Role of Histidine 64 in the Catalytic Mechanism of Human Carbonic Anhydrase II Studied with a Site-Specific Mutant†

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ABSTRACT: To test the hypothesis that histidine 64 in the active site of human carbonic anhydrase II functions as a proton-transfer group in the catalysis of CO_2 hydration, we have studied a site-specific mutant having histidine 64 replaced by alanine, which cannot transfer protons. The steady-state kinetics of CO_2 hydration has been measured as well as the exchange of ^{18}O between CO_2 and water at chemical equilibrium. The results show that the rate of exchange between CO_2 and HCO_3^- at chemical equilibrium is essentially unaffected by the amino acid substitution at pH >7.0 and slightly decreased in the mutant at pH <7.0 (by a factor of 2 at pH 6.0). However, in the absence of buffer the rate of release from the active site of water bearing substrate oxygen is smaller by as much as 20-fold for the mutant as compared to unmodified enzyme. Furthermore, in the unmodified enzyme water release is inhibited by micromolar concentrations of Cu^{2+} ions, but no such inhibition is observed with the alanine 64 variant. These results suggest that the mutation has specifically affected the rate of proton transfer between the active site and the reaction medium. This kinetic defect in the mutant can be overcome by increasing the concentration of certain buffers, such as imidazole and 1-methylimidazole, but not by other buffers, such as MOPS or HEPES. Similarly, the maximal rate of CO_2 hydration at steady state catalyzed by the alanine 64 variant is very low in the presence of MOPS or TAPS buffers but considerably higher in the presence of imidazole derivatives. The main conclusion derived from these results is that histidine 64 in native carbonic anhydrase II functions as a proton-shuttle group, providing an efficient pathway for the transfer of protons between the active site and buffer molecules in solution. An alternative pathway involving direct proton transfer between the metal site and buffer can also contribute significantly to the overall catalytic rate, but the efficiency of this pathway depends on the chemical nature of the buffer compound.

The catalytic mechanism of carbonic anhydrase is of interest because of the very wide occurrence of this enzyme in plants and animals, because of its role in several physiological processes involving CO_2, and because of its very rapid turnover in the catalytic hydration of CO_2, as great as 10^6 s^{-1} for human isozyme II found in red blood cells and many other tissues (Maren, 1967). The catalytic mechanism for the hydration of CO_2 has been shown to occur in two distinct reaction sequences, a topic that has been reviewed (Silverman & Lindskog, 1988; Lindskog, 1983). The major catalytic group for the first reaction sequence is a zinc-bound hydroxide ion that reacts with CO_2 to form HCO_3^-. The pK_a of the zinc-bound water molecule is near 7 for carbonic anhydrase II.

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

The steps involved in eq 1 have been shown to be separate from a second series of steps: the proton transfer between the active site and the reaction medium resulting in the regeneration of the zinc hydroxide form of the enzyme and, thus, in the completion of the catalytic cycle. This second reaction sequence includes a postulated intramolecular proton transfer suggested by Steiner et al. (1975) from the solvent hydrogen isotope effects on steady-state kinetic parameters and subsequently supported by a wide body of experimental data (Silverman & Lindskog, 1988; Lindskog, 1983). This intramolecular H^+ transfer occurs between the zinc-bound water molecule and another residue in the active site and appears to limit the maximal rate of CO_2 hydration. Histidine 64 in isozyme II has been widely assumed to be the "proton-shuttle group" involved in the transfer of a proton between the metal-bound water and buffer molecules in solution, as indicated in eq 2. The solvent-exposed imidazole ring of His

$$\text{His64–EZnH}_2\text{O} \rightleftharpoons {}^+\text{H–His64–EZnOH}^- \overset{\text{buffer}}{\rightleftharpoons} \text{His64–EZnOH}^- + {}^+\text{H–buffer} \quad (2)$$

64 in isozyme II is located about 7.5 Å from the zinc ion (Eriksson et al., 1988). This side chain has a pK_a of 7.1 as determined by NMR (Campbell et al., 1975).

Further support for the proton-shuttle hypothesis was obtained from measurements of the exchange of ^{18}O between CO_2 and water catalyzed by carbonic anhydrase. Silverman et al. (1979) observed that ^{18}O abstracted from substrate HCO_3^- transiently labels the active site and showed that the rate constants for ^{18}O exchange measured at chemical equilibrium can be used to obtain the rates of two steps in catalysis. The first is designated R_1, the rate of interconversion of CO_2 and HCO_3^- at chemical equilibrium (eq 1). The second is designated R_{H_2O}, the rate of release from the enzyme of water bearing substrate oxygen. Both the pH profile and the solvent hydrogen isotope effects of R_{H_2O} are consistent with a rate-limiting, intramolecular proton transfer between two groups with pK_a values both near 7, presumably zinc-bound water and

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the side chain of His 64 (Tu & Silverman, 1985).

This hypothesis gained further support from the finding that Cu^{2+} and Hg^{2+} ions at concentrations below 1 μM are inhibitors of R_{H_2O} without having an effect on R_1 (Tu et al., 1981). The properties of this inhibition suggested the coordination of these metal ions to the imidazole side chain of His 64, blocking its role in proton transfer. These suggestions were confirmed by Eriksson et al. (1986), who used X-ray diffraction to determine that Hg^{2+} is indeed bound to the side chain of His 64.

Forsman et al. (1988a,b) used site-specific mutagenesis to prepare variants of human carbon anhydrase II having lysine, glutamic acid, glutamine, or alanine at sequence position 64. Initial kinetic results measured at pH 8.8 in 50 mM 1,2-dimethylimidazole buffers showed that all these variants have high CO_2 hydration activities under these conditions, contrary to expectations based on the proposed role of His 64 as a proton shuttle. For example, the variant H64A,[1] having His 64 replaced by alanine that cannot take part in proton transfer, yielded a value of the turnover number, k_{cat}, in CO_2 hydration of nearly 60% of that of unmodified, cloned isozyme II (Forsman et al., 1988b).

We have expanded these initial studies and now report steady-state and equilibrium kinetic properties of the H64A variant over a range of pH values. The results show that H64A isozyme II is indeed a defective catalyst of CO_2 hydration, in agreement with the proton-shuttle hypothesis. Certain buffers, however, such as imidazole and 1,2-dimethylimidazole, can partially substitute for His 64 as agents of proton transfer between the enzymic active site and the reaction medium.

Materials and Methods

Enzymes. Unmutated and mutated human carbonic anhydrase II cDNA were expressed in cured Y1090 *lon⁻ Escherichia coli* (Young & Davis, 1983) or in the *lon⁻ E. coli* strain SG 20043 (Triesler & Gottesman, 1984) from a plasmid described previously (Forsman et al., 1988a). In vitro site-directed mutagenesis and cell growth conditions have been described (Forsman et al., 1988b). Modified and unmodified carbonic anhydrases were purified by affinity chromatography essentially according to the method of Khalifah et al. (1977). All carbonic anhydrase concentrations were estimated from absorbance measurements at 280 nm by using a molar absorptivity of 5.48×10^4 M^{-1} cm^{-1} (Nyman & Lindskog, 1964) on the basis of M_r = 29 300 (Henderson et al., 1976).

Steady-State Kinetics. Initial rates of CO_2 hydration were measured in a Hi-Tech stopped-flow spectrophotometer at 25 °C by the changing pH–indicator method (Khalifah, 1971; Steiner et al., 1975). Buffer–indicator pairs used were 1-methylimidazole, imidazole, or 3-(*N*-morpholino)propanesulfonic acid (MOPS) with 4-nitrophenol measured at 400 nm and 1,2-dimethylimidazole or 3-[[tris(hydroxymethyl)-methyl]amino]propanesulfonic acid (TAPS) with *m*-cresol purple measured at 578 nm. The ionic strength was kept at 0.1 M by using Na_2SO_4. To avoid inhibition by adventitious metal ions, 0.1 mM EDTA was included in the MOPS and TAPS buffers.

^{18}O-Exchange Kinetics. Oxygen-18 exchange experiments were carried out as described previously (Silverman et al., 1979; Silverman, 1982). This method is based on the exchange of ^{18}O between ^{12}C- and ^{13}C-containing species of CO_2 and water that occurs because of the hydration–dehydration reaction. The rate constants describing the change of ^{18}O atom fraction in ^{12}C- and ^{13}C-containing CO_2 are measured as catalysis proceeds at chemical equilibrium. Previous papers describe in detail how these rate constants are used to obtain the rates of the steps in the hydration–dehydration of CO_2 catalyzed by carbonic anhydrase. R_1 is the catalyzed rate of exchange at chemical equilibrium of CO_2 and HCO_3^- (eq 1) and R_{H_2O} is the rate of release from the enzyme of water bearing substrate oxygen (Silverman et al., 1979; Silverman, 1982).

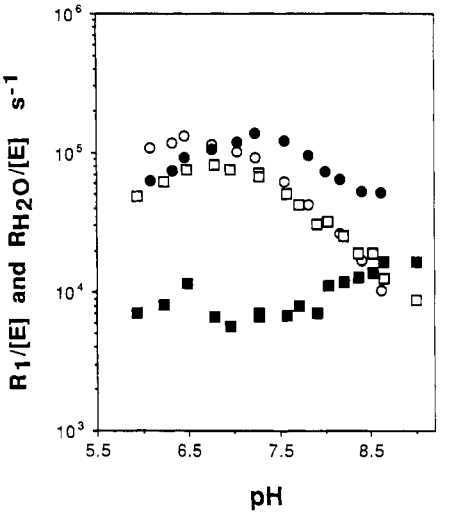

FIGURE 1: Dependence of $R_1/[E]$ and $R_{H_2O}/[E]$ on pH for unmodified and H64A human carbonic anhydrase II. Solutions contained 25 mM total concentration of CO_2 and HCO_3^-, and no buffers were used. Experiments were carried out at 10 °C with the total ionic strength maintained at 0.2 M by adding the appropriate amounts of Na_2SO_4. The rates for unmodified enzyme are (○) $R_1/[E]$ and (●) $R_{H_2O}/[E]$. The rates for the H64A variant are (□) $R_1/[E]$ and (■) $R_{H_2O}/[E]$. The concentration of unmodified and variant enzymes was 6 nM.

The calculation of R_{H_2O} requires division by a rate constant for the exchange of ^{18}O between ^{12}C- and ^{13}C-containing CO_2, a value that is sometimes small with an experimental uncertainty that is propagated into R_{H_2O} making a standard deviation of 10–30% in R_{H_2O} depending on the experimental conditions. This is a source of scatter of points observed especially for the larger values of R_{H_2O}. The precision in R_1 was somewhat better at 3–10%. To improve accuracy, some experiments were carried out at 10 °C rather than at the standard temperature of 25 °C.

All ^{18}O-exchange experiments were carried out by using solutions at a total ionic strength of 0.2 M maintained with Na_2SO_4, unless otherwise indicated. Before experiments, all glassware and the inlet vessel were washed with 10 μM EDTA and rinsed thoroughly. To sequester any metal contaminants, the ^{18}O-exchange experiments were performed by using solutions containing 10 μM EDTA, except those experiments in which Cu^{2+} ions were purposely added.

Results

The rates of catalytic interconversion of CO_2 and HCO_3^-, R_1, measured by ^{18}O exchange at 10 °C and chemical equilibrium in the absence of buffer at a total substrate concentration of 25 mM, are very similar in magnitude and pH dependence for unmodified and mutant H64A human carbonic anhydrase II (Figure 1). The values of R_1 for the H64A variant are somewhat lower than for the unmodified enzyme at pH <7. However, R_{H_2O} for the H64A variant is less by about 10-fold for 6 < pH < 8 (20-fold at pH 7) when com-

[1] Abbreviations: H64A, site-specific mutant of human carbonic anhydrase II with alanine for histidine 64; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; TAPS, 3-[[tris(hydroxymethyl)methyl]amino]propanesulfonic acid.

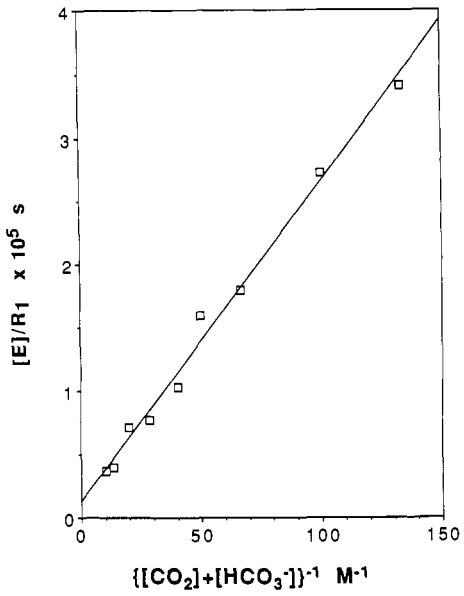

FIGURE 2: Double-reciprocal plot of the dependence of R_1 catalyzed by H64A carbonic anhydrase II on total substrate concentration. Experiments were carried out at pH 7.3 and 25 °C with the total ionic strength adjusted to 0.2 M with Na_2SO_4. No buffers were added, and the concentration of the H64A variant was 3.0 nM.

pared with that for the unmodified enzyme.

The substrate concentration dependence of R_1 for H64A at 25 °C and pH 7.3 is shown in Figure 2. The results are in accordance with eq 3 in which k_{cat}^{ex} is the maximal interconversion rate constant, K_{eff}^{S} is an apparent substrate dissociation constant, [S] is the substrate concentration, and [E] is total enzyme concentration (Koenig et al., 1974; Simonsson et al., 1979). Fitting the data of Figure 2 to eq 3 by a non-

$$R_1/[E] = k_{cat}^{ex}[S]/(K_{eff}^{S} + [S]) \quad (3)$$

linear least-squares method, we obtain $k_{cat}^{ex} = 1000 \pm 350$ ms^{-1} and $K_{eff}^{S} = 260 \pm 110$ mM (S = $[CO_2] + [HCO_3^-]$). Expressed on the basis of CO_2 concentration, $k_{cat}^{ex}/K_{eff}^{S} = 53 \pm 5$ μM^{-1} s^{-1} is obtained by taking an apparent $pK_a = 6.2$ for CO_2.

Addition of $CuSO_4$ to solutions of unmodified carbonic anhydrase II at 25 °C causes a large decrease in R_{H_2O} with a comparatively minor change in R_1 (Figure 3), as had been previously observed with human red cell carbonic anhydrase II (Tu et al., 1981). This inhibition curve for unmodified enzyme (Figure 3) is displaced to the right somewhat compared with our previous paper probably because of a weak binding of Cu^{2+} to the 25 mM HEPES buffer that was used in the present experiments; our previous experiments were carried out using no buffers. The effect of the addition of $CuSO_4$ on H64A carbonic anhydrase II is also shown in Figure 3. The effect on R_1 is minor, and there is no measurable inhibition of R_{H_2O}. The mean and standard deviation of the six values of $R_{H_2O}/[E]$ in Figure 3 for the H64A mutant is 23 ± 7 ms^{-1}.

The effects of varying the concentrations of three buffers on R_1 and R_{H_2O} catalyzed by the H64A variant at pH 7.25 and 10 °C are shown in Figure 4: imidazole, $pK_a = 7.0$; 1-methylimidazole, $pK_a = 7.2$; and MOPS, $pK_a = 7.2$. In each case there are only minor effects on R_1, but a slight inhibition by imidazole is observed. There is a very different effect of these buffers on R_{H_2O}, with imidazole causing a large increase in R_{H_2O} and MOPS having no effect up to 200 mM. The buffer HEPES ($pK_a = 7.5$) gave the same results as MOPS under the conditions of Figure 4. For red cell carbonic anhydrase II measured under the same conditions, $R_{H_2O}/[E]$ in the absence of buffer was 140 ms^{-1} and in the presence of 25 mM imidazole was 190 ms^{-1}, with no further increase of R_{H_2O} as the imidazole concentration was increased further. Pyrazole ($pK_a = 2.5$), which has a structure homologous to that of imidazole but is not a buffer in the pH range of these experiments, had no effect on $R_{H_2O}/[E]$ for the H64A variant up to pyrazole concentrations of 200 mM, but did cause a slight inhibition of $R_1/[E]$ similar to that observed with imidazole (data not shown).

Initial rates of CO_2 hydration catalyzed by cloned, unmodified carbonic anhydrase II and the H64A variant were measured at pH 7.2 and 25 °C in three buffer systems, imidazole, 1-methylimidazole, and MOPS. As shown in Table I the catalytic behavior of the unmodified enzyme is almost independent of the chemical structure of these buffers. This as well as the observed buffer concentration dependences

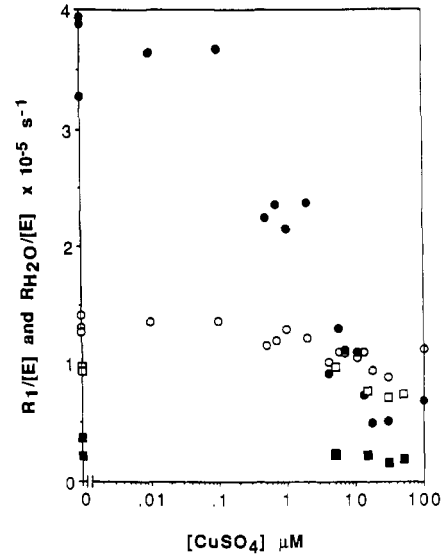

FIGURE 3: Dependence of $R_1/[E]$ and $R_{H_2O}/[E]$ on the concentration of $CuSO_4$ for unmodified and H64A human carbonic anhydrase II. Solutions contained 25 mM HEPES at pH 7.1 with the total concentration of CO_2 and HCO_3^- at 25 mM. The total ionic strength was maintained at 0.2 M by adding the appropriate amount of Na_2SO_4; the temperature was 25 °C. The rates for unmodified enzyme are (○) $R_1/[E]$ and (●) $R_{H_2O}/[E]$. The rates for the H64A variant are (□) $R_1/[E]$ and (■) $R_{H_2O}/[E]$.

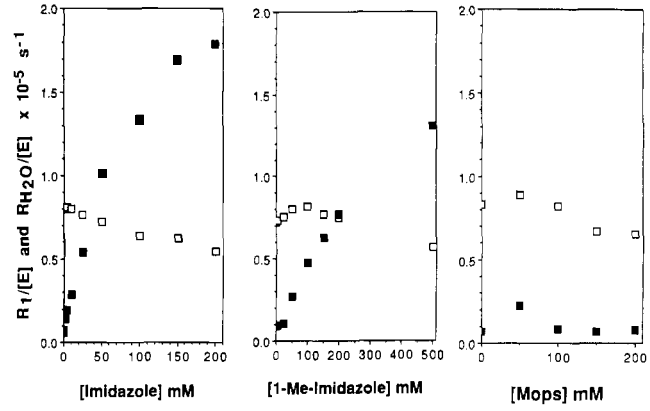

FIGURE 4: Dependence on the concentrations of three buffers of $R_1/[E]$ (□) and $R_{H_2O}/[E]$ (■) catalyzed by H64A carbonic anhydrase II. In each case the concentration of H64A was 5.0 nM, the pH was 7.25, and the temperature was 10 °C. The total concentration of substrate CO_2 and HCO_3^- was 25 mM, and the total ionic strength of solutions was maintained at 0.2 M by addition of the appropriate amounts of Na_2SO_4 (except for buffer concentrations 100 mM and greater for which no Na_2SO_4 was added but the pH was adjusted with NaOH and H_2SO_4).

Table I: Michaelis–Menten Parameters for the CO_2 Hydration Activity of Cloned, Unmodified Human Carbonic Anhydrase II (His 64) and the H64A Variant (Ala 64) in Different Buffers at pH 7.2 and 25 °C[a]

		Ala 64			His 64		
buffer	concn (mM)	k_{cat} (ms^{-1})	K_m (mM)	k_{cat}/K_m (μM^{-1} s^{-1})	k_{cat} (ms^{-1})	K_m (mM)	k_{cat}/K_m (μM^{-1} s^{-1})
imidazole	5	39 ± 1	0.9 ± 0.1	45	210 ± 5	4.2 ± 0.2	49
	50	120 ± 3	4.7 ± 0.4	25	470 ± 13	11.3 ± 0.6	42
	100	160 ± 5	8.9 ± 0.6	18	510 ± 30	13.7 ± 1.4	37
1-methylimidazole	5	15 ± 0.2	0.3 ± 0.02	45	180 ± 7	3.5 ± 0.3	52
	50	69 ± 1	2.2 ± 0.2	31	420 ± 11	9.2 ± 0.5	45
	100	110 ± 3	4.0 ± 0.3	26	510 ± 22	10.1 ± 0.9	50
MOPS	5	9	–	–	160 ± 2	3.0 ± 0.1	52
	50	13	–	–	350 ± 10	6.8 ± 0.4	51
	100	16	–	–	420 ± 14	7.8 ± 0.6	54

[a] The ionic strength was kept at 0.1 M by using Na_2SO_4. Values of k_{cat} and K_m ± SD were estimated by a nonlinear least-squares procedure. For MOPS buffers, k_{cat} values were estimated by a linear extrapolation of the Eadie–Hofstee plots at high CO_2 concentrations (Figure 5). Dash means not determined due to curved plot (Figure 5).

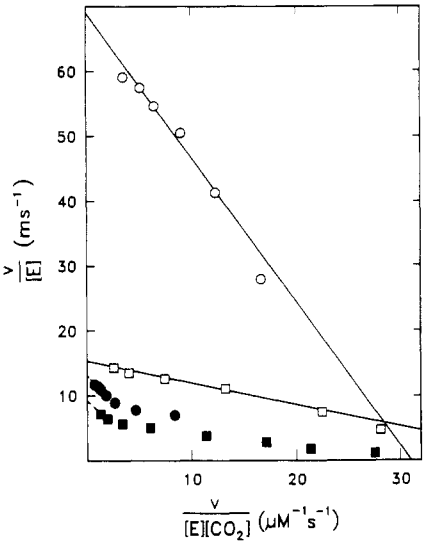

FIGURE 5: Eadie–Hofstee plots of initial rates of CO_2 hydration catalyzed by H64A carbonic anhydrase II at pH 7.2 and 25 °C. (Open symbols) 1-Methylimidazole/H_2SO_4 buffers; (solid symbols) MOPS/NaOH buffers. Buffer concentrations: (circles) 50 mM; (squares) 5 mM. The ionic strength was maintained at 0.1 M by the addition of Na_2SO_4.

agrees well with earlier observations (Jonsson et al., 1976; Rowlett & Silverman, 1982). However, the k_{cat} (and K_m) values obtained with MOPS are probably significantly smaller than those obtained with imidazole and 1-methylimidazole.

The H64A variant gave considerably smaller values of the maximal velocity than the unmodified enzyme in all three buffer systems. However, much lower rates were obtained with MOPS than with the other two buffers, and there is only a rather small dependence on MOPS concentration (Figure 5). Furthermore, when MOPS buffers are used, the H64A variant shows deviations from Michaelis–Menten behavior for reasons that are not yet understood, and the k_{cat} values given in Table I are obtained by linear extrapolation of the Eadie–Hofstee plots at high CO_2 concentrations (cf. Figure 5). With 100 mM buffers the k_{cat} values for the H64A variant are 31%, 22%, and 4% of those for unmodified enzyme in imidazole, 1-methylimidazole, and MOPS, respectively.

Imidazole seems to behave formally not only as an activator of k_{cat} but also as an inhibitor of k_{cat}/K_m. This inhibition is more pronounced with the H64A variant than with unmodified isozyme II. In contrast to the unmodified enzyme the variant seems to be inhibited also by high concentrations of 1-methylimidazole.

A few initial rate measurements of CO_2 hydration were carried out at pH 8.8 with unmodified isozyme II and the H64A variant in TAPS (pK_a = 8.4) buffers. The apparent value of k_{cat}/K_m for the H64A variant is smaller than for unmodified enzyme and seems to decrease with increasing TAPS concentration, indicating at most a 4-fold inhibition in 50 mM buffer. However, non-Michaelis–Menten kinetics, such as observed with MOPS buffers at pH 7.2, might apply, although undetected within the range of CO_2 concentrations used. (^{18}O exchange experiments carried out with the H64A variant at pH 8.4 indicated no effect of TAPS up to 200 mM on either R_1 or R_{H_2O}). More importantly, the k_{cat} value for the H64A variant in 50 mM TAPS buffer is only 3% of that for unmodified enzyme (19 and 660 ms^{-1}, respectively). This can be contrasted with previously reported results obtained in 50 mM 1,2-dimethylimidazole (pK_a = 8.2) buffers, k_{cat} = 430 ms^{-1} for the H64A variant, which is 57% of k_{cat} = 760 ms^{-1} for cloned, unmodified isozyme II (Forsman et al., 1988b).

Discussion

The recent findings by Forsman et al. (1988b), implying that site-specific variants of human carbonic anhydrase II with histidine 64 replaced by lysine, glutamic acid, glutamine, or alanine can catalyze CO_2 hydration quite efficiently, appeared to contradict the seemingly well established hypothesis involving the imidazole side chain of His 64 as a proton-transfer group. To test the proton-shuttle hypothesis further, we have carried out detailed kinetic studies on the H64A variant, since its side chain cannot participate in proton transfer. Some kinetic experiments have also been performed with the other variants (data not shown), and the results so far are similar to those obtained with H64A isozyme II.

First we discuss the rate of interconversion between CO_2 and HCO_3^- at chemical equilibrium (eq 1). This rate is estimated in ^{18}O-exchange experiments as the parameter R_1, the substrate concentration dependence of which is given in eq 3. Considering the relatively large experimental uncertainties, the value of k_{cat}^{ex} obtained for the H64A variant at pH 7.3 and 25 °C (Figure 2) is probably not significantly different from the corresponding value for red cell isozyme II under similar conditions, 1600 ms^{-1}, estimated by Simonsson et al. (1979) from NMR line broadenings of $^{13}CO_2$ and $H^{13}CO_3^-$ resonances. Thus, the amino acid substitution seems to have caused no significant change of the maximal rate of CO_2/HCO_3^- exchange at chemical equilibrium. In analogy with red cell isozyme II, k_{cat}^{ex} for the H64A variant is larger than k_{cat} for CO_2 hydration turnover at this pH (Table I), showing that steps in the exchange pathway do not limit the rate of turnover.

When [S] ≪ K_m, there must be an equilibrium distribution of enzyme forms also in the steady state. Therefore, it is in accordance with theory (Simonsson et al., 1979) that the data

in Figure 2 give a value of k_{cat}^{ex}/K_{eff}^{S} (S = CO_2) that is similar to values of k_{cat}/K_m for CO_2 hydration turnover at low (noninhibiting) buffer concentrations. Moreover, these k_{cat}/K_m values for the H64A variant are similar to those obtained for unmodified isozyme II at pH 7.2 (Table I). Thus, the replacement of His 64 by alanine has no significant effect on this CO_2/HCO_3^- interconversion parameter. This conclusion is amplified by the data in Figure 1 showing that values of R_1 for unmodified isozyme II and the H64A variant are very similar in magnitude and pH dependence when [S] < K_{eff}^{S}. The pH dependence of R_1 reflects mainly the ionization of the major catalytic group, the zinc-bound water molecule (Silverman & Lindskog, 1988; Lindskog, 1983) and the pH-dependent equilibrium between CO_2 and HCO_3^-. Forsman et al. (1988b) have previously shown that the pK_a value of the zinc-bound water molecule, as reflected in the pH profile of the 4-nitrophenyl acetate hydrolase activity, is only 0.2 unit larger for the H64A variant than for unmodified isozyme II. It is apparent from these results that the alteration of the enzyme in the H64A variant has resulted in only small changes of the rates of the reaction steps involved in CO_2/HCO_3^- exchange.

Now we turn to the steps involving proton transfer between the zinc site and the reaction medium. The same ^{18}O exchange experiments that show a similarity in R_1 show also major differences in the magnitudes of R_{H_2O} between unmodified enzyme and the H64A variant (Figure 1). In the absence of buffer, R_{H_2O} for the H64A variant is less by at least 10-fold between pH 6 and 8 when compared with that for unmodified enzyme. The magnitude and pH dependence of R_{H_2O} for the red cell enzyme has been explained by a rate-limiting, intramolecular proton transfer to the metal-bound hydroxide from His 64 (Silverman et al., 1979; Tu & Silverman, 1985). The current data are consistent with this explanation because such a proton transfer in the H64A variant is not possible.

This conclusion is also in agreement with studies of effects of Cu^{2+} and Hg^{2+} on catalysis. Eriksson et al. (1986) have shown by X-ray crystallography that Hg^{2+} binds to His 64. With Hg^{2+} bound in this manner His 64 would not be able to act as a proton shuttle. This binding has no significant effect on R_1 for red cell isozyme II at about 1 μM Cu^{2+} or Hg^{2+}, but there is a substantial inhibition of R_{H_2O} (Tu et al., 1981). Neither R_1 nor R_{H_2O} catalyzed by the H64A variant are inhibited by Cu^{2+} concentrations in this range (Figure 3), and this is consistent with the suggested role of His 64 as both proton-shuttle residue and binding site for Cu^{2+} and Hg^{2+}. In fact, the values of $R_{H_2O}/[E]$ for red cell isozyme II, maximally inhibited by Cu^{2+}, and for the H64A mutant (pH 7.1, 25 °C) are of the same order of magnitude. The significance of these values is unclear, but possibly they reflect rates of proton transfer from bulk water to zinc hydroxide in the enzymic active site (see below).

The key to the apparent inconsistency between the low R_{H_2O} values observed for the H64A variant in the absence of buffers and the previously reported (Forsman et al., 1988b) high CO_2 hydration activities is provided by the results of the kinetic studies with different buffer compounds. Thus, the magnitude of R_{H_2O} for the H64A mutant increases greatly by the addition of imidazole or 1-methylimidazole, while MOPS or HEPES has no such effect (Figure 4). Similarly, high CO_2 hydration turnover rates are observed with imidazole or 1-methylimidazole buffers, but not with MOPS buffer (Figure 5 and Table I). The compounds 1,2-dimethylimidazole and TAPS constitute another contrasting pair of buffers at higher pH. The modification of the active site in the H64A variant has resulted in a buffer specificity of proton transfer, whereas the unmodified enzyme functions almost independently of the chemical structure of the buffer. The imidazole derivatives apparently are able to transfer a proton directly between the metal-bound H_2O/OH^- and solution without the proton-shuttle group of His 64. It is not clear why MOPS, HEPES, or TAPS cannot do this; perhaps it is due to their considerably bulkier size not allowing them to reach the metal site or perhaps to their weak affinity for metal ions. The observed inhibition by imidazole and 1-methylimidazole of k_{cat}/K_m for CO_2 hydration catalyzed by the H64A variant does indicate binding by these compounds, presumably to the metal site. Pyrazole, although homologous to imidazole in structure, has a pK_a of 2.5 and cannot function as a proton-transfer agent in the pH range of these experiments. This explains the lack of effect of pyrazole on R_{H_2O} for the H64A variant.

The buffer specificity of the H64A variant allows us to speculate about the relative importance of different paths of proton transfer between the active site and the reaction medium in intact carbonic anhydrase II. The small k_{cat} value of 9 ms^{-1} obtained for the H64A variant at pH 7.2 with 5 mM MOPS buffer might approximately represent a rate constant for direct proton transfer between the metal site and bulk water. We pointed out above that Cu^{2+} inhibition of native isozyme II results in a $R_{H_2O}/[E]$ value of the same order of magnitude. Moreover, bovine carbonic anhydrase III has a buffer-independent value of k_{cat} for CO_2 hydration of about 3 ms^{-1} at pH 7 and 25 °C (Silverman & Tu, 1986; Ren et al., 1988), and this has previously been taken to imply that protons are transferred directly between the active site and bulk water without the participation of buffer. In 5 mM TAPS buffer at pH 8.8 the H64A variant gave the same k_{cat} value of 9 ms^{-1} as observed in 5 mM MOPS buffer at pH 7.2 (TAPS data not shown). This suggests that direct proton transfer from metal-bound H_2O to OH^- ions is negligible in this pH range.

Since MOPS apparently is not able to interact directly with the metal site, the k_{cat} values obtained for unmodified enzyme in the presence of MOPS buffers presumably represent proton transfer mainly via His 64. The k_{cat} values obtained for unmodified enzyme in the presence of imidazole or 1-methylimidazole buffers are about 20% larger than those obtained with MOPS (Table I). Similarly, at pH 8.8 the k_{cat} value obtained for unmodified enzyme with 50 mM 1,2-dimethylimidazole buffer is about 15% larger than that obtained with 50 mM TAPS buffer. It is tempting to suggest that these differences are due to contributions to the overall catalytic rate from proton transfer between metal site and imidazole-type buffers. When His 64 is absent, it appears that this alternate proton-transfer pathway can be particularly effective in 1,2-dimethylimidazole buffers, since Forsman et al. (1988b) found high CO_2 hydration activities in this buffer for all their variants at sequence position 64 including those with Lys 64 and Gln 64. Therefore, it does not seem likely that the alternate pathway involves binding of the imidazole derivatives at the position of the side chain of His 64. Instead, we favor an explanation of the observed saturation behavior in terms of a model involving proton transfer, perhaps through intervening water bridges, between the metal site and buffer molecules located at various positions in the active site not available to MOPS, HEPES, or TAPS.

Buffer-dependent proton transfer via His 64 in native carbonic anhydrase II is characterized by apparent K_m values for buffer in the range 1–10 mM (Jonsson et al., 1976; Rowlett & Silverman, 1982). Thus, the His 64 mediated pathway proceeds at nearly maximal rate at 100 mM buffer (cf. Table

I). However, the proton-transfer pathway in the H64A variant does not. Preliminary data on the buffer concentration dependence of rates of CO_2 hydration catalyzed by the H64A variant in 1-methylimidazole buffer at pH 7.3 and 25 °C suggest an apparent buffer K_m value of about 80 mM and a maximal k_{cat} value at infinite buffer concentration of about 175 ms^{-1}. This k_{cat} value is about 30% of the corresponding value for unmodified enzyme, suggesting that even at very high concentrations of 1-methylimidazole this alternate pathway is less efficient than the pathway involving His 64. The R_{H_2O} data in Figure 4 yield a somewhat different picture. By fitting these data to a rectangular hyperbola by a nonlinear least-squares method, we obtain buffer K_m values of about 100 mM and 500 mM for imidazole and 1-methylimidazole, respectively. The maximal values of $R_{H_2O}/[E]$ for the variant are about 250 ms^{-1} (10 °C) in both buffers, while the corresponding value for unmodified enzyme is about 200 ms^{-1}. Thus, by this criterion, proton transfer by the alternate pathway involving buffer in the H64A variant appears to be at least as effective as proton-transfer mediated by His 64 in unmodified enzyme.

In summary, our main conclusion is that His 64 in carbonic anhydrase II provides an efficient pathway for proton transfer between the active site and the reaction medium. This pathway probably dominates in the native enzyme under physiological conditions. However, our studies of the H64A variant have shown that His 64 is not an absolute requisite for efficient catalysis. Alternative proton-transfer pathways can operate, and these can also be rapid under special conditions. Thus, the function of His 64 in carbonic anhydrase II may be described as that of an effective, intramolecular buffer rendering catalysis independent of the chemical nature of surrounding buffer molecules.

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REFERENCES

Campbell, I. D., Lindskog, S., & White, A. I. (1975) *J. Mol. Biol. 98*, 597–614.

Eriksson, E. A., Jones, T. A., & Liljas, A. (1986) in *Zinc Enzymes* (Bertini, I., Luchinat, C., Maret, M., & Zeppezauer, M., Eds.) pp 317–328, Birkhäuser, Boston.

Eriksson, E. A., Jones, T. A., & Liljas, A. (1988) *Protein: Struct., Funct., Genet. 4*, 274–282.

Forsman, C., Behravan, G., Osterman, A., & Jonsson, B.-H. (1988a) *Acta Chem. Scand. B42*, 314–318.

Forsman, C., Behravan, G., Jonsson, B.-H., Liang, Z., Lindskog, S., Ren, X., Sandström, J., & Wallgren, K. (1988b) *FEBS Lett. 229*, 360–362.

Henderson, L. E., Henriksson, D., & Nyman, P. O. (1976) *J. Biol. Chem. 251*, 5457–5463.

Jonsson, B.-H., Steiner, H., & Lindskog, S. (1976) *FEBS Lett. 64*, 310–314.

Khalifah, R. G. (1971) *J. Biol. Chem. 246*, 2561–2573.

Khalifah, R. G., Strader, D. J., Bryant, S. H., & Gibson, S. M. (1977) *Biochemistry 16*, 2241–2247.

Koenig, S. H., Brown, R. D., London, R. E., Needham, T. E., & Matwiyoff, N. A. (1974) *Pure Appl. Chem. 40*, 103–113.

Lindskog, S. (1983) in *Zinc Enzymes* (Spiro, T. G., Ed.) pp 78–121, Wiley, New York.

Maren, T. H. (1967) *Physiol. Rev. 47*, 595–781.

Nyman, P. O., & Lindskog, S. (1964) *Biochim. Biophys. Acta 85*, 141–151.

Ren, X., Jonsson, B.-H., Millqvist, E., & Lindskog, S. (1988) *Biochim. Biophys. Acta 953*, 79–85.

Rowlett, R. S., & Silverman, D. N. (1982) *J. Am. Chem. Soc. 104*, 6737–6741.

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.

Triesler, P., & Gottesman, S. (1984) *J. Bacteriol. 160*, 184–191.

Tu, C. K., & Silverman, D. N. (1985) *Biochemistry 24*, 5881–5887.

Tu, C. K., Wynns, G. C., & Silverman, D. N. (1981) *J. Biol. Chem. 256*, 9466–9470.

Young, R. A., & Davis, R. W. (1983) *Science 222*, 778–782.