1984 (Bray, R. C., Engel, P. C., & Mayhew, S. G., Eds.) pp 513-526, Walter de Gruyter, Berlin.
Lederer, F. (1990) in *Chemistry and Biochemistry of Flavoenzymes* (Müller, F., Ed.) CRC Press, Inc., Boca Raton, FL (in press).
Lederer, F., & Mathews, F. S. (1987) in *Flavins and Flavoproteins*, Proceedings of the Ninth International Symposium, Atlanta, GA, 1987 (Edmondson, D. E., & McCormick, D. B., Eds.) pp 133-142, Walter de Gruyter, Berlin.
Mathews, F. S., & Xia, Z. X. (1987) in *Flavins and Flavoproteins*, Proceedings of the Ninth International Symposium, Atlanta, GA, 1987 (Edmondson, D. E., & McCormick, D. B., Eds.) pp 123-132, Walter de Gruyter, Berlin.
Mulet, C., & Lederer, F. (1977) *Eur. J. Biochem. 73*, 443-447.
Pompon, D., & Lederer, F. (1979) *Eur. J. Biochem. 96*, 571-579.
Pompon, D., & Lederer, F. (1982) *Eur. J. Biochem. 129*, 143-147.
Pompon, D., & Lederer, F. (1985) *Eur. J. Biochem. 148*, 145-154.
Pompon, D., Iwatsubo, M., & Lederer, F. (1980) *Eur. J. Biochem. 104*, 479-488.
Reid, G. A., White, S., Black, M. T., Lederer, F., Mathews, F. S., & Chapman, S. K. (1988) *Eur. J. Biochem. 178*, 329-333.
Schonbrunn, A., Abeles, R. H., Walsh, C. T., Ghisla, S., Ogata, H., & Massey, V. (1976) *Biochemistry 15*, 1798-1807.
Somlo, M., & Slonimski, P. (1966) *Bull. Soc. Chim. Biol. 48*, 1221-1249.
Tegoni, M., Janot, J. M., & Labeyrie, F. (1986) *Eur. J. Biochem. 155*, 491-503.
Thomas, K. A., Smith, G. M., Thomas, T. B., & Feldman, R. J. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 4843-4847.
Urban, P., & Lederer, F. (1984) *Eur. J. Biochem. 144*, 345-351.
Urban, P., & Lederer, F. (1985) *J. Biol. Chem. 260*, 11115-11122.
Urban, P., Alliel, P. M., & Lederer, F. (1983) *Eur. J. Biochem. 134*, 275-281.
Urban, P., Chirat, I., & Lederer, F. (1988) *Biochemistry 27*, 7365-7371.
Volokita, M., & Somerville, C. R. (1987) *J. Biol. Chem. 262*, 15825-15828.
Xia, Z. X., & Mathews, F. S. (1990) *J. Mol. Biol. 212*, 837-863.
Xia, Z. X., Shamala, N., Bethge, P. H., Lim, L. W., Bellamy, H. D., Xuong, N. H., Lederer, F., & Mathews, F. S. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 2629-2633.
Yun, S. L., & Suelter, C. H. (1978) *Anal. Biochem. 85*, 437-441.

Buffer Enhancement of Proton Transfer in Catalysis by Human Carbonic Anhydrase III†

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ABSTRACT: Among the isozymes of carbonic anhydrase, isozyme III is the least efficient in the catalysis of the hydration of CO_2 and was previously thought to be unaffected by proton transfer from buffers to the active site. We report that buffers of small size, especially imidazole, increase the rate of catalysis by human carbonic anhydrase III (HCA III) of (1) ^{18}O exchange between HCO_3^- and water measured by membrane-inlet mass spectrometry and (2) the dehydration of HCO_3^- measured by stopped-flow spectrophotometry. Imidazole enhanced the rate of release of ^{18}O-labeled water from the active site of wild-type carbonic anhydrase III and caused a much greater enhancement, up to 20-fold, for the K64H, R67H, and R67N mutants of this isozyme. Imidazole had no effect on the rate of interconversion of CO_2 and HCO_3^- at chemical equilibrium. Steady-state measurements showed that the addition of imidazole resulted in increases in the turnover number (k_{cat}) for the hydration of CO_2 catalyzed by HCA III and for the dehydration of HCO_3^- catalyzed by R67N HCA III. These results are consistent with the transfer of a proton from the imidazolium cation to the zinc-bound hydroxide at the active site, a step required to regenerate the active form of enzyme in the catalytic cycle. Like isozyme II of carbonic anhydrase, isozyme III can be enhanced in catalytic rate by the presence of small molecule buffers in solution.

Human carbonic anhydrase III (HCA III)[1] is found predominantly in skeletal muscle. It has a maximal turnover number in the hydration of CO_2 near 1×10^4 s^{-1}, about 100-fold less than that of red cell carbonic anhydrase II. There is a significant body of evidence suggesting that, like isozyme II, catalysis by HCA III of CO_2 hydration and HCO_3^- dehydration occurs in two separate and distinct steps (Silverman & Lindskog, 1988). The first step is the conversion of HCO_3^-

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[1] Abbreviations: HCA III, human carbonic anhydrase III; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 4-morpholineethanesulfonic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid.

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to CO_2 for which zinc-bound water is the reactive form of the catalytic site of the enzyme:

$$HCO_3^- + EZnH_2O \rightleftharpoons CO_2 + H_2O + EZnOH^- \quad (1)$$

To complete the catalytic cycle, the active form of the enzyme is regenerated in a second stage of the pathway involving the transfer of a proton from solution to the zinc-bound hydroxide:

$$EZnOH^- + BH^+ \rightleftharpoons EZnH_2O + B \quad (2)$$

Here BH^+ can be buffer in solution, water in the active site, or possibly a residue near the active site. In human carbonic anhydrase II, this proton transfer is facilitated by histidine-64 which shuttles a proton from buffer in solution to zinc-bound hydroxide with a rate at least as rapid as the turnover number of 10^6 s^{-1} (Silverman & Lindskog, 1988; Tu et al., 1989). The corresponding residue at position 64 in HCA III is lysine (Lloyd et al., 1986), which is not an efficient proton transfer agent for the catalysis, and it is believed that the proton in this case may be transferred directly from water with a rate constant near 10^3 s^{-1} (Silverman & Lindskog, 1988).

Although HCA III has a lysine residue at position 64, it has many similarities in its catalytic pathway with isozyme II (Silverman & Lindskog, 1988). We report here another similarity, the effect of certain buffers on the rate of catalysis by HCA III. We have measured the initial rate of the hydration–dehydration reactions of CO_2 using stopped-flow spectrophotometry and the equilibrium rate of exchange of ^{18}O between CO_2 and water using membrane-inlet mass spectrometry. In addition, several site-specific mutants of HCA III were prepared, including one in which the lysine at position 64 was replaced with histidine. Previously, Kararli and Silverman (1985) reported that the addition of the buffers Mops and Hepes[1] up to 25 mM caused no change in either the initial velocity or the ^{18}O exchange rate catalyzed by feline carbonic anhydrase III and it was thought that there was no buffer enhancement of catalysis by this enzyme. We now report that catalysis by HCA III is enhanced by the addition of imidazole and other buffers of small size. This enhancement is qualitatively similar to that observed for HCA II and suggests that the active-site cavity of HCA III is able to accommodate a small buffer such as imidazole to facilitate proton transfer from the zinc-bound water to solution.

Materials and Methods

Enzymes. A bacterial expression vector containing a wild-type HCA III gene was derived from the cDNA clone of Lloyd et al. (1986) using the pET-8 vector (a gift from Dr. F. William Studier, Brookhaven National Laboratory). This class of expression vectors is described by Rosenberg et al. (1987). The site-specific mutants K64H, R67H, and R67N and the double mutant K64H–R67N were constructed according to the method of Kunkel (1985) and transferred into the same pET-8 vector. Modified and unmodified carbonic anhydrases III were expressed in *Escherichia coli* strain BL21(DE3)pLysS (Rosenberg et al., 1987) and purified by gel filtration followed by ion-exchange chromatography as described by Tu et al. (1986). The resulting enzymes were greater than 98% pure, determined by polyacrylamide gel electrophoresis. The concentrations of wild-type HCA III and mutants were determined from the molar absorptivity of 6.2×10^4 M^{-1} cm^{-1} at 280 nm (Sanyal et al., 1982), identical with the value found for bovine isozyme III (Engberg et al., 1985).

^{18}O Exchange Kinetics. Membrane-inlet mass spectrometry was used to measure the uncatalyzed and carbonic anhydrase catalyzed exchange of ^{18}O between CO_2 and water which occurs during the hydration–dehydration cycle. The exchange of ^{18}O between ^{12}C- and ^{13}C-labeled CO_2 was also measured; this occurs because the catalyzed dehydration results in a transitory labeling of the active site with ^{18}O which then reacts with $^{13}CO_2$ (Silverman et al., 1979; Silverman, 1982). Two rates for the catalysis at chemical equilibrium can be obtained from these rates of ^{18}O exchange. The first rate, R_1, is the catalyzed rate of interconversion of CO_2 and HCO_3^-, a rate which for carbonic anhydrase II agrees with that obtained by ^{13}C NMR (Silverman et al., 1979). The second rate, R_{H_2O}, is the rate of release from the enzyme of ^{18}O-labeled water. The transient labeling of the active site with ^{18}O also provides this measure of the rate of release of ^{18}O-labeled water from the enzyme, a proton transfer dependent process since ^{18}O-labeled hydroxide is not expected to exchange rapidly (eq 3; Silverman et al., 1979; Silverman & Lindskog, 1988). Here

$$EZn^{18}OH^- + BH^+ \rightleftharpoons EZn^{18}OH_2 + B \overset{H_2O}{\rightleftharpoons} EZnH_2O + H_2{}^{18}O + B \quad (3)$$

BH^+ can represent buffer in solution or water in the active site; for HCA II, BH^+ represents mainly histidine-64. Previous reports describe in detail how ^{18}O exchange results are used to obtain the rates R_1 and R_{H_2O} (Silverman et al., 1979; Silverman, 1982). The precision in R_{H_2O} was 10–30%, with the poorest precision at higher values of R_{H_2O}. The precision in R_1 was 3–10%.

Unless otherwise indicated, all ^{18}O exchange experiments were carried out in solutions containing 25 μM EDTA and all glassware and the inlet vessel to the mass spectrometer were washed with 25 μM EDTA. In a previous report of ^{18}O exchange catalyzed by bovine carbonic anhydrase III, we described a range of substrate concentrations for which the catalyzed rate of ^{18}O depletion from CO_2 was biphasic (Silverman & Tu, 1986). To avoid this complication, the ^{18}O exchange data presented here were obtained by using a total concentration of substrate ($[CO_2] + [HCO_3^-]$) of 100 mM, which yielded rates of depletion of ^{18}O from CO_2 that could be described by a single exponential.

Steady-State Kinetics. The initial velocities of the catalyzed hydration of CO_2 and the dehydration of HCO_3^- were measured by a changing pH indicator method (Khalifah, 1971) using a Durrum-Gibson stopped-flow spectrophotometer according to procedures described earlier (Rowlett & Silverman, 1982). Solutions of CO_2 were prepared from CO_2-saturated water (34 mM at 25 °C; Pocker & Bjorkquist, 1977). The buffer–indicator pairs used were imidazole ($pK_a = 7.0$) with *p*-nitrophenol ($pK_a = 7.1$) and Mops ($pK_a = 7.2$) also with *p*-nitrophenol. The observed wavelength in the stopped-flow experiments was 400 nm. All steady-state experiments were carried out at 25 °C.

In both the stopped-flow and ^{18}O exchange experiments, the total ionic strength of solution was adjusted to 0.2 M by adding Na_2SO_4, except when the ionic strength due to substrate and buffer exceeded 0.2 M, in which case Na_2SO_4 was omitted.

Results

We have measured the effect of various concentrations of the buffer imidazole on the rate of exchange of ^{18}O between CO_2 and water catalyzed by wild-type HCA III and four mutants of HCA III: K64H, R67H, R67N, and the double mutant K64H–R67N. The addition of imidazole caused an increase in R_{H_2O} in each case with the smallest increase for wild type and the largest increase for R67N HCA III (Figure 1). These increases appeared to be saturable for each enzyme, resulting in close to 20-fold enhancement in R_{H_2O} for the R67N mutant.

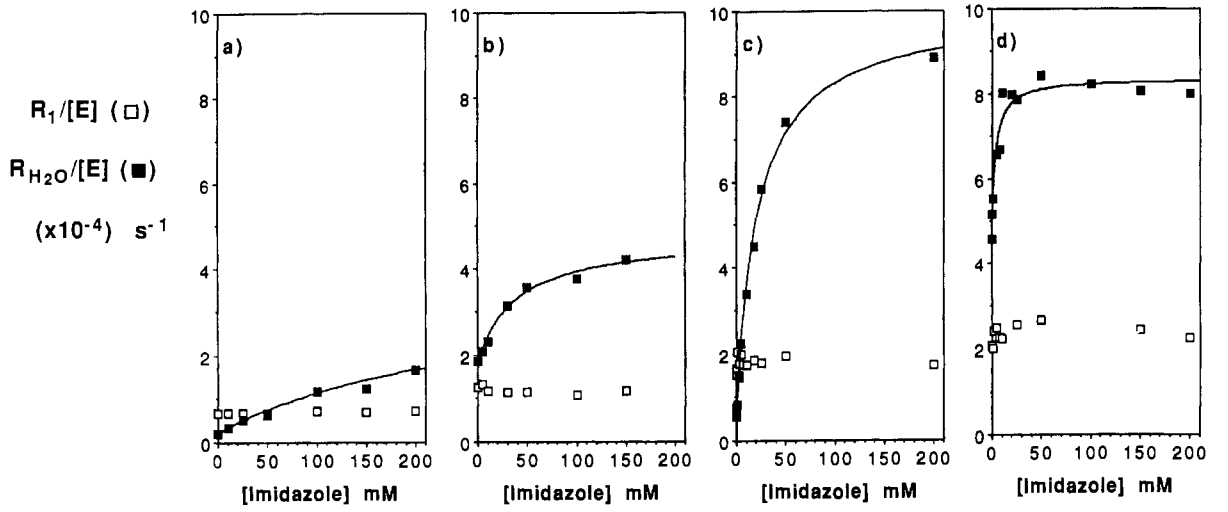

FIGURE 1: Dependence of R_1/[E] (□) and R_{H_2O}/[E] (■) on imidazole concentration for (a) wild-type carbonic anhydrase III, (b) K64H HCA III, (c) R67N HCA III, and (d) the double mutant K64H–R67N HCA III. Here [E] is the total concentration of enzyme. These experiments were carried out at 100 mM total concentration of CO_2 and HCO_3^-, 100 nM enzyme, and 25 μM EDTA, pH 7.3, 25 °C. The solid lines are nonlinear least-squares fits of the data and yield the constants given in Table I.

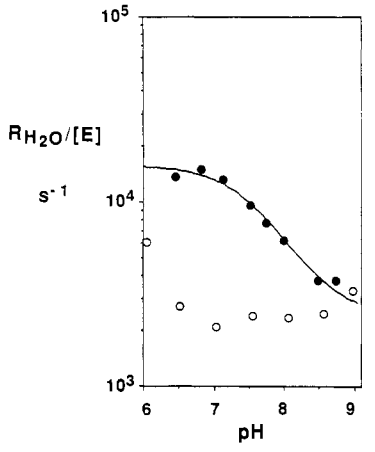

FIGURE 2: pH dependence of R_{H_2O}/[E] catalyzed by human carbonic anhydrase III: (○) in the absence of buffer; (●) in the presence of 150 mM imidazole. These experiments were carried out at 100 mM total concentration of CO_2 and HCO_3^-, 25 μM EDTA, and 25 °C. The solid line is the titration curve for a group with a pK_a of 7.6 with R_{H_2O}/[E] having a minimum of 2300 s^{-1}.

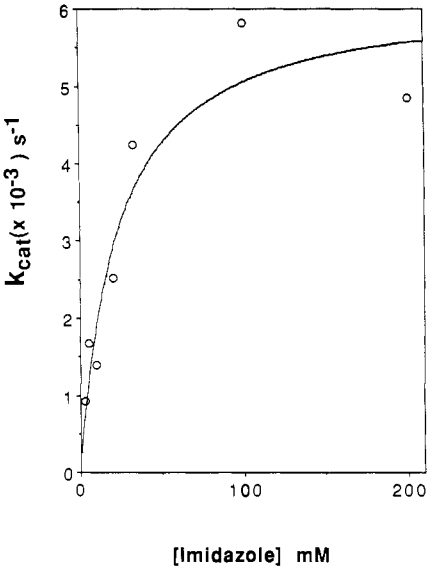

FIGURE 3: Dependence on imidazole concentration of k_{cat} for the hydration of CO_2 catalyzed by human carbonic anhydrase III at steady state. The pH was 6.8 and temperature 25 °C. Measurements were made by stopped-flow spectrophotometry at 400 nm using the indicator *p*-nitrophenol (5.7 × 10^{-5} M). The solid line is a fit to the data described by $(k_{cat})_{max}$ = 6200 s^{-1} and $(K_m)_{buffer}$ = 23 mM.

The buffers phosphate (pK_2 = 7.2) and 4-nitrophenyl phosphate (pK_2 = 5.0), each at 50 mM, caused a 6-fold and 10-fold increase, respectively, in R_{H_2O} catalyzed by HCA III with no effect on R_1. The buffers *N*-methylmorpholine (pK_a = 7.4), 2,4-lutidine (pK_a = 6.8), Hepes (pK_a = 7.5), and Mops (pK_a = 7.2) at concentrations up to 200 mM had no effect on either R_1 or R_{H_2O} catalyzed by the HCA III under the conditions of Figure 1. Under these same conditions, however, 150 mM *N*-methylmorpholine caused a 50% increase in R_{H_2O}, and 150 mM 2,4-lutidine caused a 200% increase in R_{H_2O} for R67N HCA III, with both buffers having no effect on R_1. Concentrations up to 200 mM pyrazole (pK_a = 2.5), which has a structure similar to imidazole but is not a buffer at the pH of these experiments, caused no change in R_1 or R_{H_2O} for any of these enzymes.

The effect of the single replacements we made at positions 64 and 67 had no major effect on R_1 (Figure 1). The largest effect was observed in R_1 for the double mutant K64H–R67N which was greater by 3-fold compared with wild-type HCA III. The addition of up to 150 mM imidazole had no effect on R_1 for any of the enzymes studied here (Figure 1).

In the absence of buffer, R_{H_2O} did not vary with pH in the range of pH 6–9 for wild-type feline CA III (Tu et al., 1983), or for wild-type HCA III (Figure 2). In the presence of a large concentration (150 mM) of imidazole, R_{H_2O} for HCA III varied with pH in a manner consistent with a titration curve with a pK_a of 7.6 (Figure 2), suggesting that the imidazolium cation is responsible for the enhancement of R_{H_2O}.

Initial velocity measurements of the hydration of CO_2 catalyzed by wild-type HCA III in the presence of 50 mM Mops at pH 7.1 and 25 °C yielded the steady-state rate constants k_{cat} = (2.1 ± 0.5) × 10^3 s^{-1} and k_{cat}/K_m = (2.9 ± 0.5) × 10^5 M^{-1} s^{-1}, in reasonable agreement with measurements made on bovine (Ren et al., 1988a; Engberg et al., 1985) and feline carbonic anhydrase III (Kararli & Silverman, 1985; Sanyal et al., 1982).

The turnover number k_{cat} for the hydration of CO_2 was enhanced upon addition of imidazole and approached a value of 6 × 10^3 s^{-1} at concentrations of imidazole 100 mM and greater (Figure 3). This is a larger value than that obtained with the more bulky buffers Mops, Mes, and 1,2-dimethylimidazole used in the results of Ren et al. (1988a) and Kararli

Table I: Rate Constants for the Dependence on Imidazole Concentration of the Release of $H_2{}^{18}O$ from Mutant and Wild-Type Human Carbonic Anhydrases III and II[a]

enzyme	$k_{H_2O}{}^B/K_{eff}{}^B$ ($\times 10^{-5}$ M^{-1} s^{-1})	$k_{H_2O}{}^B$ ($\times 10^{-4}$ s^{-1})	k_{H_2O} ($\times 10^{-4}$ s^{-1})
wild-type HCA III	1.2 ± 0.2	3.3 ± 1.0	0.2
K64H HCA III	9.0 ± 1.7	2.5 ± 0.2	1.8
R67H HCA III	18 ± 2	6.3 ± 0.3	0.8
R67N HCA III	43 ± 1	9.4 ± 0.3	0.6
K64H–R67N HCA III	120 ± 40	3.5 ± 0.2	4.6
H64A HCA II[b]	27 ± 3	25 ± 4	0.7
wild-type HCA II[b]	>200	5 ± 0.4	14

[a] Experimental conditions are described in the legend to Figure 1, and the rate constants are defined in eq 4. Data are nonlinear least-squares values and standard errors in a fit to eq 4. [b] From Tu et al. (1989). These data were obtained at 10 °C.

Table II: Observed Steady-State Constants and Solvent Hydrogen Isotope Effects for the Dehydration of HCO_3^- Catalyzed by the Mutant of Human Carbonic Anhydrase III Containing an Asparagine at Position 67 (R67N HCA III)[a]

	15 mM imidazole	30 mM imidazole	150 mM imidazole
k_{cat} ($\times 10^{-4}$ s^{-1})	0.29 ± 0.02	1.6 ± 0.1	2.9 ± 0.1
$^Dk_{cat}{}^b$	2.2 ± 0.3	1.8 ± 0.3	1.2 ± 0.1
k_{cat}/K_m ($\times 10^{-5}$ M^{-1} s^{-1})	3.3 ± 0.5	2.7 ± 0.1	2.2 ± 0.2
$^Dk_{cat}/K_m{}^b$	1.4 ± 0.5	0.9 ± 0.1	0.9 ± 0.2

[a] Experiments were performed using the conditions described in the legend to Figure 4. These experiments were carried out at an uncorrected pH meter reading of 6.8, relying on the fact that the correction of a pH meter reading in 100% D_2O is approximately offset by the change in ionization state of buffer in D_2O (Schowen, 1978). [b] The superscript D is used to indicate the solvent hydrogen isotope effect on the designated constant. Thus, $^Dk_{cat}$ is $(k_{cat})_{H_2O}/(k_{cat})_{D_2O}$.

and Silverman (1985). Upon addition of imidazole, at the concentrations shown in Figure 3, the value of k_{cat}/K_m for the hydration of CO_2 showed a hyperbolic increase from the lowest value of 6×10^4 M^{-1} s^{-1} at 5 mM imidazole to the highest of 2×10^5 M^{-1} s^{-1} at imidazole concentrations of 100 mM and above.

Initial velocity measurements of the dehydration of HCO_3^- catalyzed by R67N HCA III showed that k_{cat} increased up to a limit of 3×10^4 s^{-1} (Figure 4) and k_{cat}/K_m at $(3.1 \pm 0.4) \times 10^5$ M^{-1} s^{-1} did not vary as imidazole concentration increased. Solvent hydrogen isotope effects were measured to help interpret these steady-state constants and are presented in Table II. Ren et al. (1988a) have reported large deviations from Michaelis–Menten behavior in the dehydration of HCO_3^- catalyzed by bovine CA III; because of this, the steady-state parameters for this catalysis are not reported. No such deviations were observed in the dehydration catalyzed by R67N HCA III for concentrations of HCO_3^- greater than 8 mM. The turnover number for dehydration catalyzed by R67N HCA III did exhibit an increase upon addition of Mops, from 3×10^3 s^{-1} at 25 mM Mops to 8×10^3 s^{-1} at 50 mM Mops. Larger concentrations of Mops did not further enhance k_{cat}.

Discussion

Carbonic anhydrase III was hitherto thought not to be enhanced in catalytic activity by buffers acting as proton transfer agents in solution. We have now observed enhancement by specific buffers of small size, especially imidazole, of catalysis by HCA III and some site-directed mutants. Figure 1 shows that there is no significant effect of the buffer imidazole on R_1, the rate of interconversion of CO_2 and HCO_3^- at chemical equilibrium. This is consistent with evidence for both HCA II and HCA III that the catalysis of the steps in the interconversion of CO_2 and HCO_3^- involves no rate-contributing proton transfer (Simonsson et al., 1979; Kararli & Silverman, 1985). It is interesting that the replacement of both lysine-64 and arginine-67 in HCA III did not have a large effect on R_1 (Figure 1); these residues are probably not responsible for the lower catalytic activity of HCA III. Moreover, R_1 did not decrease for these mutants, indicating that the amino acid replacements caused no gross structural changes in the enzyme.

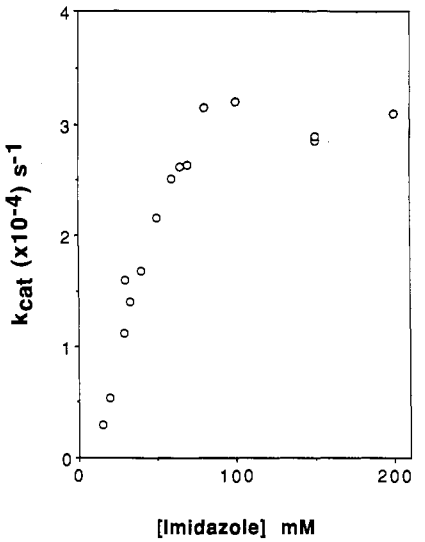

FIGURE 4: Dependence on imidazole concentration of k_{cat} for the dehydration of HCO_3^- catalyzed by R67N human carbonic anhydrase III at steady state. The pH was 6.8 and temperature 25 °C. Measurements were made by stopped-flow spectrophotometry at 400 nm using the indicator *p*-nitrophenol (3.0×10^{-5} M).

The most straightforward explanation for the enhancement of R_{H_2O} observed for wild-type HCA III and four site-specific mutants is that imidazole is able to enter the active-site cavity, transfer a proton to the zinc-bound ^{18}O-labeled hydroxide, and facilitate the release of ^{18}O from the enzyme as $H_2{}^{18}O$. This is represented in eq 3 in which BH^+ is imidazole. The pH dependence of R_{H_2O} in Figure 2 suggests that the imidazolium cation is causing this enhancement. At pH 7.3, the value of R_{H_2O} for R67N HCA III in the presence of 200 mM imidazole is near 1×10^5 s^{-1} (Figure 1); this can be compared with the maximal value of R_{H_2O} of 8×10^5 s^{-1} observed under the same conditions for the efficient wild-type HCA II. The active-site cavity of HCA III is more sterically constrained than in HCA II due to phenylalanine-198 and isoleucine-207 and is more positively charged due to lysine-64 and arginine-67 (Eriksson, 1989; in HCA II, these residues are leucine-198, valine-207, histidine-64, and asparagine-67). This difference may explain the observation that the bulkier buffers 2,4-lutidine and *N*-methylmorpholine caused increases in R_{H_2O} with R67N HCA III and HCA II (Silverman et al., 1979) but not with the wild-type HCA III. Pyrazole, with a structure similar to imidazole but an inefficient proton transfer agent because of its low pK_a of 2.5, had no effect on R_{H_2O}, indicating that the increased values of R_{H_2O} seen with imidazole are not caused by structural changes due to binding without proton transfer. These observations and their explanation are similar to those made for the enhancement by imidazole of R_{H_2O} catalyzed by HCA II and some site-directed mutants (Tu et al., 1989).

A quantitative understanding of the effect of imidazole on the enhancement of R_{H_2O} was obtained by using eq 4 which

$$R_{H_2O} = k_{H_2O}[E] + k_{H_2O}{}^B[E][B]/(K_{eff}{}^B + [B]) \quad (4)$$

provides a satisfactory fit to the data of Figure 1. Equation 4 represents the rate of release of ^{18}O-labeled water, R_{H_2O}, as the sum of two parallel processes (Yagil & Hoberman, 1969):

water release in the absence of buffer and water release facilitated by buffer. Here [E] and [B] represent the concentrations of total enzyme and total buffer, k_{H_2O} is the rate constant for release of ^{18}O-labeled water in the absence of buffer, and $k_{H_2O}^B$ is the maximal rate constant for that component of water release enhanced by buffer. K_{eff}^B is an apparent binding constant for buffer. The first term, $k_{H_2O}[E]$, describes the release of ^{18}O-labeled water in the absence of buffer and could arise from proton transfer from water in the active-site cavity or from intramolecular proton transfer involving a residue near the active site. The second term of eq 4 describes the saturable increase in R_{H_2O} upon addition of buffer and represents the phase of the catalysis shown in eq 3 in which buffer transfers a proton to the zinc hydroxide facilitating the release of water. Table I presents values of the constants of eq 4 obtained by a nonlinear least-squares fit to the data of Figure 1 as well as data obtained from other forms of carbonic anhydrase.

The R67N mutant had the largest net increase in R_{H_2O} caused by the addition of imidazole, indicated by $k_{H_2O}^B$ in Table I, presumably because the replacement of the bulky and charged arginine with the smaller and uncharged asparagine permitted a more efficient proton transfer from the imidazolium cation to the zinc hydroxide. The values of $k_{H_2O}^B$ for the mutants with a histidine at position 64, K64H and the double mutant K64H–R67N HCA III, are not as great as for R67N HCA III probably because the histidine of position 64 is an internal proton transfer group. This suggestion is supported by the report of Tu et al. (1989) that the pH dependence of R_{H_2O} catalyzed by HCA II is consistent with proton transfer from histidine-64 to zinc hydroxide. That histidine-64 is an intramolecular proton donor also explains the observation in Figure 1 that, in the absence of the buffer imidazole, R_{H_2O} values for the enzymes containing a histidine at residue 64 are larger than R_{H_2O} for the other variants of isozyme III (see also k_{H_2O} of Table I).

The values of $k_{H_2O}^B/K_{eff}^B$ are the apparent second-order rate constants for proton transfer from imidazole to enzyme at low imidazole concentrations. The large values of this constant for the K64H–R67N double mutant and wild-type HCA II indicate the accessibility of the buffer imidazole to the proton shuttle group of histidine-64 which is located in the active-site cavity near the surface of the protein. The suggested function of this residue is to be accessible to solution and shuttle protons to the active site. The data in Table I also indicate that the replacement of arginine at position 67 in HCA III with asparagine or histidine has increased significantly, compared with the wild-type enzyme, this apparent second-order rate constant for proton transfer from imidazole to the active site.

To observe the effect of imidazole on catalysis at steady state, we used HCA III and the R67N mutant of HCA III; in both cases, imidazole caused a large increment in activity (Figures 3 and 4). For R67N HCA III, we examined the dehydration reaction, since it is most analogous to the direction of ^{18}O depletion as shown in eq 1–3. Similar to our observations with R_{H_2O}, we found an increment in k_{cat} upon addition of imidazole (Figure 4); there was no effect on k_{cat}/K_m caused by the addition of up to 200 mM imidazole. This result is consistent with the proposal that catalysis occurs in two stages. The ratio k_{cat}/K_m contains rate constants for steps from the binding of HCO_3^- to the first irreversible step, the release of CO_2 (eq 1), and does not contain rate constants for the subsequent steps which involve transfer of a proton to the zinc-bound hydroxide (eq 2). The values of k_{cat}/K_m which do not change as imidazole is added confirm that imidazole plays no role in the steps involved in the interconversion of CO_2 and HCO_3^-. This is again analogous to descriptions of catalysis by HCA II and H64A HCA II which also show little or no effect on k_{cat}/K_m but significant increases in k_{cat} for hydration and dehydration upon addition of buffer (Tu et al., 1989; Pocker et al., 1986; Rowlett & Silverman 1982; Jonsson et al., 1976). For wild-type HCA III, there was nearly a 3-fold increase in k_{cat}/K_m for hydration of CO_2 upon addition of imidazole which presumably indicates some role of imidazole in enhancing the interconversion of CO_2 and HCO_3^-.

By comparing the steady-state and ^{18}O exchange results, we can provide an explanation for our observations in Figure 4 of k_{cat} for dehydration catalyzed by R67N HCA III. The steady-state constant k_{cat} cannot exceed the first-order rate constant of any forward step in the dehydration pathway; k_{cat} contains steps found in both R_1 and R_{H_2O} and has the proton transfer of eq 2 as a rate-limiting step (Silverman & Lindskog, 1988). This consideration and the data in Figures 1 and 4 imply that at low imidazole concentrations the predominant rate-contributing step for k_{cat} is the proton transfer from the imidazolium cation to the zinc-bound hydroxide approximated by $R_{H_2O}/[E]$, and at large imidazole concentrations, the predominant rate-contributing step is in the HCO_3^- to CO_2 conversion approximated by $R_1/[E]$. Hence, the values of k_{cat} in Figure 4 suggest a change in the rate-limiting step as the imidazole concentration increased. This comparison between steady-state and equilibrium ^{18}O exchange must remain qualitative because of the differences inherent in the two methods [i.e., for ^{18}O exchange, there is an equilibrium concentration of zinc hydroxide at the active site which can be very different from the steady-state concentrations discussed by Ren et al. (1988b)].

The proposal that a change in the rate-limiting step in k_{cat} for dehydration catalyzed by R67N HCA III is occurring is supported by measurement of solvent hydrogen isotope effects (SHIE). For both HCA II and III, the SHIE on the steps in the interconversion of CO_2 and HCO_3^- (eq 1) were determined to be unity (Simonsson et al., 1979; Kararli & Silverman, 1985), and the SHIE on the buffer-facilitated proton transfer steps (eq 2) ranged from 2.5 to 3.8 (Steiner et al., 1975; Kararli & Silverman, 1985). The data for R67N HCA III in Table II show an SHIE for k_{cat} near unity for 150 mM imidazole, consistent with the conversion of HCO_3^- to CO_2 (eq 1) being the predominant rate-contributing step. The SHIE for k_{cat} is much greater than unity at 15 mM imidazole, consistent with the intermolecular proton transfer step (eq 2) being the predominant rate-contributing step.

Conclusion. The exchange of ^{18}O between CO_2 and water catalyzed by carbonic anhydrase III and four site-directed mutants was enhanced by buffers of small size, especially imidazole, in a manner strongly suggesting proton transfer from the buffer to zinc-bound hydroxide at the active site. Initial velocity experiments showed increases in k_{cat} upon addition of imidazole for hydration of CO_2 catalyzed by HCA III and for dehydration of HCO_3^- catalyzed by R67N HCA III. These properties are qualitatively similar to those observed with carbonic anhydrase II and clarify the rate-limiting role of proton transfer in the catalytic pathway of carbonic anhydrase III.

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REFERENCES

Engberg, P., Millqvist, E., Pohl, G., & Lindskog, S. (1985) *Arch. Biochem. Biophys. 241*, 628–638.
Eriksson, A. E. (1989) Doctoral Dissertation, Uppsala University.
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.
Lloyd, J., McMillan, S., Hopkinson, D., & Edwards, Y. H. (1986) *Gene 41*, 233–239.
Pocker, Y., & Bjorkquist, D. W. (1977) *Biochemistry 16*, 5698–5707.
Pocker, Y., Janic, N., & Miao, C. H. (1986) in *Zinc Enzymes* (Bertini, I., Luchinat, C., Maret, W., & Zeppezauer, M., Eds.) pp 341–356, Birkhauser, Boston, MA.
Ren, X., Jonsson, B.-H., Millqvist, E., & Lindskog, S. (1988a) *Biochim. Biophys. Acta 953*, 79–85.
Ren, X., Sandstrom, A., & Lindskog, S. (1988b) *Eur. J. Biochem. 173*, 73–78.
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.
Sanyal, G., Swenson, E. R., Pessah, N. I., & Maren, T. H. (1982) *Mol. Pharmacol. 22*, 211–220.
Schowen, K. B. (1978) in *Transition States of Biochemical Processes* (Gandour, R. D., & Schowen, R. L., Eds.) Plenum, 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., 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.
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.
Yagil, G., & Hoberman, H. D. (1969) *Biochemistry 8*, 352–360.

Measurement of Active-Site Homology between Potato and Rabbit Muscle α-Glucan Phosphorylases through Use of a Linear Free Energy Relationship†

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ABSTRACT: The Michaelis–Menten parameters (V_{max} and K_m) for turnover of an extensive series of deoxy and deoxyfluoro derivatives of α-D-glucopyranosyl phosphate by the α-glucan phosphorylase from potato tuber have been determined. Very large rate reductions are observed as a consequence of each substitution, primarily due to losses in specific binding interactions, most likely hydrogen bonding, at the enzymic transition state. Comparison of the V_{max}/K_m values so determined with those measured for rabbit muscle α-glucan phosphorylase [Street et al. (1989) *Biochemistry 28*, 1581] reveals an astonishingly similar specificity, especially in light of the phylogenetic separation of their host organisms. This indicates that very similar hydrogen-bonding interactions between the enzyme and the substrate must be present at the transition states for the two enzymic reactions; therefore, they have very similar active sites. Quantitation of this similarity is achieved by plotting the logarithm of the V_{max}/K_m value for each substrate analogue with the potato enzyme against the same parameter for the muscle enzyme, yielding straight lines (ρ = 0.998 and 0.999) of slope 1.0 and 1.2 for the deoxy and deoxyfluoro substrates, respectively. Since the correlation coefficient of such plots is a direct measure of the similarity of the two transition-state complexes, thus of the enzyme active sites, it can be used as a measure of active-site homology between the two enzymes. The extremely high homology observed in this case is consistent with the observed sequence homology at the active site.

Few methods currently exist for comparing the structures and properties of two similar, possibly evolutionarily related, enzymes and providing an index of their similarity. The most effective and widely used such parameter is the degree of sequence homology derived from comparison of amino acid sequence data. This is a very valuable measure of structural and functional similarity, especially as it can be used to inspect individual parts of the overall sequence in isolation from the remainder. However, it clearly requires prior determination of the amino acid sequence, a nontrivial task. An alternative approach to such comparisons, which would probe similarities in the active-site region, might involve a comparison of the catalytic capabilities of the enzymes in question. This could

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