Similarly, in CA I bicarbonate dissociation limits $k_{cat}^{CO_2}$ (Simonsson et al., 1982). These results suggest that the active site of CA IV stabilizes bound anions better than CA II, presumably a consequence of the increased positive charge on the enzyme as illustrated in the structure (Stams et al., 1996) as well as the lower pI value of CA IV (pI = 5.6−5.9) (Zhu & Sly, 1990) compared to CA II (pI = 7.3) (Funakoshi & Deutsch, 1970).

Along with stabilizing the bicarbonate complex, CA IV stabilizes the transition state for bicarbonate dehydration by >1 kcal/mol compared to CA II. Both of these factors contribute to the increased second-order rate constant for bicarbonate dehydration. The correlation between stabilization of the catalytic transition state and stabilization of zinc-bound hydroxide has been observed previously with CA variants (Kiefer & Fierke, 1994; Krebs et al., 1993) and suggests that zinc−hydroxide retains significant negative charge in the transition state. These data are also consistent with a small intrinsic ^{13}C isotope effect for bicarbonate dehydration catalyzed by CA II that suggests a very late transition state (Paneth & O'Leary, 1987).

Finally, given that $k_{cat}^{CO_2}$ is comparable in CA IV and CA II (Table 1) while bicarbonate dissociation is partially rate-limiting, the rate constant for proton transfer from zinc-bound water to His-64 is increased 2−3-fold compared to CA II (Figure 7). This increase is consistent with the Brönsted relationship describing the dependence of proton transfer on the relative pK_a of the donor and acceptor groups (Silverman

et al., 1993). The decreased proton transfer between the proton shuttle group and buffer in solution is unexpected given that the pK_a values of both the proton shuttle group and the final intramolecular proton acceptors are similar (Rowlett & Silverman, 1982); however, recent studies of mutations at Ala-65 in CA II indicate that the rate constant for intermolecular proton transfer can also be decreased by steric hindrance (Jackman et al., 1996).

Recent experiments suggest that the properties of CA IV isolated from rodent sources are altered somewhat from the kinetic properties of human CA IV, despite the 37% sequence identity with CA II (Hurt et al., 1997). In particular, the k_{cat} for CO_2 hydration catalyzed by murine CA IV is approximately 10-fold lower than human CA IV at pH 7, suggesting that the proton shuttle is partially blocked (Hurt et al., 1997). This difference in the proton transfer rate constant has been proposed to be caused by the presence of a Gln at position 63 in rat and mouse CA IV rather than the conserved Gly observed in the high-activity human, bovine, and rabbit CA IVs, as well as the CA II isozyme (Tamai et al., 1996). This proposal is confirmed by the increased activity of the Gln 63→Gly substitution in murine CA IV. Therefore, the bulkier Gln side chain either strictly occludes or causes a conformational shift of His-64 to decrease the rate constant for proton transfer (Tamai et al., 1996), similar to the results obtained for substitutions at Ala-65 in CA II (Jackman et al., 1996).

Anion Inhibition. Anions inhibit carbonic anhydrase activity to varying degrees depending on the isozyme. For example, CA I, the more abundant carbonic anhydrase found in erythrocytes, is estimated to be 85% inhibited due to the relatively high chloride concentration in erythrocytes ($K_I^{Cl} \approx 6$ mM) while CA II, the other carbonic anhydrase isozyme found in red blood cells, is not inhibited at these concentrations ($K_I^{Cl} \approx 200$ mM) and is less sensitive to inhibition by halides in general (Maren et al., 1976). In this respect, CA IV is more sensitive to inhibition by halides than CA II, with K_I values as much as 5-fold lower (Table 2; Liljas et al., 1994; Maren & Sanyal, 1983). These decreases are consistent with the increased stability of anion complexes bound to CA IV previously discussed.

Additionally, CA IV is more sensitive to inhibition by anions that are structurally similar to bicarbonate, such as formate, acetate, and sulfate, than CA II (Table 2). The crystal structure of formate complexed with CA II suggests that the binding mode of formate is similar, but not identical, to that of bicarbonate (Hakansson et al., 1992); one of the oxygen atoms of formate accepts a hydrogen bond from the amide nitrogen of Thr-199 while the other oxygen is 2.5 Å from zinc but does not displace the zinc-bound water, as observed for bicarbonate complexes. The increased ≈0.8 kcal/mol affinity of these anions is consistent with the previous conclusion that CA IV binds bicarbonate more tightly than CA II, likely due to the increased positive potential in the active site. CA I has similarly been observed to bind bicarbonate more tightly than CA II, due mainly to the substitution of a positively charge histidine (at low pH) for Thr-200 in CA II (Behravan et al., 1990, 1991).

Even though CA IV is generally inhibited by anions, we observed activation of k_{cat}/K_M for bicarbonate dehydration upon addition of low concentrations of Cl^-, Br^-, and $H_2PO_4^-$ (Figure 6). This effect is different from the activation of CA III at high substrate concentration caused by the addition of $H_2PO_4^-$, where the phosphate ion facilitates proton transfer between zinc-bound water and solvent (Rowlett et al., 1991). In CA IV, anions increase $k_{cat}/K_M^{HCO_3^-}$ which reflects mainly the rate constant for binding bicarbonate to form the $E{\cdot}HCO_3^-$ complex (Tables 1 and 4). The structure of CA IV reveals that the C-terminus of this enzyme contains a number of positively charged residues that are not compensated by hydrogen bonds or negatively charged groups on the protein surface (Stams et al., 1996), suggesting that these positive side chains facilitate the interaction of CA IV with the negatively charged phosphate groups of the phospholipid membrane. It is plausible that the positively charged residues on the surface of CA IV may affect the trajectory of bicarbonate association and decrease the association rate constant with the active site zinc. As anions are added, the positive charges might be shielded so that bicarbonate is directed toward the active site cavity. At higher concentrations, anions become inhibitory by binding to the active site (Liljas et al., 1994). This observation that phosphate (and other anions) activates CA IV toward bicarbonate dehydration suggests that the association between the negative phosphates of the phospholipid membrane and CA IV may also activate the enzyme.

Sulfonamide Inhibitors. Unlike the increased affinity of anions, the affinity of CA IV for the anionic sulfonamide inhibitors is decreased by 0.15−2.5 kcal/mol compared to CA II (Table 3) with the affinity of DNSA affected the least. The smaller effect on DNSA affinity is likely related to the differential position of the hydrophobic side chain observed in the X-ray crystal structure of the CA II·DNSA complex; the naphthyl ring of DNSA is situated in the hydrophobic pocket of CA II (Nair et al., 1996), rather than extending out into the active site cleft as observed for other sulfonamides (Jain et al., 1994; Vidgren et al., 1990, 1993).

The side chains of the amino acids in this pocket, Val-121, Val-142, Leu-198, and Trp-209, are conserved in both CA II and CA IV. However, significant sequence changes occur in the region of residue 131; Phe-131 in CA II is substituted with Val in CA IV and Gly-132 is replaced by Lys (Okuyama et al., 1992), leading to significant structural deviations in CA IV (Stams et al., 1996). We speculate that these changes may account for the decreased affinity of certain sulfonamides (Table 3). Furthermore, the large differences in the affinity of sulfonamide inhibitors with mainly hydrophobic character are consistent with the decreased esterase activity of CA IV, suggesting that phenyl compounds do not form favorable interactions with the active site of CA IV. Finally, the large disparity in the sulfonamide dissociation constants for these two isozymes indicates that the design of isozyme-specific inhibitors is possible.

Physiology. The most obvious structural difference between the two high-activity isozymes is that CA IV is localized extracellularly by a GPI-anchor in certain organs while CA II is a ubiquitous cytosolic isozyme (Brown et al., 1990; Ghandour et al., 1992; Hageman et al., 1991; Sato et al., 1990). Physiological studies have shown that CA IV is the isozyme that is primarily responsible for bicarbonate reabsorption in the proximal tubule of the kidney (Lucci et al., 1983) and may also be the isozyme in the lung that is responsible for dehydration of bicarbonate before expiration of CO_2 (Zhu & Sly, 1990). Our *in vitro* results show that

CA IV is indeed more efficient than CA II in HCO_3^- dehydration, thereby providing biochemical support for the physiological data. In addition to the increased efficiency of bicarbonate dehydration, CA IV is activated by relatively low concentrations of certain anions, including phosphate. As mentioned previously, the proposed role of the positively charged residues in the C-terminal sequence of CA IV is facilitation of association with the phospholipid anchor. Our finding that $H_2PO_4^-$ is an activator of HCO_3^- dehydration suggests that the phospholipid anchor may serve dual roles as an extracellular tether and a protein activator. However, a more detailed examination of these interactions will be required to confirm this possibility.

ACKNOWLEDGMENT

We thank Dr. David Christianson for helpful discussions.

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BI962663S