

- Pearl, L. H., & Taylor, W. R. (1987) *Nature (London)* 329, 351-354.
- Polgar, L. (1987) *FEBS Lett.* 219, 1-4.
- Polgar, L. (1989) *Mechanisms of Protease Action*, pp 157-182, CRC Press, Inc., Boca Raton, FL.
- Rich, D. H. (1985) *J. Med. Chem.* 28, 263-273.
- Rich, D. H., Green, J., Toth, M. V., Marshall, G. R., & Kent, S. B. H. (1990) *J. Med. Chem.* 33, 1285-1288.
- Richards, A. D., Phylip, L. H., Farmerie, W. G., Scarborough, P. E., Alvarez, A., Dunn, B. M., Hirel, P.-H., Konvalinka, J., Strop, P., Pavlickova, L., Kostka, V., & Kay, J. (1990) *J. Biol. Chem.* 265, 7733-7736.
- Schowen, K. B. J. (1978) in *Transition State of Biochemical Processes* (Gandour, R. D., & Schowen, R. L., Eds.) pp 225-279, Plenum Press, New York.
- Schowen, R. L. (1977) in *Isotope Effects in Enzyme-Catalyzed Reactions* (Cleland, W. W., O'Leary, M. H., & Northrop, D. B., Eds.) pp 64-99, University Park Press, Baltimore, MD.
- Suguna, K., Padlan, E. A., Smith, C. W., Carlson, W. D., & Davies, D. R. (1987) *Proc. Natl. Acad. Sci. U.S.A.* 84, 7009-7013.
- Swain, A. L., Miller, M. M., Green, J., Rich, D. H., Kent, S. B. H., & Wlodawer, A. (1990) *Proc. Natl. Acad. Sci. U.S.A.* 87, 8805-8809.
- Tang, J., Ed. (1977) *Acid Proteases, Structure, Function, and Biology*, pp 61-81, Plenum Press, New York.
- Tomaszek, T. A., Jr., Magaard, V. W., Bryan, H. G., Moore, M. L., & Meek, T. D. (1990) *Biochem. Biophys. Res. Commun.* 168, 274-280.
- Venkatasubban, K. S., & Schowen, R. L. (1985) *CRC Crit. Rev. Biochem.* 17, 1-41.
- Viola, R. E. (1984) *Arch. Biochem. Biophys.* 228, 415-424.
- Wlodawer, A., Miller, M., Jaskolski, M., Sathyanarayana, B. K., Baldwin, E., Weber, I. T., Selk, L. M., Clawson, L., Schneider, J., & Kent, S. B. H. (1989) *Science* 245, 616-621.

## Catalytic Enhancement of Human Carbonic Anhydrase III by Replacement of Phenylalanine-198 with Leucine<sup>†</sup>

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**ABSTRACT:** Carbonic anhydrase III, a cytosolic enzyme found predominantly in skeletal muscle, has a turnover rate for CO<sub>2</sub> hydration 500-fold lower and a K<sub>i</sub> for inhibition by acetazolamide 700-fold higher (at pH 7.2) than those of red cell carbonic anhydrase II. Mutants of human carbonic anhydrase III were made by replacing three residues near the active site with amino acids known to be at the corresponding positions in isozyme II (Lys-64 → His, Arg-67 → Asn, and Phe-198 → Leu). Catalytic properties were measured by stopped-flow spectrophotometry and <sup>18</sup>O exchange between CO<sub>2</sub> and water using mass spectrometry. The triple mutant of isozyme III had a turnover rate for CO<sub>2</sub> hydration 500-fold higher than wild-type carbonic anhydrase III. The binding constants, K<sub>i</sub>, for sulfonamide inhibitors of the mutants containing Leu-198 were comparable to those of carbonic anhydrase II. The mutations at residues 64, 67, and 198 were catalytically independent; the lowered energy barrier for the triple mutant was the sum of the energy changes for each of the single mutants. Moreover, the triple mutant of isozyme III catalyzed the hydrolysis of 4-nitrophenyl acetate with a specific activity and pH dependence similar to those of isozyme II. Phe-198 is thus a major contributor to the low CO<sub>2</sub> hydration activity, the weak binding of acetazolamide, and the low pK<sub>a</sub> of the zinc-bound water in carbonic anhydrase III. Intramolecular proton transfer involving His-64 was necessary for maximal turnover.

Carbonic anhydrase III is a major protein of skeletal muscle where it comprises as much as 20% of cytosolic protein (Gros & Dodgson, 1988). Carbonic anhydrase II is found in red cells and secretory tissues. These isozymes of carbonic anhydrase

are useful for investigations of catalytic mechanisms using site-directed mutagenesis because they have large differences in activity with very similar backbone structures. Isozyme III of carbonic anhydrase has a steady-state turnover number, *k*<sub>cat</sub>, for hydration of CO<sub>2</sub> that is 500-fold smaller than that of isozyme II, and the binding constant for inhibition by acetazolamide is much weaker for isozyme III compared with isozyme II (Sanyal et al., 1982; Tu et al., 1983; Kararli & Silverman, 1985; Engberg et al., 1985). Moreover, isozyme III has a pK<sub>a</sub> for the main activity-controlling group, zinc-bound water, that is lower by at least 1 pK<sub>a</sub> unit compared

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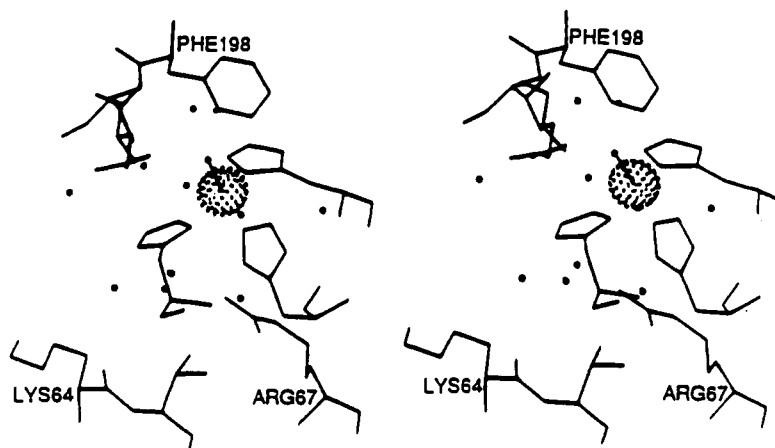


FIGURE 1: Stereodiagram of residues near the zinc (dotted sphere) in bovine carbonic anhydrase III from the crystal structure of A. E. Eriksson and A. Liljas (Eriksson, 1988). Water molecules are shown as individual dots, and the zinc-water bond is represented as a line connecting the two.

with that of isozyme II (Engberg & Lindskog, 1984; Kararli & Silverman, 1985).

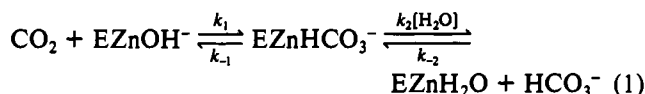
On the other hand, the backbone conformations of human carbonic anhydrase II (HCA II)<sup>1</sup> and bovine CA III are quite similar; the root mean square difference in location of main chain atoms is 0.92 Å, and is lower for many residues near the active site (Eriksson, 1988). There is a 58% amino acid identity between isozymes II and III (Tashian, 1989); among the major differences are three residues in the active-site cavity: Lys-64, Arg-67, and Phe-198 (their positions relative to the zinc are shown in Figure 1). Among the five isozymes of vertebrate carbonic anhydrase sequenced to date, these three residues are unique to isozyme III. In HCA II, these are His-64, Asn-67, and Leu-198. We have replaced these three residues in the active-site cavity of HCA III with the amino acids present at the corresponding positions of HCA II.

The replacements Lys-64 → His and Arg-67 → Asn in HCA III, as reported previously, result in mutants with modest (3-fold or less) enhancement in  $k_{\text{cat}}/K_m$  for hydration of CO<sub>2</sub> (Jewell et al., 1991). Neither of these replacements at positions 64 and 67 causes the pK<sub>a</sub> for the zinc-bound water to increase into the pH range above 6 or to enhance the very weak catalytic hydrolysis of 4-nitrophenyl acetate. Histidine at position 64, which occurs naturally in HCA II and has been placed in HCA III, is necessary for maximal turnover in the hydration of CO<sub>2</sub> by providing a pathway for proton transfer between zinc-bound water and buffers in solution (Tu et al., 1989; Jewell et al., 1991).

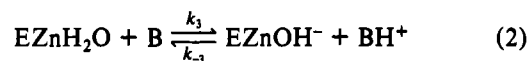
There have been no previous kinetic experiments that reflect on the specific function or role of Phe-198 in catalysis by HCA III. We have approached this problem using the site-specific mutant in which Phe-198 is replaced with Leu, the residue that appears in this position in HCA II. In addition, the double and triple mutants with replacements also at positions 64 and 67 were studied to detect interactions between these positions in catalysis. Catalysis of the hydration of CO<sub>2</sub> was measured by stopped-flow spectrophotometry and the exchange of <sup>18</sup>O between CO<sub>2</sub> and water by mass spectrometry. The replacement Phe-198 → Leu in HCA III caused major increases in kinetic constants for CO<sub>2</sub> hydration and 4-nitrophenyl acetate hydrolysis and in the tightness of binding of some

sulfonamide inhibitors. Thus, Phe-198 is a significant contributor to some of the unique properties of carbonic anhydrase III.

The catalytic pathway for the hydration of CO<sub>2</sub> is quite similar for isozymes II and III (Silverman & Lindskog, 1988). The substrate, CO<sub>2</sub>, reacts with the active site in which hydroxide is present as a ligand of the zinc. The subsequent release of product, HCO<sub>3</sub><sup>-</sup>, results in water bound to the metal:



To regenerate the form of the enzyme active in hydration, a proton-transfer step follows in a separate and rate-contributing stage of the catalysis shown in eq 2 where B is buffer in



solution or water (Silverman & Lindskog, 1988). This is an essential step in the catalysis and requires that the proton transfer occurs at least as rapidly as the maximal turnover number.

For isozyme II, there are many studies to show that His-64 acts as a proton shuttle, enhancing activity by increasing the rate described in eq 2 (Steiner et al., 1975; Tu et al., 1989). This proton transfer is believed to proceed through at least two hydrogen-bonded water molecules situated between the zinc-bound water and the imidazole of the side chain of His-64 (Venkatasubban & Silverman, 1980). Wild-type HCA III has a lysine at this position which is not a significant proton shuttle at physiological pH, but data suggest that it does shuttle protons at pH > 8 (Rowlett et al., 1991; Jewell et al., 1991). The low and pH-independent CO<sub>2</sub> hydration activity of HCA III at pH < 8 is consistent with proton transfer probably proceeding from zinc-bound water to water in the active site (Silverman & Lindskog, 1988).

#### MATERIALS AND METHODS

**Enzymes.** Bacterial expression vectors containing the human CA III coding region [derived from the cDNA clone of Lloyd et al. (1986)] were constructed by using vectors of the pET series or their derivatives (a gift from Dr. F. William Studier, Brookhaven National Laboratory). Rosenberg et al. (1987) have described this class of expression vectors. The method of Kunkel (1985) was used to insert site-specific mutants into the desired coding regions. All mutants were verified by DNA sequencing to ensure that the designated sites

<sup>1</sup> Abbreviations: HCA III, human carbonic anhydrase III; Ches, 2-(*N*-cyclohexylamino)ethanesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid; Taps, 3-[[tris(hydroxymethyl)methyl]amino]propanesulfonic acid.

were appropriately modified. The enzymes were expressed in *Escherichia coli* strain BL21(DE3)pLysS (Rosenberg et al., 1987). HCA III and its mutants were purified by exclusion and ion-exchange chromatography (Tu et al., 1986) and were greater than 98% pure, determined by polyacrylamide gel electrophoresis. The concentrations of HCA III and its mutants were determined from the molar absorptivity of  $6.2 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$  at 280 nm (Sanyal et al., 1982), identical with the value found for bovine isozyme III (Engberg et al., 1985). For the triple mutant K64H-R67N-F198L HCA III, this molar absorptivity was confirmed by titration with the tight binding inhibitor ethoxzolamide to estimate the active-site concentration using a Henderson plot (Segel, 1975).

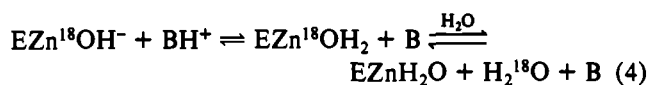
**Steady-State Kinetics.** Initial velocities for hydration of  $\text{CO}_2$  were determined by stopped-flow spectrophotometry (Applied Photophysics) using a pH-indicator method (Khalifah, 1971; Rowlett & Silverman, 1982). By bubbling  $\text{CO}_2$  into water, we prepared saturated solutions (34 mM  $\text{CO}_2$  at 25 °C; Pocker & Bjorkquist, 1977), and after dilution, final  $\text{CO}_2$  concentrations ranged from 0.1 to 28 mM. The buffer-indicator pairs (with the wavelengths observed) were the following: Mes<sup>1</sup> ( $pK_a$  6.1) and chlorophenol red ( $pK_a$  6.3, 574 nm); Mops ( $pK_a$  7.2) and *p*-nitrophenol ( $pK_a$  7.1, 400 nm); Taps ( $pK_a$  8.4) and *m*-cresol purple ( $pK_a$  8.3, 578 nm); Ches ( $pK_a$  9.3) and thymol blue ( $pK_a$  8.9, 590 nm). All steady-state experiments were carried out at 25 °C with the total ionic strength of the solution maintained at a minimum of 0.1 M using  $\text{Na}_2\text{SO}_4$ . Sulfate inhibits carbonic anhydrase II with a  $K_i$  of 9 mM at pH 5.2 with no inhibition at pH 6.5 and higher (Simonsson & Lindskog, 1982). Sodium sulfate is a partial hyperbolic inhibitor of bovine CA III, reducing catalysis to 58% of uninhibited activity with a  $K_i$  of 1.1 mM at pH 7.0 (Rowlett et al., 1991). The initial velocity of hydration was determined by least-squares analyses of a minimum of six traces of indicator absorbance vs time, each comprising less than 10% of the complete reaction. The enzyme-catalyzed rate for hydration of  $\text{CO}_2$  was determined for each buffered solution at each pH by subtracting the uncatalyzed rate measured in the absence of enzyme from the rate observed with enzyme.

Initial velocities of the hydrolysis of 4-nitrophenyl acetate were measured (Beckman DU7 spectrophotometer) by the method of Verpoorte et al. (1967) in which the increase in absorbance was followed at 348 nm, the isosbestic point of nitrophenol and the conjugate nitrophenolate ion. Measurements were made at 25 °C, and ionic strength was maintained at 0.1 M with  $\text{Na}_2\text{SO}_4$ . Solutions contained 50 mM of one of the buffers used in the  $\text{CO}_2$  measurements.

**$^{18}\text{O}$  Exchange Kinetics.** The measurement of the rates of the hydration-dehydration cycles of  $\text{CO}_2$  by the exchange of  $^{18}\text{O}$  between  $\text{CO}_2$  and water is based on a method used by Mills and Urey (1940). The application of this method to the catalysis of  $\text{CO}_2$  hydration by carbonic anhydrase has been described (Silverman et al., 1979; Silverman, 1982). We measured the exchange of  $^{18}\text{O}$  between  $\text{CO}_2$  and water by membrane-inlet mass spectrometry. We also measured the exchange of  $^{18}\text{O}$  between  $^{12}\text{C}$ - and  $^{13}\text{C}$ -labeled  $\text{CO}_2$  which occurs because the catalyzed dehydration of labeled  $\text{HCO}_3^-$  results in a transitory labeling of the active site with  $^{18}\text{O}$  which then reacts with  $^{13}\text{CO}_2$  [eq 3; see also Silverman (1982)].

$$\text{HCOO}^{18}\text{O}^- + \text{EZnH}_2\text{O} \rightleftharpoons \text{EZn}^{18}\text{OH}^- + \text{CO}_2 + \text{H}_2\text{O} \quad (3)$$

Two rates for the catalysis at chemical equilibrium were determined from the  $^{18}\text{O}$  exchange results. The first,  $R_1$ , is the rate of interconversion of  $\text{CO}_2$  and  $\text{HCO}_3^-$  at chemical equilibrium (eq 3). The second,  $R_{\text{H}_2\text{O}}$ , is the rate of release of  $^{18}\text{O}$ -labeled water from the active site (eq 4).  $R_{\text{H}_2\text{O}}$  involves



proton transfer to the zinc-bound hydroxide, forming a zinc-bound water which is readily exchangeable with solvent water (Tu & Silverman, 1985). Oxygen-18-containing water is greatly diluted by  $\text{H}_2^{16}\text{O}$ , resulting in net depletion of  $^{18}\text{O}$  from species of  $\text{CO}_2$ . In eq 4,  $\text{BH}^+$  can be buffer in solution, water in the active site, or an amino acid side chain of the enzyme such as the imidazolium of histidine-64. Previous reports describe in detail how the rate constants for  $^{18}\text{O}$  exchange are used to obtain the rates  $R_1$  and  $R_{\text{H}_2\text{O}}$  (Silverman et al., 1979; Silverman, 1982). The rate  $R_1$  measured at a total substrate concentration that is small compared with its apparent equilibrium dissociation constant at the active site provides a rate constant equivalent to  $k_{\text{cat}}/K_m$  (Simonsson et al., 1979).

Experiments were performed at 25 °C, and the total ionic strength of solution was maintained at a minimum of 0.2 M by the addition of the appropriate amount of  $\text{Na}_2\text{SO}_4$ . When the contributions of buffer and substrate to the ionic strength exceeded 0.2 M, no  $\text{Na}_2\text{SO}_4$  was added. The standard deviations in  $R_{\text{H}_2\text{O}}$  were 10–25% with the poorest precision at higher values of  $R_{\text{H}_2\text{O}}$ . The standard deviations in  $R_1$  were less than 10%.

In a report of  $^{18}\text{O}$  exchange catalyzed by bovine carbonic anhydrase III in the absence of buffers, Silverman and Tu (1986) described a biphasic depletion of  $^{18}\text{O}$  from  $\text{CO}_2$  which was attributed to positional  $^{18}\text{O}$  exchange in an intermediate complex. Consistent with other examples of positional isotope exchange (Raushel & Villafranca, 1988), this exchange was abolished at high substrate and product concentration,  $S_{\text{tot}} = [\text{CO}_2] + [\text{HCO}_3^-] = 100 \text{ mM}$ , for both bovine (Silverman & Tu, 1986) and human CA III (this work, data not shown). All  $^{18}\text{O}$  exchange reactions catalyzed by wild-type HCA III reported here were carried out with 100 mM total substrate for which  $^{18}\text{O}$  depletion from  $\text{CO}_2$  can be described by a single exponential. The mutants considered here with the replacement Phe-198 → Leu showed  $^{18}\text{O}$  exchange as a single exponential at all total substrate and product concentrations (5 mM  $\leq S_{\text{tot}} \leq 150 \text{ mM}$  at pH 7.2). Positional isotope exchange indicates partitioning among intermediates in the catalytic pathway (Raushel & Villafranca, 1988), and the absence of biphasic  $^{18}\text{O}$  exchange in mutants containing the replacement Phe-198 → Leu may reflect a larger ratio  $k_2/k_{-1}$  of eq 1 compared with wild type. Work on this topic is in progress.

## RESULTS

The steady-state parameters for the hydration of  $\text{CO}_2$  catalyzed by mutants of HCA III with replacements at positions 64, 67, and 198 were measured by stopped-flow spectrophotometry. The pH dependence of  $k_{\text{cat}}/K_m$  could be described by a single ionization with a maximum at high pH; values of this ratio measured by  $^{18}\text{O}$  exchange at low total substrate concentration were identical with values measured at steady state.<sup>2</sup> The replacement Phe-198 → Leu resulted in a large enhancement of  $k_{\text{cat}}/K_m$  for the hydration of  $\text{CO}_2$ , about 20-fold compared with wild-type HCA III (Table I). The further replacement of Arg-67 to form the double mutant R67N-F198L HCA III resulted in a 70-fold increase com-

<sup>2</sup> The ratio  $k_{\text{cat}}^{\text{exch}}/K_{\text{eff}}^{\text{CO}_2}$  described by Simonsson et al. (1979) for catalysis at chemical equilibrium is in theory equivalent to  $k_{\text{cat}}/K_m$  for hydration of  $\text{CO}_2$ . Values of the former ratio were obtained in this work by using  $^{18}\text{O}$  exchange and varying the total substrate concentration and were the same within experimental error as the values of  $k_{\text{cat}}/K_m$  obtained at initial velocity.

Table I: Maximal (pH-Independent) Steady-State Constants and Values of the Apparent  $pK_a$  for the Hydration of  $CO_2$  and Hydrolysis of 4-Nitrophenyl Acetate Catalyzed by Carbonic Anhydrase and Mutants<sup>a</sup>

enzyme	CO <sub>2</sub> hydration			4-nitrophenyl acetate hydrolysis	
	$k_{cat}$ ( $\times 10^{-4}$ s <sup>-1</sup> )	$k_{cat}/K_m$ ( $\times 10^{-6}$ M <sup>-1</sup> s <sup>-1</sup> )	$pK_a$	$k_{cat}/K_m$ ( $\times 10^{-3}$ M <sup>-1</sup> s <sup>-1</sup> )	$pK_a$
wild-type HCA III <sup>b</sup>	0.2	0.3	<6.0	0.01	
F198L HCA III	2.2 $\pm$ 0.3	7.4 $\pm$ 0.3	6.9	1.0 $\pm$ 0.1	6.4
R67N-F198L HCA III	2.1 $\pm$ 0.4	21 $\pm$ 1	6.9	2.1 $\pm$ 0.1	6.6
K64H-R67N-F198L HCA III	220 $\pm$ 25	18 $\pm$ 2	6.8	2.1 $\pm$ 0.1	6.6
wild-type HCA II <sup>c</sup>	140	150	7.0	2.7	6.9

<sup>a</sup> Steady-state constants were obtained as described in the text. Values of the apparent  $pK_a$  were determined from  $k_{cat}/K_m$  with standard errors in the  $pK_a$  of 0.2 for  $CO_2$  hydration and 0.1 for ester hydrolysis. <sup>b</sup> The value of  $k_{cat}$  for  $CO_2$  hydration omits values at pH >8 which approached  $1 \times 10^4$  s<sup>-1</sup> (Jewell et al., 1991). The very small value for ester hydrolysis may not occur at the active site for  $CO_2$  hydration (Tu et al., 1986). <sup>c</sup> Khalifah (1971) for  $CO_2$  hydration and Steiner et al. (1975) for 4-nitrophenyl acetate hydrolysis.

Table II: Inhibition Constants,  $K_i$  (Micromolar), Were Determined from  $R_i$ , the Catalyzed Rate of Interconversion of  $CO_2$  and  $HCO_3^-$  at Chemical Equilibrium<sup>a</sup>

inhibitor	$K_i$ ( $\mu$ M)			
	HCA III	F198L HCA III	R67N-F198L HCA III	HCA II
acetazolamide	40 (0.4) <sup>b</sup>	0.6 (0.08)	0.03 (0.007)	0.06 (0.02)
ethoxzolamide	8 (0.08)	0.1 (0.02)	0.004 (0.0007)	0.008 (0.003)
OCN <sup>-</sup>	30 (0.3)	50 (7)	30 (7)	30 (10)
I <sup>-</sup>	30000 (300)	30000 (5000)	30000 (6000)	30000 (9000)

<sup>a</sup> Measurements were made at pH 7.2 with conditions as described in the legend to Figure 2. No buffers were used. <sup>b</sup> Values in parentheses are the estimated, pH-independent values for  $K_i$  describing the binding of inhibitors to the zinc-bound water form of the enzymes. These values were calculated as explained in the text using the values of  $pK_a$  determined from the esterase activities (Table I). For HCA III, these values were calculated by assuming a  $pK_a$  of 5.2 for the zinc-bound water.

pared with the value of  $3 \times 10^5$  M<sup>-1</sup> s<sup>-1</sup> for wild-type HCA III. There was no further enhancement in this parameter caused by the replacement Lys-64  $\rightarrow$  His to form the triple mutant (Table I). Maximal values of  $k_{cat}/K_m$  for these and two additional mutants are given in Figure 4. Each of the mutants containing Leu-198 showed changes in  $k_{cat}/K_m$  consistent with an ionization of  $pK_a$  near 7, assumed to be that of the zinc-bound water with similarities to HCA II (Table I; Khalifah, 1971).

The presence of an ionization affecting catalysis was confirmed by the measurement of catalytic hydrolysis of 4-nitrophenyl acetate. The resulting pH-rate profiles can be described as dependent on a single ionization with values of  $pK_a$  between 6.4 and 6.9 (Table I). The catalytic rate of this hydrolysis by the wild-type bovine isozyme III is very small (the maximal value of  $k_{cat}/K_m$  is  $11$  M<sup>-1</sup> s<sup>-1</sup>; Tu et al., 1986). By this measure, the double and triple mutants of HCA III listed in Table I have activity nearly equivalent to that of isozyme II.

The steady-state turnover number  $k_{cat}$  for hydration catalyzed by the single mutant F198L (Table I) did not vary in the pH range 6.5–8.5 and was approximately 10-fold higher than for the wild-type HCA III which is  $2 \times 10^3$  s<sup>-1</sup> in this pH range (Jewell et al., 1991). The double mutant R67N-F198L HCA III had values of  $k_{cat}$  similar to the single mutant, except at pH >8.5 for which  $k_{cat}$  increased with pH. This effect may be caused by proton transfer involving Lys-64. A similar effect was observed in the single mutant R67N HCA III (Jewell et al., 1991). There was further enhancement observed for the triple mutant for which  $k_{cat}$  attained values comparable to those for HCA II of  $1.4 \times 10^6$  s<sup>-1</sup> (Khalifah, 1971).

The proton-transfer-dependent rate of release of water from the active site,  $R_{H_2O}/[E]$ , was measured in the absence of buffers other than the substrate itself,  $CO_2$  and its hydrated forms.  $R_{H_2O}/[E]$  varied with pH for the triple mutant (Figure 2) in a manner similar to HCA II (Tu & Silverman, 1985). The maximal value of  $R_{H_2O}/[E]$  for the triple mutant,  $9 \times 10^4$  s<sup>-1</sup>, can be compared to the maximal value, near  $5 \times 10^5$  s<sup>-1</sup>,

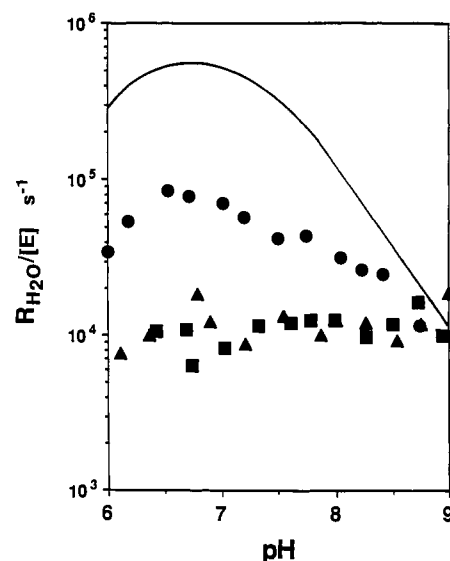


FIGURE 2: pH dependence of  $R_{H_2O}/[E]$  for (▲) F198L HCA III, (■) R67N-F198L HCA III, and (●) K64H-R67N-F198L HCA III. The values for HCA II are represented by the solid line [see Tu and Silverman (1985)]. Solutions contained 100 mM total concentration of  $CO_2$  and  $HCO_3^-$ , and no buffers were used. Experiments were carried out at 25 °C with the total ionic strength of the solution maintained at 0.2 M with  $Na_2SO_4$ .

for HCA II. The values of  $R_{H_2O}/[E]$  for F198L and R67N-F198L HCA III are similar, near  $10^4$  s<sup>-1</sup>, and rather independent of pH (Figure 2). For wild-type HCA III, the values of  $R_{H_2O}/[E]$  are independent of pH at  $2 \times 10^3$  s<sup>-1</sup>. Maximal values of  $R_{H_2O}/[E]$  for additional mutants are given in Figure 5.

Upon addition of the buffer imidazole, there was considerable enhancement of  $R_{H_2O}/[E]$  for the single and double mutants (Figure 3). Enhancement of  $R_{H_2O}$  by addition of imidazole is a characteristic of HCA III and the K64H and R67N single mutants (Tu et al., 1990) as well as HCA II and some mutants (Tu et al., 1989). This has been interpreted as evidence for proton transfer from the imidazolium cation to

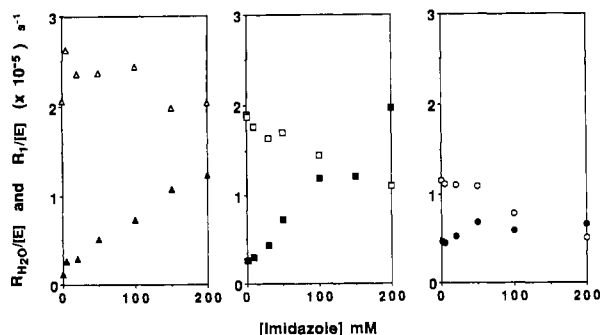


FIGURE 3: Dependence on the imidazole concentration of (filled symbols)  $R_{H_2O}/[E]$  and (open symbols)  $R_I/[E]$  catalyzed by (left) F198L HCA III, (middle) R67N-F198L HCA III, and (right) K64H-R67N-F198L HCA III. The pH was 7.2 at 25 °C with the total concentration of all species of  $CO_2$  at 100 mM. The total ionic strength of solution was maintained at 0.2 M by the addition of  $Na_2SO_4$ .

Table III: Solvent Hydrogen Isotope Effects on  $R_I$  and  $R_{H_2O}$  Catalyzed by HCA III, HCA II, and Two Mutants<sup>a</sup>

	HCA III	F198L HCA III	K64H- R67N- F198L HCA III	HCA II
$(R_I)_{H_2O}/(R_I)_{D_2O}$	$1.03 \pm 0.05^b$	$0.96 \pm 0.08$	$1.05 \pm 0.07$	$1.05 \pm 0.03^c$
$R_{H_2O}/R_{D_2O}$	$2.4 \pm 0.4^b$	$2.7 \pm 0.7$	$4.5 \pm 0.5$	$8.0 \pm 0.7^c$

<sup>a</sup> Data were obtained at an uncorrected pH meter reading of 7; other conditions were as described in the legend to Figure 2. <sup>b</sup> Kararli and Silverman (1985). <sup>c</sup> Tu and Silverman (1982); this measurement was at an uncorrected pH meter reading of 6.6.

the zinc hydroxide, increasing the rate of release of  $H_2^{18}O$  as shown in eq 4. For three mutants of isozyme III containing the replacement Phe-198 → Leu, the rate  $R_I$  of the interconversion of  $CO_2$  and  $HCO_3^-$  at chemical equilibrium appears to be slightly inhibited by the addition of imidazole, as determined from the negative slopes of the plots in Figure 3.

The inhibition of two mutants and wild-type carbonic anhydrases II and III by two anions and the sulfonamides acetazolamide and ethoxzolamide is compared in Table II. The reported values of  $K_I$  were obtained from  $^{18}O$  exchange experiments and are the concentrations of inhibitor required to reduce  $R_I$  to 50% of its uninhibited value under the condition that the total substrate concentration ( $[CO_2] + [HCO_3^-] = 25$  mM) is much less than the apparent binding constant for substrate  $K_{eff}$ . At the pH of these experiments, 7.2,  $K_{eff}$  is about 200 mM for HCA II (Simonsson et al., 1979) and greater than 200 mM for HCA III and the two mutants in Table I as determined by substrate dependence studies (data not shown). The values of  $K_I$  determined from  $R_{H_2O}$  in the same manner agreed within experimental uncertainty ( $\pm 25\%$ ) with the values determined from  $R_I$ . For the tightly bound inhibitors ethoxzolamide and acetazolamide, we accounted for the mutual depletion of free enzyme and inhibitor (Segel, 1975).

Solvent hydrogen isotope effects (SHIE) observed for  $R_I/[E]$  are unity for isozymes II and III and two mutants of HCA III (Table III). This is consistent with a wide body of data indicating no rate-contributing proton transfer in the steps of the interconversion of  $CO_2$  and  $HCO_3^-$  shown in eq 1 (Silverman & Lindskog, 1988). The SHIE for  $R_{H_2O}/[E]$  are greater than 2.0 for each case in Table III, consistent with proton transfer as a rate-contributing step in the pathway for the release of water from the active site (eq 4). For comparison,  $k_{cat}$  for hydration of  $CO_2$  has SHIE of 3.8 and 2.5

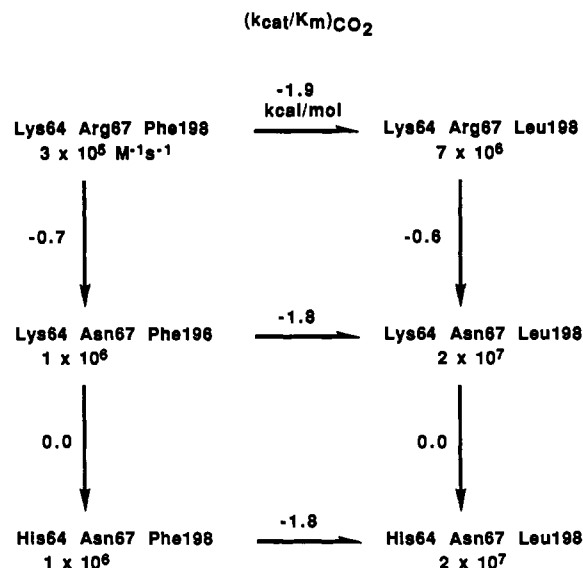


FIGURE 4: Comparisons of  $k_{cat}/K_m$  for hydration of  $CO_2$  catalyzed by variants of HCA III obtained by site-directed mutagenesis at positions 64, 67, and 198 in the active-site cavity of HCA III. Values of  $k_{cat}/K_m$  in  $M^{-1} s^{-1}$  appear beneath each designated mutant. The values in kilocalories per mole adjacent to the arrows are the changes in free energy barriers for the catalysis corresponding to the designated mutations. Free energy changes are determined by using  $\Delta G = -RT \ln [(k_{cat}/K_m)_{mut2}/(k_{cat}/K_m)_{mut1}]$ .

for HCA II and III, respectively (Steiner et al., 1975; Kararli & Silverman, 1985).

## DISCUSSION

The most significant structural differences near the active sites of isozymes II and III of human carbonic anhydrase are at positions 64, 67, and 198. We have prepared mutants of isozyme III in which each of these residues is replaced by the amino acid present at the corresponding position of isozyme II. The aim of this work is to determine the catalytic role of these residues and the source of the large differences in catalytic and inhibition properties between HCA II and III.

**Interconversion of  $CO_2$  and  $HCO_3^-$ .** The replacements Arg-67 → Asn and Phe-198 → Leu affected  $k_{cat}/K_m$  independently; the lowered energy barrier for the double mutant was the algebraic sum of the energy changes for each of the single mutants. This is demonstrated in Figure 4 in which the change in energy barriers for  $k_{cat}/K_m$  of the catalysis is given by the value on the arrows, in the manner described by Carter et al. (1984). The replacement Lys-64 → His had no effect on  $k_{cat}/K_m$  [except at pH > 8; see Jewell et al. (1991)]. These results can be understood by the rather widely separated positions of these residues in the active-site cavity of isozyme III (Figure 1). Phe-198 is on the hydrophobic side of the active-site cavity, and Lys-64 and Arg-67 are on the hydrophilic side. Moreover, these two positively charged residues are separated by 8.8 Å, the distance between the side-chain N-ζ of Lys-64 and the C-ζ of Arg-67 in the crystal structure of bovine CA III (Eriksson 1988). The distance between these positions and the zinc is 12.6 and 9.2 Å, respectively.

We have also observed a pH dependence in  $k_{cat}/K_m$  for hydration of  $CO_2$  in the HCA III mutants containing the Phe-198 → Leu replacement. This is in contrast to the values of  $k_{cat}/K_m$  for wild-type CA III which are independent of pH in the range of pH 6–8 (Tu et al., 1983; Engberg et al., 1985; Kararli & Silverman, 1985). The lack of a pH dependence is attributed to the low  $pK_a$  of the zinc-bound water. The visible absorption spectrum of Co(II)-substituted bovine CA III (Engberg & Lindskog, 1984) provides additional evidence

that for wild-type HCA III the  $pK_a$  of the zinc-bound water is less than 6.0. The pH dependence of  $k_{cat}/K_m$  in the Leu-198-containing mutants suggests that the  $pK_a$  of the zinc-bound water is above 6 and closer to the value of  $pK_a$  7 for HCA II.

The pH dependence of catalysis for the Leu-198-containing mutants was supported by observation of the hydrolysis of 4-nitrophenyl acetate in which the effect of an ionization with a  $pK_a$  near 6.5 is apparent. There is very strong evidence that the  $pK_a$  obtained from ester hydrolysis reflects the ionization of the catalytically important metal-bound water (Simonsson & Lindskog, 1982; Pocker & Sarkanen, 1978). We conclude that the Phe-198  $\rightarrow$  Leu replacement has increased the  $pK_a$  of the zinc-bound water from a value below 6.0 in wild-type HCA III (the exact value has not been determined because the enzyme denatures before the ionization is reached) to a value near 6.5 for the Leu-198-containing mutants. The values of  $pK_a$  from the esterase data are a more precise estimate than those obtained from  $k_{cat}/K_m$  for  $CO_2$  hydration.

The increases in  $k_{cat}/K_m$  for the mutants of HCA III containing Leu-198 (Table I and Figure 4) can be attributed to the steps in eq 1. The ratio  $k_{cat}/K_m$  for hydration of  $CO_2$  contains rate constants in the pathway up to and including the release of  $HCO_3^-$  from the enzyme; in terms of the simplified pathway of eq 1,  $k_{cat}/K_m = k_1 k_2 / (k_{-1} + k_2)$ . This ratio is unaffected by the intra- and intermolecular proton-transfer steps described in eq 2 [see, for example, Jonsson et al. (1976) and Tu et al. (1990)]. Unlike HCA II in which  $k_{cat}/K_m$  is dominated by  $k_1$  (Lindskog, 1984), isozyme III probably has significant contributions to  $k_{cat}/K_m$  of all three constants,  $k_1$ ,  $k_{-1}$ , and  $k_2$  (Rowlett et al., 1991). Thus, the Phe-198  $\rightarrow$  Leu replacement could enhance  $k_{cat}/K_m$  at one or more of three steps:  $CO_2$  binding, the interconversion on the enzyme, and the dissociation rate of product  $HCO_3^-$ . To emphasize the complexity in explaining these changes, we note that the mutant of the efficient carbonic anhydrase II made by the replacement Leu-198  $\rightarrow$  Phe (that is, L198F HCA II) has a value of  $k_{cat}/K_m$  that is about as great as that observed for wild-type HCA II.<sup>3</sup>

The increased value of the apparent  $pK_a$  of the catalysis upon the replacement Phe-198  $\rightarrow$  Leu is not due significantly to changes involving the two positively charged side chains of Lys-64 and Arg-67 which have minor effects on this  $pK_a$  (Table I). It is interesting that this increase is caused by the replacement of a hydrophobic residue by another hydrophobic residue. This increase in  $pK_a$  is very difficult to explain since it could arise, for example, from rather small conformational changes that affect the alignment or distance of the hydrogen bonds to zinc-bound water and that stabilize the zinc-bound water over zinc-bound hydroxide [see, for example, Scheiner and Hillenbrand (1985)]. It is known from the structures of isozymes II and III that the zinc-bound hydroxide (and zinc-bound water in the low-pH form) is hydrogen-bonded with the side chain of Thr-199 and with two water molecules in the active site, forming part of a partially ordered network of at least nine hydrogen-bonded waters (Eriksson et al., 1988). One of these waters forms a hydrogen bond with the  $\pi$ -electron cloud of the phenyl ring of Phe-198 in HCA III (Eriksson, 1988), suggesting that the Phe-198  $\rightarrow$  Leu mutation may affect this water structure.

**Proton Transfer.** The replacement Phe-198  $\rightarrow$  Leu caused up to 10-fold increases in the  $k_{cat}$  for hydration (Table I) and  $R_{H_2O}$ , the proton-transfer-dependent rate of release of water from the active site (Figure 2), without introducing a residue

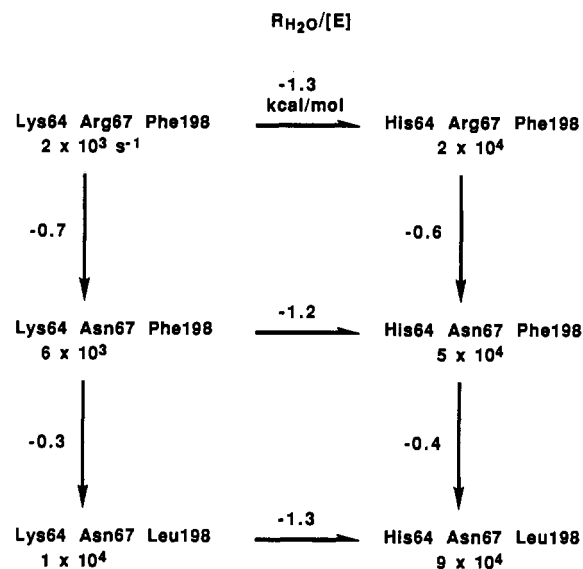


FIGURE 5: Comparisons of the water off rates  $R_{H_2O}/[E]$  (maximal values under the conditions of Figure 2) for variants of HCA III at positions 64, 67, and 198 in the active-site cavity of HCA III. Values of  $R_{H_2O}/[E]$  in  $s^{-1}$  appear beneath each designated mutant, and the values in kilocalories per mole adjacent to the arrows are the free energy changes for the barriers in catalysis for the corresponding variants.

capable of proton transfer. For the single mutant F198L and the double mutant R67N-F198L HCA III, the values of  $k_{cat}$  are the same and generally independent of pH at  $2 \times 10^4 s^{-1}$ , except at pH  $> 8.5$  for the double mutant which may be due to the ionization of Lys-64. The values of  $R_{H_2O}/[E]$  are also the same for this single and double mutant at about  $1 \times 10^4 s^{-1}$ , again independent of pH. These properties of  $k_{cat}$  and  $R_{H_2O}/[E]$  are similar to those observed for H64A HCA II, a mutant of the efficient isozyme in which the internal proton-transfer group of His-64 is replaced with alanine, a side chain which cannot transfer protons. For H64A HCA II,  $k_{cat}$  is near  $1 \times 10^4 s^{-1}$  (pH 7.2 with a noninteracting buffer such as Mops; Tu et al., 1989), and  $R_{H_2O}/[E]$  is independent of pH near  $1 \times 10^4 s^{-1}$ . The solvent hydrogen isotope effects of Table III are consistent with a rate-contributing proton transfer in  $R_{H_2O}$  catalyzed by two mutants of HCA III containing Leu-198. A reasonable hypothesis is proton transfer between the zinc-bound water and water in the active site.

Both  $k_{cat}$  and  $R_{H_2O}/[E]$  were further increased for the triple mutant;  $k_{cat}$  increased to a value near  $1 \times 10^6 s^{-1}$  and  $R_{H_2O}/[E]$  to  $1 \times 10^5 s^{-1}$  (Table I, Figure 2). This triple mutant contains His-64 which increases these parameters because of the introduction of an internal proton shuttle, the imidazole of the side chain, to facilitate proton transfer between zinc-bound water and solution. This is supported not only by the increased values of  $k_{cat}$  and  $R_{H_2O}/[E]$  in Table I and Figure 2 but also roughly by the pH dependence of these parameters (Steiner et al., 1975; Tu et al., 1989; Jewell et al., 1991). The solvent hydrogen isotope effect in  $R_{H_2O}$  for the triple mutant (Table III) indicates a considerable contribution of proton transfer to the overall rate of  $R_{H_2O}$ .

We give in Figure 5 the changes in energy barriers for  $R_{H_2O}/[E]$  upon mutations in HCA III. Each of the enzymes in the first column contains Lys-64, and each in the second column contains His-64; therefore, the energy changes associated with the horizontal arrows of Figure 5 pertain to the introduction of the internal proton shuttle. This decrease in the energy barrier of 1.3 kcal/mol is independent of the replacements we made at positions 67 and 198. The intramo-

<sup>3</sup> Sven Lindskog, personal communication.

molecular proton transfer between His-64 and the zinc-bound hydroxide is believed to occur through intervening hydrogen-bonded water (Venkatasubban & Silverman, 1980), and our results suggest that the replacements Arg-67 → Asn and Phe-198 → Leu do not influence this process. The measurements of  $R_{H_2O}$  were made in the absence of added buffers so that the additional pathway for proton transfer involving buffer in solution (Tu et al., 1989) is avoided in these measurements.

Both  $k_{cat}$  and  $R_{H_2O}$  for isozyme III are believed to be dominated by proton transfer to water (Rowlett et al., 1991; Kararli & Silverman, 1985). Proton-transfer energetics involving water are sensitive to changes in the distance of the hydrogen bond and deformations of the O—H...O angle. For example, increasing the O...O distance by 0.1 Å can increase the energy barrier for the proton transfer  $(H_2O-H-H_2O)^+$  by as much as 5 kcal/mol (Hillenbrand & Scheiner, 1986). One other consideration is the frequent observation that the energy barrier for proton transfer is directly related to the difference in  $pK_a$  of the proton donor and acceptor groups. On this basis alone, we would predict that the mutants containing Leu-198 would have  $k_{cat}$  for hydration less than for wild type because of the greater proton affinity of the zinc-bound water in the mutants ( $pK_a \approx 6.5$ ) compared with the wild type ( $pK_a < 6.0$ ). However, the opposite was observed (Table I). Consequently, changes in the structure of hydrogen-bonded water caused by the different side chains in the mutants may be more important than the initial and final energy levels in explaining the proton-transfer rate between zinc-bound water and water in the active site.

Another effect of the Phe-198 → Leu replacement in HCA III is to enhance the apparent second-order rate constant  $k_{H_2O}^B/K_{eff}^B$  [see Tu et al. (1990)] for the transfer of protons from imidazole in solution to the enzyme. For F198L HCA III, this constant is  $5 \times 10^5 M^{-1} s^{-1}$ , obtained from Figure 3, and for the double mutant R67N-F198L HCA III, it is  $9 \times 10^5 M^{-1} s^{-1}$ . The constant  $k_{H_2O}^B/K_{eff}^B$  for the triple mutant is difficult to estimate because of experimental scatter, but is quite low, presumably because this enzyme has the internal proton-transfer group of His-64. The value of  $k_{H_2O}^B/K_{eff}^B$  for wild-type HCA III is  $1 \times 10^5 M^{-1} s^{-1}$  (Tu et al., 1990). The increase in  $k_{H_2O}^B/K_{eff}^B$  caused by the replacement Phe-198 → Leu could be due to enhanced accessibility of the small buffer imidazole to the active site caused by the removal of the steric hindrance of Phe-198 or to some more subtle structural change. It is possible that proton transfer between imidazole and zinc-bound water occurs through the intervening water structure just as proposed for His-64 (Silverman & Lindskog, 1988).

**Inhibition.** Table II presents the apparent inhibition constants  $K_i$  at pH 7.2 for two sulfonamides and two anions determined by inhibition of  $^{18}O$  exchange catalyzed by HCA II, HCA III, and two mutants. It is known that anions such as  $SCN^-$  and halides bind directly to the metal in carbonic anhydrase, displacing water or forming a fifth ligand (Lindskog, 1982; Eriksson et al., 1988). The binding of anions to the zinc hydroxide form of isozyme II is very weak compared with that of the zinc-bound water form (approximately 100-fold weaker; Tibell et al., 1984). This has not been shown specifically for isozyme III but is almost certainly the case. The pH dependence of the binding of acetazolamide indicates that a similar situation pertains (Lindskog & Thorslund, 1968).

We can convert the apparent values of  $K_i$  in Table II to pH-independent binding constants for the zinc-bound water form of the enzymes by dividing each value of the apparent

$K_i$  by  $(1 + K_a/[H^+])$  using the values of  $pK_a$  determined from the hydrolysis of 4-nitrophenyl acetate (Table I). These estimates of pH-independent  $K_i$  are given in parentheses in Table II. The replacement Phe-198 → Leu caused a considerable increase in the tightness of binding for both acetazolamide and ethoxzolamide, resulting in values of  $K_i$  closer to that for HCA II. Eriksson et al. (1988) have shown that the thiadiazole ring of acetazolamide bound to HCA II is in van der Waals' contact with the side chain of Leu-198. It is most likely an unfavorable steric interaction between the thiadiazole ring and Phe-198 that contributes to the weak binding of acetazolamide to HCA III. On the other hand, the Phe-198 → Leu replacement in HCA III caused a weaker binding of  $OCN^-$  and  $I^-$ , consistent with the known weaker binding of anions to HCA II compared with HCA III (Table II; Sanyal et al., 1982).

**Conclusion.** It was previously suggested (Kararli & Silverman, 1985) that Lys-64 and Arg-67 were responsible for the unique catalytic and inhibition properties of carbonic anhydrase III, specifically its low activity in  $CO_2$  hydration and ester hydrolysis, low  $pK_a$  for zinc-bound water, and weak binding of sulfonamides. The present work complements Jewell et al. (1991) in showing that Lys-64 and Arg-67 have relatively minor roles in determining these properties but that Phe-198 can be identified as a major contributor to these unique features of carbonic anhydrase III.

#### ACKNOWLEDGMENTS

The guidance and many helpful discussions of Dr. Susan M. Tanhauser are gratefully acknowledged. We thank Professor Anders Liljas and Dr. Elisabeth Eriksson for making available details of the structure of bovine carbonic anhydrase III.

**Registry No.** HCA III, 9001-03-0; Phe, 63-91-2; Leu, 61-90-5; Arg, 74-79-3; Lys, 56-87-1; His, 71-00-1; Asn, 70-47-3;  $CO_2$ , 124-38-9;  $OCN^-$ , 661-20-1;  $I^-$ , 20461-54-5; acetazolamide, 59-66-5; ethoxazolamide, 452-35-7; 4-nitrophenyl acetate, 830-03-5.

#### REFERENCES

- Carter, P. J., Winter, G., Wilkinson, A. J., & Fersht, A. R. (1984) *Cell* 38, 835-840.
- Engberg, P., & Lindskog, S. (1984) *FEBS Lett.* 170, 326-330.
- Engberg, P., Millqvist, E., Pohl, G., & Lindskog, S. (1985) *Arch. Biochem. Biophys.* 241, 628-638.
- Eriksson, A. E. (1988) Doctoral Dissertation, Uppsala University.
- Eriksson, A. E., Kylsten, P. M., Jones, T. A., & Liljas, A. (1988) *Proteins: Struct., Funct., Genet.* 4, 283-293.
- Gros, G., & Dodgson, S. J. (1988) *Annu. Rev. Physiol.* 50, 669-694.
- Hillenbrand, E. A., & Scheiner, S. (1986) *J. Am. Chem. Soc.* 108, 7178-7186.
- Jewell, D. A., Tu, C. K., Paranawithana, S. R., Tanhauser, S. M., LoGrasso, P. V., Laipis, P. J., & Silverman, D. N. (1991) *Biochemistry* 30, 1484-1490.
- Jonsson, B.-H., Steiner, H., & Lindskog, S. (1976) *FEBS Lett.* 64, 310-314.
- Kararli, T., & Silverman, D. N. (1985) *J. Biol. Chem.* 260, 3484-3489.
- Khalifah, R. G. (1971) *J. Biol. Chem.* 246, 2561-2573.
- Kunkel, T. (1985) *Proc. Natl. Acad. Sci. U.S.A.* 82, 488-492.
- Lindskog, S. (1982) *Adv. Inorg. Biochem.* 4, 115-170.
- Lindskog, S. (1984) *J. Mol. Catal.* 23, 357-368.
- Lindskog, S., & Thorslund, A. (1968) *Eur. J. Biochem.* 3, 453-460.
- Lloyd, J., McMillan, S., Hopkinson, D., & Edwards, Y. H. (1986) *Gene* 41, 233-239.



- Mills, G. A., & Urey, H. C. (1940) *J. Am. Chem. Soc.* 62, 1019-1026.
- Pocker, Y., & Bjorkquist, D. W. (1977) *Biochemistry* 26, 5698-5707.
- Pocker, Y., & Sarkanen, S. (1978) *Adv. Enzymol. Relat. Areas Mol. Biol.* 47, 149-274.
- Raushel, F. M., & Villafranca, J. J. (1988) *CRC Crit. Rev. Biochem.* 23, 1-26.
- Rosenberg, A. H., Lade, B. N., Chui, D.-S., Lin, S.-W., Dunn, J. J., & Studier, F. W. (1987) *Gene* 56, 125-135.
- Rowlett, R. S., & Silverman, D. N. (1982) *J. Am. Chem. Soc.* 104, 6737-6741.
- Rowlett, R. S., Gargiulo, N. J., Santoli, F. A., Jackson, J. M., & Corbett, A. H. (1991) *J. Biol. Chem.* 266, 933-941.
- Sanyal, G., Swenson, E. R., Pessah, N. I., & Maren, T. H. (1982) *Mol. Pharmacol.* 22, 211-220.
- Scheiner, S., & Hillenbrand, E. A. (1985) *Proc. Natl. Acad. Sci. U.S.A.* 82, 2741-2745.
- Segel, I. H. (1975) *Enzyme Kinetics*, pp 150-159, John Wiley & Sons, New York.
- Silverman, D. N. (1982) *Methods Enzymol.* 87, 732-752.
- Silverman, D. N., & Tu, C. K. (1986) *Biochemistry* 25, 8402-8408.
- Silverman, D. N., & Lindskog S. (1988) *Acc. Chem. Res.* 21, 30-36.
- Silverman, D. N., Tu, C. K., Lindskog, S., & Wynns, G. C. (1979) *J. Am. Chem. Soc.* 101, 6734-6740.
- Simonsson, I., & Lindskog, S. (1982) *Eur. J. Biochem.* 123, 29-36.
- Simonsson, I., Jonsson, B.-H., & Lindskog, S. (1979) *Eur. J. Biochem.* 93, 409-417.
- Steiner, H., Jonsson, B.-H., & Lindskog, S. (1975) *Eur. J. Biochem.* 59, 253-259.
- Tashian, R. E. (1989) *BioEssays* 10, 186-192.
- Tibell, L., Forsman, C., Simonsson, I., & Lindskog, S. (1984) *Biochim. Biophys. Acta* 789, 302-310.
- Tu, C. K., & Silverman, D. N. (1985) *Biochemistry* 24, 5881-5887.
- Tu, C. K., & Silverman, D. N. (1982) *Biochemistry* 21, 6353-6360.
- Tu, C. K., Sanyal, G., Wynns, G. C., & Silverman, D. N. (1983) *J. Biol. Chem.* 258, 8867-8871.
- Tu, C. K., Thomas, H. G., Wynns, G. C., & Silverman, D. N. (1986) *J. Biol. Chem.* 261, 10100-10103.
- Tu, C. K., & Silverman, D. N., Forsman, C., Jonsson, B. H., & Lindskog, S. (1989) *Biochemistry* 28, 7913-7918.
- Tu, C. K., Paranawithana, S. R., Jewell, D. A., Tanhauser, S. M., LoGrasso, P. V., Wynns, G. C., Laipis, P. J., & Silverman, D. N. (1990) *Biochemistry* 29, 6400-6405.
- Venkatasubban, K. S., & Silverman, D. N. (1980) *Biochemistry* 19, 4984-4989.
- Verpoorte, J. A., Mehta, S., & Edsall, J. T. (1967) *J. Biol. Chem.* 242, 4221-4229.

## Stepwise Improvements in Catalytic Effectiveness: Independence and Interdependence in Combinations of Point Mutations of a Sluggish Triosephosphate Isomerase<sup>†</sup>

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**ABSTRACT:** Second-site suppressor changes that improve the catalytic potency of a sluggish mutant of the enzyme triosephosphate isomerase have been examined both individually and in combination. Each of the second-site mutations increases the specific catalytic activity of a triosephosphate isomerase in which the catalytic base, glutamate-165, has been changed to aspartate. These second-site suppressors are G10S, S96P, S96T, E97D, V167D, and G233R. Not one of these changes enhances the value of  $k_{\text{cat}}/K_m$  for the wild-type enzyme, which is consistent with the knowledge that the reaction catalyzed by the wild-type enzyme is already diffusion-controlled. Indeed, two of the changes, S96P and V167D, are catalytically deleterious to the wild-type isomerase. When pairs of second-site suppressors are combined with the primary lesion E165D, six pairs show additive independence while the effects of eight other pairs are less than additive. The sites fall into two clusters: pairs within a cluster always interfere with one another and do not produce additive improvements in catalytic activity, whereas combinations of changes from different clusters tend to be additive in their effects. No combination of second-site suppressor mutations behaves synergistically, though there seems to be no a priori reason to exclude this possibility. Since the catalytic potency of each of the six second-site suppressor mutants can be further improved by the introduction of (at least) one of the other five changes, it is evident that none of the double mutants lies at a local catalytic maximum. In these cases, therefore, the opportunity exists for at least two "steps" of monotonic catalytic improvement along each of six different "paths" in protein space.

**T**he development of techniques for the directed and random mutagenesis of a target gene has brought some of the goals

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of protein engineering within reach. Manipulation of the nucleic acid sequence encoding a protein of interest permits the analysis of the contribution of any of its amino acids to stability, binding, specificity, or catalysis. Thus, proteins of increased thermal stability (Shortle & Lin, 1985; Hecht et al., 1985; Matthews et al., 1987; Pantoliano et al., 1989; Alber,