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Enhancement of the Catalytic Properties of Human Carbonic Anhydrase III by Site-Directed Mutagenesis†

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ABSTRACT: Among the seven known isozymes of carbonic anhydrase in higher vertebrates, isozyme III is the least efficient in catalytic hydration of CO_2 and the least susceptible to inhibition by sulfonamides. We have investigated the role of two basic residues near the active site of human carbonic anhydrase III (HCA III), lysine 64 and arginine 67, to determine whether they can account for some of the unique properties of this isozyme. Site-directed mutagenesis was used to replace these residues with histidine 64 and asparagine 67, the amino acids present at the corresponding positions of HCA II, the most efficient of the carbonic anhydrase isozymes. Catalysis by wild-type HCA III and mutants was determined from the initial velocity of hydration of CO_2 at steady state by stopped-flow spectrophotometry and from the exchange of ^{18}O between CO_2 and water at chemical equilibrium by mass spectrometry. We have shown that histidine 64 functions as a proton shuttle in carbonic anhydrase by substituting histidine for lysine 64 in HCA III. The enhanced CO_2 hydration activity and pH profile of the resulting mutant support this role for histidine 64 in the catalytic mechanism and suggest an approach that may be useful in investigating the mechanistic roles of active-site residues in other isozyme groups. Replacing arginine 67 in HCA III by asparagine enhanced catalysis of CO_2 hydration 3-fold compared with that of wild-type HCA III, and the pH profile of the resulting mutant was consistent with a proton transfer role for lysine 64. Neither replacement enhanced the weak inhibition of HCA III by acetazolamide or the catalytic hydrolysis of 4-nitrophenyl acetate.

Carbonic anhydrase III (CA III)[1] is found almost entirely in skeletal muscle where it can comprise as much as 20% of soluble protein (Shiels et al., 1984). It is the least efficient of the seven isozymes of carbonic anhydrase now known to occur in higher vertebrates (Tashian, 1989). The maximal catalytic turnover number for CA III is near 1×10^4 s^{-1} (Ren et al., 1988; Kararli & Silverman, 1985), 100-fold less than that for human carbonic anhydrase II (1.4×10^6 s^{-1}; Khalifah, 1971), the most efficient of the carbonic anhydrase isozymes found in red cells and secretory tissues (Maren, 1967). Moreover, isozyme III is much less susceptible to inhibition by sulfonamides such as acetazolamide than is isozyme II (Sanyal et al., 1982). Bovine CA III and HCA II have similar amino acid sequences, with 60% of the residues identical, and nearly identical backbone structures (Eriksson, 1988). Superposition of these two structures by computerized fit of main-chain atoms showed a root mean square difference of 0.92 Å, although values in the region of the active site are considerably lower (Eriksson, 1988). The position of lysine 64 and arginine 67 with respect to the active-site zinc and its three histidine ligands in the crystal structure of bovine CA III is shown in Figure 1 (Eriksson, 1988). Lysine 64 extends away from the zinc into solution, and arginine 67 points into the active-site cavity but does not extend directly toward the zinc. The distance from the N-ζ of lysine 64 to the zinc is 12.3 Å, and the distance from the C-ζ of arginine 67 to the zinc is 9.2 Å.

In the pathway for catalysis by isozymes II and III of carbonic anhydrase, the substrate CO_2 reacts with the active site when hydroxide is present as a zinc ligand. The subsequent release of HCO_3^- leaves a water molecule on the metal (eq 1). The enzyme with zinc-bound water must transfer a proton

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

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[1] 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.

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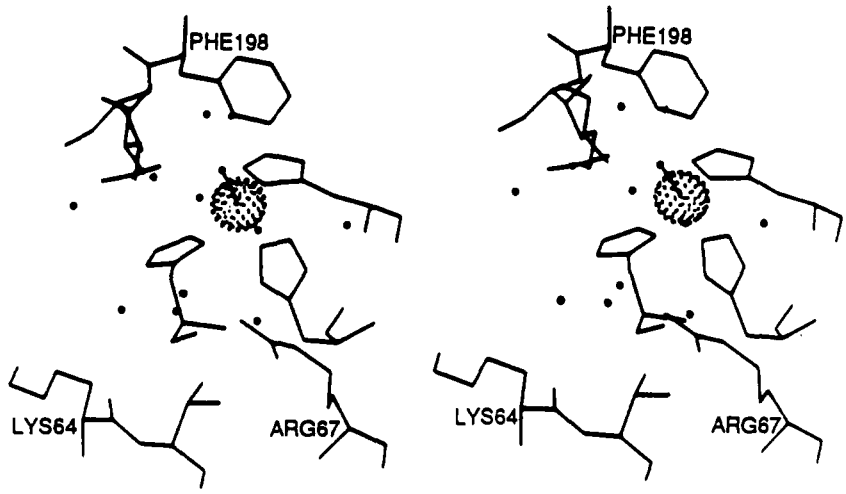

FIGURE 1: Stereo diagram showing the positions of lysine 64 and arginine 67 near the active site of bovine carbonic anhydrase III [from coordinates provided by A. E. Eriksson and A. Liljas; see Eriksson (1988)]. The zinc ion is represented as a dotted sphere, and the zinc-to-water bond is represented as a line connecting the two. Other water molecules near the zinc are shown as individual dots.

to solution to regenerate the active form of enzyme for the next hydration step. This is shown in eq 2, where B is buffer in solution or water. Since this step is essential in the catalysis,

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

the proton transfer must occur at least as fast as the maximal turnover number. There is a wide body of data supporting the hypothesis, proposed by Steiner et al. (1975), that the histidine residue at position 64 in isozyme II is involved in these steps by shuttling protons between the active site and buffer (Silverman & Lindskog, 1988; Tu et al., 1989). Wild-type HCA III has a lysine at position 64 (Lloyd et al., 1986); the lower turnover number as well as the very small pH dependence in the hydration of CO_2 for this isozyme is consistent with direct proton transfer from zinc-bound water to water in solution (Ren et al., 1988; Engberg et al., 1985; Kararli & Silverman, 1985).

As a direct test of the function of the proton shuttle in the catalytic pathway of carbonic anhydrase, we have used site-directed mutagenesis to introduce a histidine into position 64 of isozyme III. This tests the possible functions of lysine 64 and its contribution to some of the unique properties of HCA III such as its low catalytic activity and its weak binding of acetazolamide. We have also replaced arginine 67 with an asparagine, which occurs in wild-type HCA II. There have been several suggestions concerning the effects of these residues in HCA III. One hypothesis is that the Coulombic interaction between the two positively charged residues at positions 64 and 67 of HCA III and zinc-bound water might account in part for the pK_a of less than 6 of zinc-bound water in CA III compared with the corresponding pK_a near 7 for CA II (Tu et al., 1983; Engberg & Lindskog, 1984). Other hypotheses are that the presence of these two basic residues at 64 and 67 might account for the lower efficiency of HCA III by stabilization of the enzyme–bicarbonate complex and that arginine 67 because of its size limits access of substrate to the active site.

To measure catalysis by HCA III and mutants, we have determined the initial velocity of hydration of CO_2 at steady state using stopped-flow spectrophotometry and the exchange of ^{18}O between CO_2 and water at chemical equilibrium using mass spectrometry. We found the mutant of isozyme III with a histidine residue at position 64, K64H HCA III, to have enhanced catalytic activity compared with wild type, consistent with this histidine acting as a proton shuttle similar to that of wild-type isozyme II (Tu et al., 1989). The mutant R67N HCA III also had enhanced CO_2 hydration activity consistent with proton transfer between lysine 64 and the active site. Both of these mutants showed a small enhancement, 3-fold or less, on the steps of the actual interconversion of CO_2 and HCO_3^- (eq 1).

Materials and Methods

Enzyme. Bacterial expression vectors containing the human CA III coding region [derived from the cDNA clone of Lloyd et al. (1986)] and the human CA II coding region [derived from cDNA clone isolated in this laboratory (D. Jewell and P. Laipis, unpublished data)] were constructed with vectors of the pET series or their derivatives (a gift from Dr. F. William Studier, Brookhaven National Laboratory). This class of expression vectors is described by Rosenberg et al. (1987). Site-specific mutants were inserted into the coding regions with the method of Kunkel (1985). All mutants were verified by DNA sequencing to ensure that only the designated sites were 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 6.2×10^4 M^{-1} cm^{-1} at 280 nm (Sanyal et al., 1982), identical with the value found for bovine isozyme III (Engberg et al., 1985). Modified carbonic anhydrases II were purified by affinity chromatography (Khalifah et al., 1977), and concentrations were determined from the molar absorptivity of 5.34×10^4 M^{-1} cm^{-1} (Edsall et al., 1966).

Steady-State Kinetics. Initial velocities for CO_2 hydration were determined with a stopped-flow spectrophotometer (Applied Photophysics) with a pH-indicator method (Khalifah, 1971) according to procedures described by Rowlett and Silverman (1982). Solutions were prepared from CO_2-saturated water (34 mM at 25 °C; Pocker and Bjorkquist, 1977), and final CO_2 concentrations ranged from 2.8 to 28 mM. The buffer–indicator pairs (with the wavelengths observed) were as follows: Mes (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 solution maintained at a minimum of 0.2 M with Na_2SO_4. When the contributions of buffer and substrate to the ionic strength exceeded 0.2 M, no Na_2SO_4 was added. [Sulfate is an inhibitor of carbonic anhydrase II with K_I = 9 mM at a low pH of 5.2 with no inhibition above pH 6.5 (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., 1990).] 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 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. The standard errors in k_{cat}/K_m were under 10% and in k_{cat} were generally under 17%.

^{18}O-Exchange Kinetics. The uncatalyzed and carbonic anhydrase catalyzed exchanges of ^{18}O between CO_2 and water were measured by membrane-inlet mass spectrometry. We also measured the exchange of ^{18}O between ^{12}C- and ^{13}C-labeled CO_2, which occurs because the catalyzed dehydration of labeled HCO_3^- results in a transitory labeling of the active site with ^{18}O which then reacts with $^{13}CO_2$ [eq 3; see also Silverman (1982) and Silverman et al. (1979)]. Two rates

$$HCOO^{18}O^- + EZnH_2O \rightleftharpoons EZn^{18}OH^- + CO_2 + H_2O \quad (3)$$

for the catalysis at chemical equilibrium were determined from the ^{18}O exchange results. The first, R_1, is the rate of interconversion of CO_2 and HCO_3^- at chemical equilibrium (eq 3). The second, R_{H_2O}, is the rate of release of ^{18}O-labeled water from the active site (eq 4). R_{H_2O} involves a proton transfer

$$EZn^{18}OH^- + BH^+ \rightleftharpoons EZn^{18}OH_2 + B \overset{H_2O}{\rightleftharpoons} EZnH_2O + H_2{}^{18}O + B \quad (4)$$

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 $H_2{}^{16}O$, resulting in net depletion of ^{18}O from species of CO_2. In eq 4, BH^+ can be buffer in solution, water in the active site, or a side chain of the enzyme such as histidine 64. Previous reports describe in detail how the rate constants for ^{18}O exchange are used to obtain the rates R_1 and R_{H_2O} (Silverman et al., 1979; Silverman, 1982).

Measurements were carried out at 25 °C, and the total ionic strength of solution was maintained at a minumum of 0.2 M by the addition of the appropriate amount of Na_2SO_4. The standard deviations in R_{H_2O} were 10–25% with the poorest precision at higher values of R_{H_2O}. The standard deviations in R_1 were less than 10%.

In a previous report of ^{18}O exchange catalyzed by bovine carbonic anhydrase III in the absence of buffers, we described a biphasic depletion of ^{18}O from CO_2 (Silverman & Tu, 1986). Biphasic depletion is much more difficult to interpret than is an ^{18}O-exchange rate which can be described by a single exponential. However, in the presence of a large concentration of total substrate, $[CO_2] + [HCO_3^-]$ = 100 mM, or in the presence of 100 or 150 mM imidazole, which enhances proton transfer between the active site and solution, the depletion of ^{18}O from CO_2 followed a single exponential and was readily interpreted as previously described (Silverman et al., 1979). Thus, we carried out ^{18}O-exchange experiments using 100 mM total substrate concentration where no biphasic ^{18}O depletion was seen for either wild-type CA III (Silverman & Tu, 1986) or the mutants studied here.

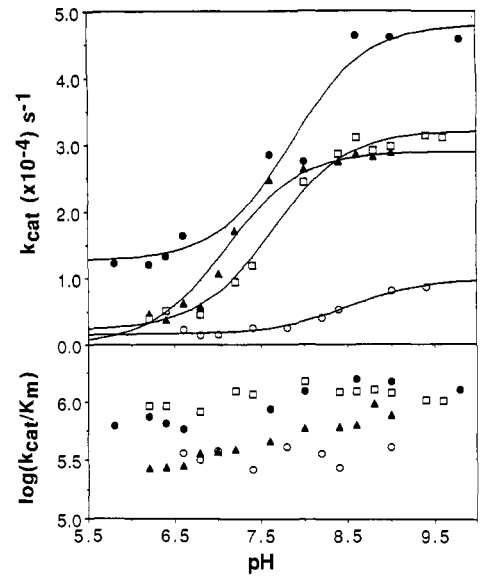

FIGURE 2: pH dependence of k_{cat} and the logarithm of k_{cat}/K_m (M^{-1} s^{-1}) for the hydration of CO_2 catalyzed by (○) wild-type HCA III, (▲) K64H HCA III, (□) R67N HCA III, and (●) K64H-R67N HCA III. The buffers used were as follows: from pH 5.5 to pH 6.8, Mes; from pH 6.8 to pH 7.5, Mops; from pH 7.5 to pH 8.8, Taps; from pH 9.0 to pH 10.0, Ches. All buffer concentrations were 50 mM, the temperature was 25 °C, and the total ionic strength of solution was maintained at a minimum of 0.2 M by addition of Na_2SO_4. The solid lines were obtained by a least-squares fit of the data with these values of pK_a: wild-type HCA III, 8.5 ± 0.1; K64H HCA III, 7.1 ± 0.1; R67N HCA III, 7.6 ± 0.1; K64H-R67N HCA III, 7.9 ± 0.2.

RESULTS

Steady State. Values of the turnover number k_{cat} for the hydration of CO_2 catalyzed by K64H, R67N, and K64H-R67N HCA III showed pH-dependent behavior with enhanced activity compared with wild-type HCA III (Figure 2). Enhancement of activity was maximal at high pH and could be roughly described as dependent on the ionization of a single group, as shown by the solid lines in Figure 2 with values of the pK_a given in the legend. The pH dependence and values of k_{cat} for HCA III are very similar to those reported by Kararli and Silverman (1985) for feline CA III, where the pK_a of the ionization curve was 9.0, and that of Ren et al. (1988) for bovine CA III. We have used sulfonic acid buffers such as Mes, Mops, and Taps which do not enhance catalysis by HCA III, indicating there is no direct proton transfer with the active site (Tu et al., 1990; Kararli & Silverman, 1985). A similar conclusion was obtained with Mops and H64A HCA II (Tu et al., 1989). However, Mops was found to enhance about 3-fold the dehydration of HCO_3^- catalyzed by R67N HCA III (Tu et al., 1990). The buffer dependence of HCA II is understood to involve proton transfer with the active site mediated by a proton shuttle (Silverman & Lindskog, 1988).

The values of k_{cat}/K_m determined by stopped flow for hydration of CO_2 catalyzed by HCA III and mutants are shown in Figure 2. The values of this ratio, in general, showed no pH dependence, although k_{cat}/K_m for K64H HCA III increased gradually by 3-fold as pH increased in the region of pH 6–9. The value of k_{cat}/K_m for HCA III varied around a mean value of (2.9 ± 0.5) × 10^5 M^{-1} s^{-1} at pH 7.0 in 50 mM Mops, in agreement with values found for feline CA III (Tu et al., 1983) and bovine CA III (Engberg et al., 1985; Ren et al., 1988). The largest values of this ratio were observed for R67N and K64H-R67N HCA III at a magnitude about 3-fold greater than for wild-type HCA III.

We also measured steady-state constants for the hydration

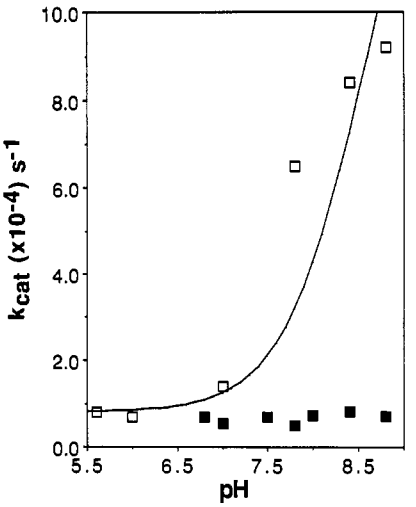

FIGURE 3: pH dependence of k_{cat} for the hydration of CO_2 catalyzed by (□) H64K HCA II and (■) H64A HCA II. Experimental conditions were as given in the legend to Figure 2.

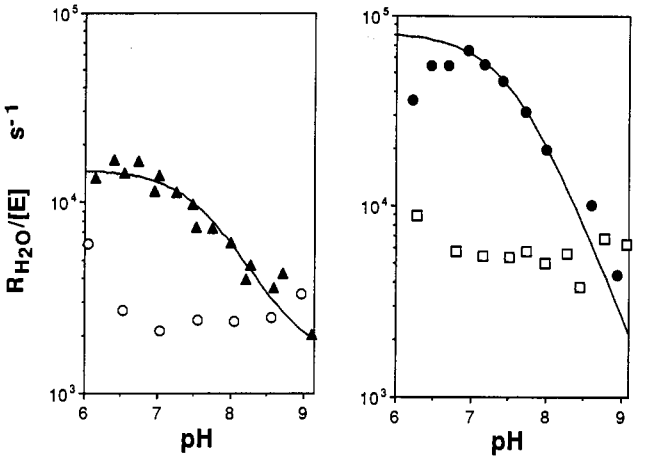

FIGURE 4: Variation with pH of $R_{H_2O}/[E]$, the rate constant for release from enzyme of water bearing substrate oxygen, catalyzed (left) by (○) wild-type HCA III and (▲) K64H HCA III and (right) by (□) R67N HCA III and (●) K64H-R67N HCA III. [E] is total enzyme concentration. Data were obtained at 25 °C with solutions containing 100 mM concentration of all species of CO_2 and with total ionic strength of solution maintained at 0.2 M with Na_2SO_4. No buffers were added. The solid lines, obtained by least-squares fit, are the titration curves for activation by the protonated form of a group with a pK_a of 7.5 ± 0.2.

of CO_2 catalyzed by two mutants of HCA II: H64K and H64A HCA II. In these cases, k_{cat} for H64K HCA II showed a strong pH dependence, but H64A did not (Figure 3).

The hydrolysis of 4-nitrophenyl acetate catalyzed by HCA III is very slow [k_{cat}/K_m = 6 M^{-1} s^{-1} at pH 6.5; see Tu et al. (1986)] compared with hydrolysis by HCA II [$k_{cat}/K_m \approx 1 \times 10^3$ M^{-1} s^{-1} at pH 6.5; see Steiner et al. (1975) and Pocker and Stone (1967)]. The mutants K64H HCA III and R67N HCA III did not have an enhanced rate of this hydrolysis compared with the wild-type HCA III (k_{cat}/K_m was 1.4 and 1.9 M^{-1} s^{-1} for K64H and R67N, respectively, at pH 6.5).

Equilibrium. The rate constant $R_{H_2O}/[E]$ describes the release from the active site of water that contains oxygen abstracted from substrate bicarbonate (eq 4). For wild-type HCA III and R67N HCA III this parameter showed no pH dependence over the pH range of 6.5–9 (Figure 4). For the mutants K64H and K64H-R67N HCA III there was a pH dependence for $R_{H_2O}/[E]$ that can be described as enhanced by the protonated form of a single ionizable group as shown by the solid lines in Figure 4 with values of pK_a = 7.5.

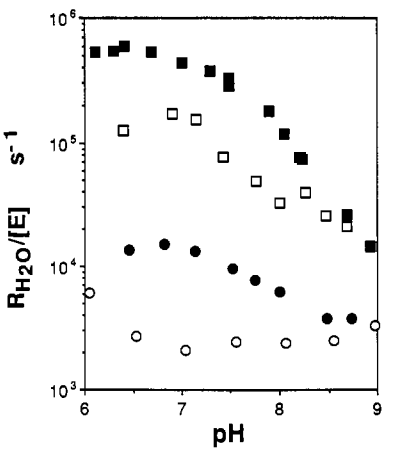

FIGURE 5: Variation with pH of $R_{H_2O}/[E]$ catalyzed by (○) wild-type HCA III with no added buffer, (●) wild-type HCA III with 150 mM imidazole, (□) R67N HCA III with 150 mM imidazole, and (■) wild-type HCA II with no added buffer. Experimental conditions were as described in the legend to Figure 4.

The values of $R_{H_2O}/[E]$ for the mutants studied here were enhanced by the buffer imidazole, as has been described by Tu et al. (1990). Figure 5 demonstrates that, for the single mutant R67N HCA III in the presence of 150 mM imidazole as a proton transfer agent, maximal levels of $R_{H_2O}/[E]$ (2×10^5 s^{-1}) were observed that approached the maximal levels for HCA II (6×10^5 s^{-1}). We note also that the presence of 150 mM imidazole and wild-type HCA III resulted in values of $R_{H_2O}/[E]$ very similar to those of K64H HCA III in the absence of buffer (compare Figures 4 and 5).

Solvent hydrogen isotope effects were measured at an uncorrected pH meter reading of 7.3 and 25 °C with 100 mM total concentration of substrate (CO_2 + HCO_3^-). Solutions contained sufficient Na_2SO_4 to make the total ionic strength 0.2 M, and the deuterated water in the reaction solution was at least 0.98 atom fraction deuterium. The solvent hydrogen isotope effects on R_1 were unity (±10%) for wild-type, K64H, R67N, and K64H-R67N HCA III; the solvent hydrogen isotope effects on R_{H_2O} were 2.4 (±25%) for each of these enzymes.

The inhibition by acetazolamide of ^{18}O exchange catalyzed by HCA III gave an inhibition constant K_I of 43 μM, determined from the inhibition of both R_1 and R_{H_2O} (pH 7.2 with conditions as in the legend to Figure 4). This inhibition of ^{18}O exchange catalyzed by K64H and R67N HCA III gave K_I values of 71 and 10 μM, respectively. The value of K_I for the inhibition of HCA II by acetazolamide is 10^{-8} M (Sanyal et al., 1982).

Discussion

We have investigated the influence of lysine 64 and arginine 67 in the active site cavity of HCA III on the catalysis of CO_2 hydration and its inhibition by acetazolamide. These two prominent, charged residues are near the zinc (Figure 1) and represent major differences in the primary structures of the most and least efficient of the carbonic anhydrases, isozymes II and III.

Proton Transfer. These two residues clearly affect R_{H_2O}, the proton transfer dependent rate of release from the enzyme of ^{18}O-labeled water (eq 4). For wild-type HCA II, the fast isozyme, $R_{H_2O}/[E]$ has a pH dependence (Figure 5) consistent with proton transfer from histidine 64 to zinc-bound hydroxide and has a maximum value near 6×10^5 s^{-1} (Tu & Silverman, 1985). $R_{H_2O}/[E]$ for wild-type HCA III is smaller at 2×10^3 s^{-1} and has no apparent pH dependence (Figure 4), consistent

with proton transfer from water to the zinc-bound hydroxide. (This lack of a pH dependence for HCA III excludes the transfer of a proton from H_3O^+ to enzyme as a significant pathway in the pH range of these studies.) Compared with wild-type HCA III, the K64H mutant had enhanced values of R_{H_2O} which could be fit with a titration curve (pK_a 7.5) with a maximum at low pH (Figure 4). This is the expected pH dependence for proton transfer from the imidazolium group ($pK_a \approx 7$) of histidine 64 to the active site to form zinc-bound water with a pK_a less than 6.0. These data and those in Figure 2 are consistent with histidine 64 in K64H HCA III having a pK_a like that in wild-type HCA II (pK_a 7.1; Campbell et al., 1975). However, we have only kinetic evidence for this pK_a and note that in another isozyme, HCA I, the pK_a of His 64 is much lower (pK_a 4.7; Campbell et al., 1974).

We have also been able to enhance the proton transfer dependent release of water, R_{H_2O}, by providing a proton transfer group externally as the buffer imidazole in solution. The presence of 150 mM imidazole with wild-type HCA III showed an enhancement of R_{H_2O} (Figure 5) nearly identical with that of the mutant K64H HCA III in the absence of buffer (Figure 4), supporting the role of the imidazole ring of histidine 64 in this process. Figure 5 also demonstrates that the single-mutant R67N HCA III in the presence of a large concentration of imidazole has a greatly enhanced value of R_{H_2O}, one that is close in magnitude to that observed for isozyme II. Thus, this single mutation of the least efficient isozyme and a proton transfer agent in solution achieves proton transfer rates nearly as rapid as in the most efficient isozyme. The addition of pyrazole (pK_a 2.5), a structural analogue of imidazole but not a proton transfer agent at pH near 7, had no effect on R_1 or R_{H_2O} of these wild-type or mutant enzymes.

The enhancement of R_{H_2O} was also observed for the double-mutant K64H-R67N HCA III (Figure 4). Here the criteria for independent interactions of side chains, as described by Carter et al. (1984), apply. The loss in transition-state energy for $R_{H_2O}/[E]$ catalyzed by the double mutant K64H-R67N compared with wild type is the sum of the energy changes calculated for each of the single mutants K64H and R67N. There is no synergistic or antagonistic interaction between the side chains we tested at residues 64 and 67 in this proton transfer dependent process.

The enhancement of R_{H_2O} in K64H HCA III (Figure 4) complements studies of isozyme II which are consistent with a catalytic role for histidine 64 as a proton shuttle based on a decrease in catalysis when this histidine was chemically modified (Pocker & Janjic, 1988; Khalifah & Edsall, 1972) or replaced with alanine by site-directed mutagenesis (Tu et al., 1989). The observed enhancement of R_{H_2O} caused by placing a histidine at position 64 in the slow isozyme is direct support for the role of this residue in intramolecular proton transfer.

This interpretation is also reached from the data for the steady-state constant k_{cat} for hydration of CO_2. Here also k_{cat} for wild-type HCA III is generally independent of pH (Figure 2); the small pH dependence of k_{cat} observed with pK_a near 8.5 could possibly be due to ionization of lysine 64, as discussed below. The pH dependence of K64H and K64H-R67N HCA III can be roughly described by the ionization of a single group with pK_a near 7.5 with maximal values at high pH. This is expected for catalysis enhanced by proton transfer from zinc-bound water to the imidazole ring of histidine 64 in the hydration direction and is consistent with the results of the ^{18}O exchange data, which pertain to the dehydration direction (eqs 3 and 4). The values of k_{cat} for K64H-R67N HCA III are greater than those for wild type at low pH (Figure 2) where the imidazole ring of histidine 64 is protonated and not able to enhance catalysis by accepting a proton from zinc-bound water. This enhancement may reflect a more rapid proton transfer from zinc–water to water in the active site of this double mutant compared with wild-type HCA III. With a low-pH value near 10^4 s^{-1}, k_{cat} for K64H-R67N HCA III is near in magnitude to k_{cat} for H64A HCA II (using the non-interacting buffer Mops), which lacks the proton shuttle (Tu et al., 1989; Figure 3).

The pH dependence of k_{cat} catalyzed by R67N HCA III (Figure 2) also appears dependent on the ionization of a group with a pK_a near 7.6, but this enzyme has no histidine to act as a proton shuttle, and the ^{18}O-exchange data of Figure 4 for R67N HCA III do not show a pH dependence. We believe, for the following reasons, that these data are consistent with lysine 64 in R67N HCA III accepting a proton from zinc–water and transferring it to solution in the hydration direction. (1) The pH dependence with a maximum at high pH (Figure 2) is appropriate for proton transfer from zinc-bound water to lysine. It may not be necessary to suggest a perturbed pK_a for lysine 64; Rowlett et al. (1990) in computer simulations of catalysis by CA III have found that the apparent pK_a observed in the pH profile for k_{cat} for hydration does not correspond to the actual pK_a of lysine 64. To account for an apparent pK_a of 8 in the pH profile of bovine CA III required a microscopic pK_a of 9 for lysine 64. In this case the apparent pK_a is influenced by a change in rate-limiting step from proton transfer between zinc–water and solvent at low pH to the interconversion of CO_2 and HCO_3^- at high pH. (2) Such a pH dependence is seen weakly and with a higher apparent pK_a of 8.5 in wild-type HCA III (Figure 2). These enhancements are not seen in R_{H_2O} catalyzed by wild-type and R67N HCA III (Figure 4) because the ^{18}O-exchange experiment depends on the dehydration reaction, where proton transfer from the basic lysine to form a very acidic zinc–water is unfavorable. (3) The mutant H64K HCA II showed an enhanced k_{cat} when compared with H64A HCA II (Figure 3), an enhancement qualitatively similar to R67N HCA III (in Figure 2). The values of k_{cat} using the mutant H64A HCA II as a control showed no pH dependence in Figure 3. Moreover, we observed that H64K HCA II had values of $R_{H_2O}/[E]$ of 2×10^4 s^{-1} at 25 °C that were independent of pH (data not shown), similar to the case with R67N HCA III.

Hence, the evidence suggests that in R67N HCA III the lysine 64 side chain is able to interact with the zinc–water by accepting a proton, perhaps through water bridges, to result in enhanced catalysis; but in the wild-type HCA III, lysine 64 does this only weakly. It is possible that the orientations of the side chain of lysine 64 in HCA III are too restricted by the presence of arginine 67 to do this. A second explanation is that arginine 67 is part of a binding site in HCA III for sulfate, which is present in our experiments to maintain ionic strength. Rowlett et al. (1990) have noted that sulfate exerts a partial hyperbolic inhibition of the hydration of CO_2 catalyzed by bovine CA III with a value of K_I near 1 mM. Their kinetic data are consistent with a model in which sulfate depresses the proton transfer between lysine 64 and zinc-bound water. The enhancement in k_{cat} observed for R67N HCA III in Figure 2 may be explained as proton transfer involving lysine 64 made more apparent by the elimination of sulfate inhibition. In fact, we observed steady-state parameters for R67N HCA III identical with those in Figure 2 when the experiments were carried out in the absence of sulfate (data not shown).

Interconversion of CO_2 and HCO_3^-. The values of k_{cat}/K_m for hydration of CO_2 were quite similar over the entire pH range studied for K64H and R67N as well as the double-mutant K64H-R67N HCA III (Figure 2). Hence, the amino acid replacements of histidine for lysine at position 64 and asparagine for arginine at 67 in HCA III have resulted in only minor changes compared with the nearly 500-fold difference in k_{cat}/K_m between wild-types HCA II and III (Silverman & Lindskog, 1988). This ratio contains rate constants for the steps up to and including the first irreversible step which in the hydration direction is the release of HCO_3^-. Furthermore, the relatively small changes in k_{cat}/K_m indicate that there have been no gross changes in the structure of isozyme III caused by these mutations. The lack of pH dependence of k_{cat}/K_m for all the enzymes in Figure 2 indicates that they all have a pK_a for zinc-bound water outside the pH range of these experiments. These residues are thus not responsible for the low pK_a of zinc-bound water in isozyme III, a pK_a which is known to be low (<6.0) for wild-type CA III (Tu et al., 1983; Engbert & Lindskog, 1984). Several suggestions concerning the possible effects on the catalysis of lysine 64 and arginine 67 have been reviewed in the introduction. These are just two of several active-site residues that are different between human isozymes II and III; others include isoleucine 91 and leucine 198 in HCA II, which are arginine 91 and phenylalanine 198 in HCA III (Tashian, 1989). Eriksson (1988) has suggested that Phe 198 is important in determining the catalytic properties of isozyme III, and preliminary evidence from this laboratory supports this idea.

The solvent hydrogen isotope effects on R_1 measured by ^{18}O exchange at pH 7.3 were close to unity for HCA III and for the mutants K64H, R67N, and K64H-R67N HCA III, indicating that in each of these enzymes there is no rate-limiting proton transfer in the catalytic interconversion of CO_2 and HCO_3^-. Thus, neither of these residue replacements has altered this fundamental aspect of the catalysis. The solvent hydrogen isotope effects for R_{H_2O} were near 2.4 for these enzymes, consistent with a limiting proton transfer in regenerating the protonated form of the active site. Each of these variants of HCA III, along with the wild-type itself, is enhanced by buffers of small size (Tu et al., 1990), indicating that proton transfer plays a significant role in the catalytic pathway. Thus, we conclude that the overall mechanisms of all of these mutants of isozyme III remain qualitatively the same as that of wild-type HCA III; that is, data are consistent with direct nucleophilic attack of zinc–hydroxide on CO_2 with a subsequent rate-limiting proton transfer from the enzyme to solution.

Conclusion. We have replaced lysine 64 and arginine 67, two prominent basic residues in the active site cavity of human carbonic anhydrase III, with the amino acids known to be in the corresponding positions of HCA II, histidine 64 and asparagine 67. We have shown that histidine 64 functions to shuttle protons between the active site and solution in carbonic anhydrase by placing it in HCA III, which in its natural form lacks this residue. The enhanced CO_2 hydration activity of the mutant K64H HCA III is direct support for the role of this histidine in the catalytic mechanism and suggests an approach that may be useful in investigating the mechanistic roles of active-site residues in other isozyme groups. Moreover, our data suggest that lysine 64 in the mutant R67N HCA III can also function in this intramolecular proton transfer. The characteristic and unique features of isozyme III such as its low and pH-independent catalytic activity in the hydration of CO_2 and its weak binding of the sulfonamide acetazolamide are only slightly influenced by replacement of lysine 64 and arginine 67.

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Ligand Binding Studies of Engineered Cytochrome P-450$_d$ Wild Type, Proximal Mutants, and Distal Mutants†

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ABSTRACT: Interactions of various axial ligands with cytochrome P-450$_d$ wild type, proximal mutants (Lys453Glu, Ile460Ser), and putative distal mutants (Glu318Asp, Thr319Ala, Thr322Ala) expressed in yeast were studied with optical absorption spectroscopy. P-450$_d$ wild type and all five mutants were purified essentially as the high-spin form, but the putative distal mutants contained about 5% low-spin form. Bindings of metyrapone and 4-phenylimidazole to the wild type and all mutants formed nitrogen-bound low-spin forms. In contrast, binding of 2-phenylimidazole to the wild type and most of mutants formed oxygen-bond low-spin forms except for the mutant Glu318Asp in which the nitrogen-bound low-spin form was formed. By analogy with the distal structure of P-450$_{cam}$, it was thus suggested that Glu318 of P-450$_d$, which corresponds with Asp251 of P-450$_{cam}$, somehow interacts with 2-phenylimidazole over the heme plane. Addition of 1-butanol and acetanilide, a substrate of P-450$_d$, to the wild type and mutants caused the spin change to the low-spin form. The order of dissociation constants of these oxygen ligands to P-450$_d$ was wild type > proximal mutants > putative distal mustants. Spectral analyses showed that the binding site of acetanilide is the same as that of another substrate, 7-ethoxycoumarin, in the putative distal mutants but is not the same in the wild type and proximal mutants. From these findings together with other spectral data, it was suggested that the region from Glu318 to Thr322 is located at the distal region of the heme in membrane-bound P-450$_d$ as suggested from the X-ray crystal structure of water-soluble P-450$_{cam}$ and amino acid alignments of P-450s.

Cytochrome P-450 (P-450)[1] is the heme enzyme that catalyzes monooxidation reactions of organic substrates (Sato & Omura, 1978; Ortiz de Montellano, 1986). Although the crystal structure of the water-soluble bacterial P-450$_{cam}$ is known (Poulos et al., 1985, 1986; Poulos & Howard, 1987), the tertiary structure of membrane-bound microsomal P-450s has not been understood. With appropriate alignments of amino acid sequences of membrane-bound P-450s together with that of P-450$_{cam}$, it is feasible to speculate the tertiary structure of membrane-bound P-450s to a certain extent (Nelson & Strobel, 1988; Gotoh & Fujii-Kuriyama, 1989). Furthermore, modern DNA recombinant techniques have enabled us to replace amino acids at the specific site of the huge protein molecule (Blow et al., 1986). Those techniques have become highly useful for studying the structure–function relationship of the enzyme. With the aim of understanding the structure of the heme environment of membrane-bound P-450, we first replaced amino acids of the conserved Cys in the carboxy-terminal region of the membrane-bound P-450$_d$ (Shimizu et al., 1988). It was proved that the conserved Cys in the carboxy-terminal region of the membrane-bound P-450 is the axial ligand of P-450 and that the structure of the proximal heme environment of the membrane-bound P-450 is similar to that of water-soluble P-450$_{cam}$. On the basis of the sequence alignment and the X-ray crystal structure, we further replaced amino acids at the putative distal region of P-450$_d$ (Furuya et al., 1989a,b). It was implied that the putative distal amino acids of the heme in P-450$_d$ are important for catalytic activities and/or substrate specificities. Nevertheless, the precise tertiary structure of membrane-bound P-450 is not known yet. It was thought necessary to obtain more direct structural information on this membrane-bound P-450$_d$ by conventional spectral methods.

Optical absorption spectra of P-450$_{cam}$ (Dawson et al., 1982) and microsomal P-450s (White & Coon, 1982) provided valuable information on the heme environment of this enzyme. To obtain more precise structural information on the heme

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[1] Abbreviations: P-450, cytochrome P-450; P-450$_d$, rat liver microsomal cytochrome P-450$_d$, which corresponds to P-450IA2; P-450$_{cam}$, cytochrome P-450 purified from *Pseudomonas putida* grown in the presence of camphor, which corresponds to P-450CI; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; ESR, electron spin resonance; 7-ethoxycoumarin, 7-ethoxy-2*H*-1-benzopyran-2-one; acetanilide, *N*-phenylacetamide; metyrapone, 2-methyl-1,2-di-3-pyridyl-1-propanone; Emulgen 913, poly(oxyethylene) *p*-nonylphenyl ether containing 13.1 oxyethylene units on average; K_d, dissociation constant.

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