THE CATALYTIC MECHANISM OF HUMAN CARBONIC ANHYDRASE C: INHIBITION OF CO_2 HYDRATION AND ESTER HYDROLYSIS BY HCO_3^-

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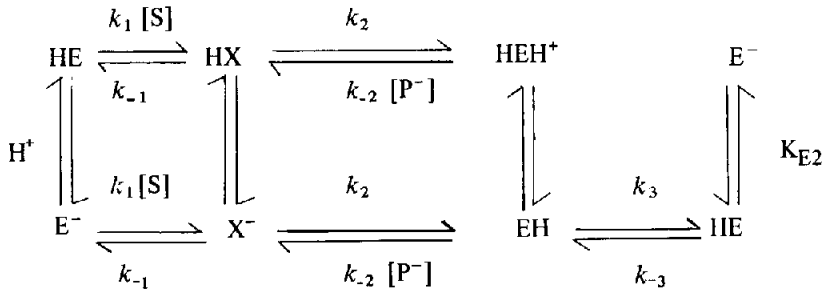
1. Introduction

The transport of H^+ between the active site and the solvent is a crucial step in the carbonic anhydrase-catalyzed interconversion of CO_2 and HCO_3^- [1,2]. Recently we reported that the steady-state parameters for both directions of the reaction are subject to substantial hydrogen isotope effects, and we put forward the hypothesis that the rate of catalysis is limited by the transfer of H^+ between a 'catalytic group' and a 'proton transfer group' [2]. The latter group was assumed to exchange H^+ rapidly with a buffered medium. The results were most easily rationalized by a reaction scheme of the following form:



(1)

In this scheme the protonated forms of the catalytic group and the proton transfer group are indicated by EH and HE, respectively, while S and P^- represent CO_2 and HCO_3^-, respectively. The transitory complexes, E^-–S and EH–P^-, are symbolized by X^-.

The intramolecular proton transfer step, EH $\rightleftharpoons$ HE, represents an isomerization of stable enzyme forms occurring between the release of product and the binding of substrate. Thus, it should be possible to test our hypothesis by studies of product inhibition [3]. In this paper we report data on the inhibition of CO_2 hydration by HCO_3^-. We have also estimated substrate binding to carbonic anhydrase C from the inhibition of the esterase activity by equilibrium mixtures of CO_2 and HCO_3^-. The results of these experiments are in accordance with the proposed mechanism and suggest that the intramolecular proton transfer step is not completely rate limiting in ^1H_2O but becomes rate limiting when the solvent is changed to ^2H_2O.

2. Materials and methods

Human carbonic anhydrase C was prepared by the method of Henderson and Henriksson [4]. Enzyme concentrations were estimated spectrophotometrically at 280 nm taking $A_{280}^{1\%} = 18.7\ cm^{-1}$ [5] and a mol. wt. of 29 300 [6]. 2(*N*-morpholino)ethanesulfonic acid (MES) and *N*-2-hydroxethylpiperazine-N'-ethanesulfonic acid (HEPES) were obtained from Sigma Chemical Co. Other chemicals were the same as used in previous investigations [2,7]. Stock solutions of CO_2 and $NaHCO_3$ were prepared as described previously [2]. The CO_2 hydration reaction was

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monitored in a Durrum-Gibson stopped-flow spectrophotometer by the 'changing pH-indicator' method [2,8]. One drive syringe contained a CO_2 solution and the other one a solution of enzyme, buffer, indicator and $NaHCO_3$. The components of the second solution were mixed by repeated transfers between interlocked syringes. Most of the experiments were performed at pH near 8, and the addition of HCO_3^- did not change the pH of the buffer significantly. In one experiment at pH 7.1 the pH of the $NaHCO_3$ stock solution was adjusted with H_2SO_4 prior to mixing with the buffer. The hydrolysis of *p*-nitrophenyl acetate was measured in the stopped-flow apparatus by monitoring the release of *p*-nitrophenol at 348 nm [7]. Because of the weak inhibition, the CO_2–HCO_3^- equilibrium mixtures were often prepared by saturation of a $NaHCO_3$ solution of the appropriate concentration with CO_2 to give the desired pH. In some cases the inhibitor solution was included in both the substrate and enzyme syringes. All measurements were performed at 25°C, and the ionic strength was kept at 0.2 with Na_2SO_4. In ^2H_2O, values of pH were estimated by the addition of 0.4 to the pH meter reading. Theoretical curves were fitted to experimental points using a Hewlett-Packard 9100B calculator with a 1925A plotter.

3. Results and discussion

3.1. *Product inhibition of CO_2 hydration*

The presence of an enzyme isomerization step on the catalytic pathway (Eq. 1) should give rise to an [S] [P⁻] term in the rate equation [3], which has the following form:

$$\frac{v_0}{[E_0]} = \frac{\frac{k^h_{cat}}{K^h_m}[S] - \frac{k^d_{cat}}{K^d_m}[P^-]}{1 + \frac{[S]}{K^h_m} + \frac{[P^-]}{K^d_m} + \frac{[S]\,[P^-]}{K_{sp}}} \qquad (2)$$

In eq. 2 the suffixes, h and d, refer to CO_2 and HCO_3^- as substrates, respectively. The [S] [P⁻] term is expected to contribute significantly to the denominator at reasonable concentrations of CO_2 and HCO_3^- unless the isomerization step is rapid compared to other steps in the catalytic cycle.

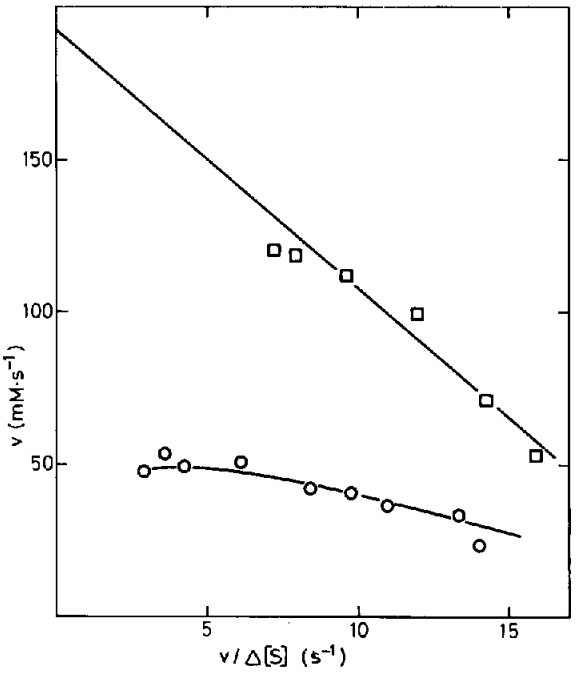

Fig.1. Product inhibition of CO_2 hydration catalyzed by human carbonic anhydrase C in ^1H_2O at pH 8.3 and 25°C. The initial velocities, v, are plotted against $v/\Delta[S]$ where $\Delta[S]$ is the initial CO_2 concentration in excess of that initially present in the HCO_3^- solution. Symbols: (□) without HCO_3^-; (○) with a constant ratio $[HCO_3^-]/\Delta[CO_2] = 12.2$. Buffer, 50 mM 1,2-dimethylimidazole-H_2SO_4 containing metacresol purple; ionic strength, 0.2; enzyme concentration, 0.21 μM. The curves were calculated using eq. 2 and $k^h_{cat} = 9.4 \times 10^5\ s^{-1}$, $K^h_m = 8$ mM, $K^d_m = 60$ mM and $K_{sp} = 2 \times 10^{-3}\ M^2$

In one set of experiments (fig.1 and fig.2) the CO_2 hydration rates were measured as a function of CO_2 concentration while the ratio $[HCO_3^-]/[CO_2]$ was kept constant. Under these conditions, [S] [P⁻] is proportional to $[S]^2$, and the expected pattern should be analogous to that of substrate inhibition. With ^2H_2O as solvent (fig.2) such a pattern is clearly observed, whereas the results obtained with ^1H_2O as solvent (fig.1) show that any contribution to the inhibition of an [S] [P⁻] term must be relatively small. However, the observed inhibition by HCO_3^- in ^1H_2O near pH 8 is significantly greater than expected from earlier kinetic schemes [9–11], because these predict that there should be no [S] [P⁻] term, and K^d_m should assume large values at alkaline pH. At

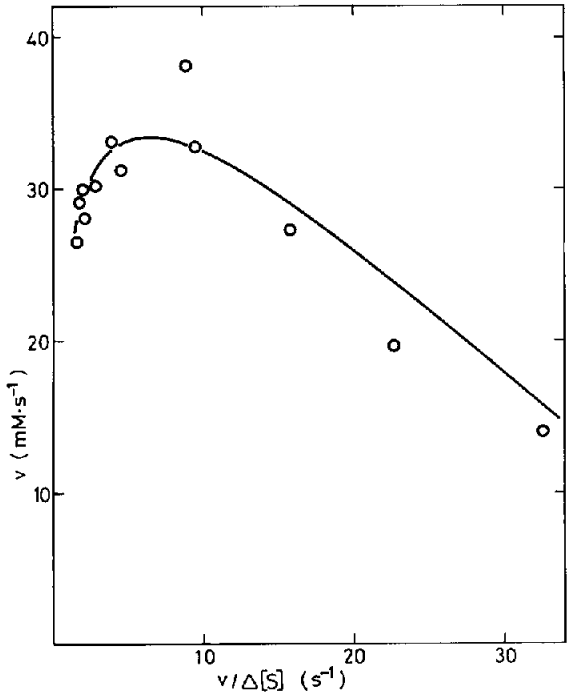

Fig.2. Product inhibition of CO_2 hydration catalysed by human carbonic anhydrase C in ^2H_2O at pH 8.4 and 25°C. The ratio $[HCO_3^-]/\Delta[CO_2]$ was 8.7. Enzyme concentration, 0.54 μM. Other conditions as in Fig.1. The curve was calculated using eq. 2 and $k_{cat}^h = 2.3 \times 10^5\ s^{-1}$, $K_m^h = 2.4$ mM, $K_m^d = 13$ mM and $K_{sp} = 2.7 \times 10^{-4}\ M^2$.

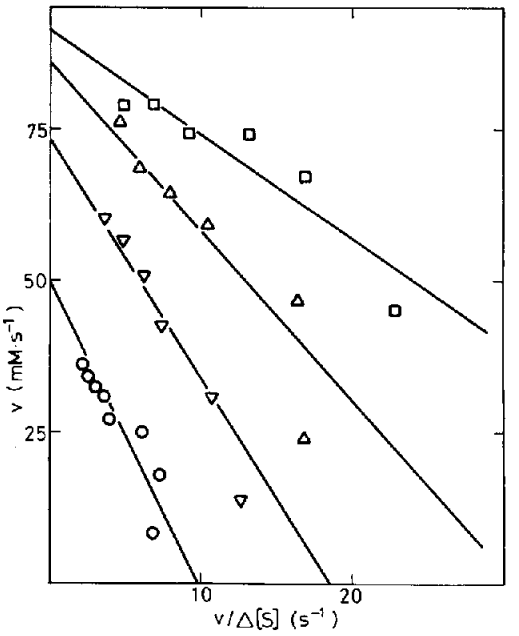

Fig.3. Product inhibition of CO_2 hydration catalyzed by human carbonic anhydrase C in ^2H_2O at pH 8.5 and 25°C. Symbols: (□) without HCO_3^-; (△) 10 mM HCO_3^-; (▽) 50 mM HCO_3^-; (○) 137 mM HCO_3^-. The enzyme concentration was 0.51 μM. Other conditions as in fig.1.

pH 7.1 K_m^d is known from previous experiments in ^1H_2O [2]. Calculations showed that the $[P^-]/K_m^d$ term is too small to account for the observed product inhibition, and an approximate value of $K_{sp} = 1 \times 10^{-3}\ M^2$ was estimated using eq. 2. Simulations of the observed inhibitions in ^1H_2O near pH 8 gave values of K_m^d about 70 mM and $K_{sp} = (1.5 \pm 0.5) \times 10^{-3}\ M^2$. The observed inhibition patterns in ^2H_2O at pH 8.3–8.4 gave K_m^d about 13 mM and $K_{sp} = (2.1 \pm 0.6) \times 10^{-4}\ M^2$. These values suggest that K_{sp} has an isotope effect which is at least as large as those previously reported for K_m^h and K_m^d [2] and possibly of the magnitude 5–10.

The significance of an $[S]\,[P^-]$ term in ^2H_2O was further tested in an experiment where $[HCO_3^-]$ was kept at different fixed values as $[CO_2]$ was varied. The results are given in fig.3 as plots of initial rates, v_0, versus $v_0/\Delta[S]$, where $\Delta[S]$ is the initial CO_2 concentration in excess of that in equilibrium with the initial HCO_3^- concentration. This transformation reduces the numerator of eq. 2 to a single term in $\Delta[S]$. The observed straight-line behaviour and the noncompetitive pattern (in Clelands terminology [12]) are in accordance with the presence of an $[S]\,[P^-]$ term and the absence of a significant $[S]^2$ term in the denominator of the rate equation. Secondary plots appeared approximately linear showing that the pattern observed in fig.2 was not caused by a $[P^-]^2$ term in the rate equation. From these secondary plots were estimated $K_m^d \approx 31$ mM and $K_{sp} = 2.9 \times 10^{-4}\ M^2$ (pH 8.5). Although this value of K_m^d is larger than those obtained in the other experiments and in our previous studies in the pH range 6–8 [2], it confirms our earlier conclusion that most of the pH dependence of the dehydration reaction must be contained in k_{cat}^d. However, it is possible that K_m^d increases by a factor of 2 or 3 between pH 6 and 8.5.

3.2. *Inhibition of the esterase reaction by HCO_3^- and CO_2*

In earlier kinetic models for carbonic anhydrase the observed pH independence of K_m^h was rationalized by the assumption that CO_2 binds independently of the ionization state of the catalytic group [1]. As shown by Kernohan [10] and by Khalifah and Edsall [11] these models predict that K_m^h should be a substrate dissociation constant. In contrast, one consequence of a rate-limiting isomerization step in eq. 1 is that K_m^h would be a kinetic parameter differing in magnitude and pH dependence from the apparent substrate dissociation constant [2].

We have attempted to estimate substrate binding by measuring the inhibition of the carbonic anhydrase-catalyzed hydrolysis of *p*-nitrophenyl acetate by equilibrium mixtures of CO_2 and HCO_3^-. The observed inhibition is quite weak, and in most cases only one, high inhibitor concentration was used. The values of K_i shown in fig.4 are based on the total concentrations of CO_2 and HCO_3^-, and they are calculated on the assumption that the enzyme forms 1:1 complexes with these substrates, and that these complexes are completely inactive in the esterase reaction. (Preliminary results with the human B enzyme, which is more strongly inhibited, suggest that these asssumptions are justified in that case.) The data are in approximate accordance with the pH dependence predicted from eq. 1, but in the calculation of the theoretical curve in fig.4 allowance has also been made for the binding of CO_2 to the acidic form of the catalytic group. This binding would be unimportant in most kinetic experiments, because it is characterized by a K_i of about 400 mM, 50-fold larger than K_m^h (8 mM in ^1H_2O [2]). Therefore, we conclude that if the inhibition of the esterase activity reflects the kinetically relevant substrate binding in the CO_2–HCO_3^- interconversion, then the earlier kinetic models must be abandoned. In addition, our results suggest that K_i has practically no isotope effect, whereas the K_m values have previously been shown to have isotope effects of 3–4 [2].

3.3. *Tentative interpretations*

The steady-state rate equation associated with eq. 1 yields relations between rate constants and the estimated parameters of eq. 2. Likewise K_i can be expressed in the rate constants of eq. 1. In principle,

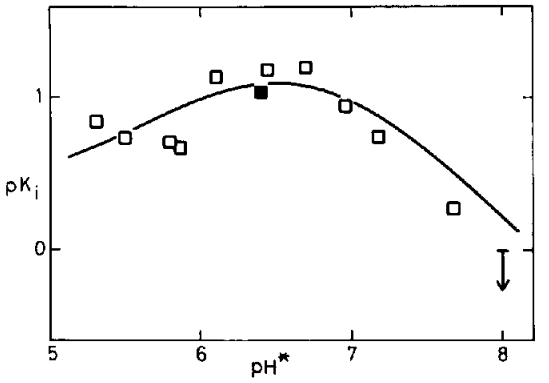

Fig.4. Inhibition by HCO_3^- and CO_2 of the hydrolysis of *p*-nitrophenyl acetate catalyzed by human carbonic anhydrase C. Values of K_i are calculated on the basis of the total concentrations of CO_2 and HCO_3^-. Open symbols, ^1H_2O; filled symbol, ^2H_2O. At pH 8 in ^1H_2O no significant inhibition was detected with 100 mM HCO_3^-. Buffers: 50 mM HEPES above pH* 7; 50 mM MES below pH* 7. The symbol pH* denotes uncorrected pH meter readings. Substrate concentration, 0.8 mM; enzyme concentration, 2 μM; ionic strength, 0.2; temperature, 25°C. The curve was calculated on the basis of eq. 1 taking apparent pK_a values for carbonic acid and the catalytic group of 6.3 and 6.8, respectively. The ratio k_2/k_{-2} (eq. 1) was taken as 35 mM, and it was further assumed that the acidic form of the catalytic group binds CO_2 with a dissociation constant of 400 mM.

these relations provide a test of how well eq. 1 describes the experimental results. Our calculations indicate that eq. 1 is probably an oversimplification, but it is possible to find rate constants and pK_a values (table 1) which are in accordance with all the data in

Table 1
Approximate rate constants and pK_a values estimated by fitting experimental results to eq. 1.

Parameter		Value in ^1H_2O	Value in ^2H_2O
k_1	$(M^{-1}s^{-1}) \times 10^{-8}$	3	3
k_{-1}	$(s^{-1}) \times 10^{-6}$	2.5	2.5
k_2	$(s^{-1}) \times 10^{-6}$	1.5	1.5
k_{-2}	$(M^{-1}s^{-1}) \times 10^{-7}$	3	3
k_3	$(s^{-1}) \times 10^{-6}$	3	0.3
k_{-3}	$(s^{-1}) \times 10^{-6}$	0.7	0.2
pK_{E1}		6.9	7.5
pK_{E2}		7.5	7.7

K_{E1} and K_{E2} are the acid dissociation constants of the postulated 'catalytic' and 'proton transfer' groups, respectively.

this paper and our previous paper on kinetic isotope effects [2] within experimental errors. Although these errors are rather large because of limitations inherent in the 'changing pH-indicator' method, it seems reasonable to conclude that an intramolecular proton transfer step is probably rate limiting in ^2H_2O, while the catalytic rate may not be governed by a single step in ^1H_2O at 25°C (table 1). Perhaps this is a clue to the cause of the apparent discrepany with respect to the pH behaviour of K_m^d between our results [2] and those of Magid [13] who worked at 2°C. At temperatures above and below 25°C different steps may be rate limiting, and this could give rise to a variation of the shapes of the pH profiles of the Michaelis–Menten parameters with temperature.

Acknowledgements

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