KINETIC PARAMETERS OF HUMAN CARBONIC ANHYDRASE B AS DETERMINED FROM NMR LINEWIDTHS OF ^{13}C IN CO_2 AND HCO_3^-*.

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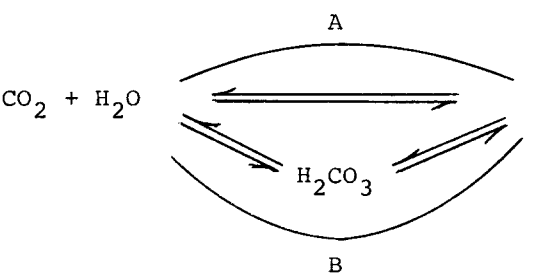
Summary: The linewidths of the ^{13}C NMR signals of CO_2 and HCO_3^-, in equilibrium aqueous solutions containing small amounts of carbonic anhydrase, are determined mainly by the rate of enzyme-induced interconversion of CO_2 and HCO_3^-. We have measured these linewidths in unbuffered solutions of human carbonic anhydrase B for several values of $[CO_2]$, at 25°C as a function of pH. From a least-squares analysis of the data, using the equations relating the linewidths to the enzyme kinetics, we have obtained values for the kinetic (Michaelis-Menten) parameters that characterize this interconversion. These preliminary results are in approximate agreement with published values for highly buffered solutions. Additionally, the results confirm that the product of the hydration reaction, and the substrate for the dehydration, is the neutral molecule H_2CO_3.

Introduction: The enzyme mechanism of carbonic anhydrase, which catalyzes the reversible interconversion of CO_2 and HCO_3^-, has been the subject of some debate (1,2). Referring to Eq. 1, the question is whether



(1)

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the enzyme catalyzes the pathway A (or what is essentially equivalent, the reaction $CO_2 + OH^- \rightleftarrows HCO_3^-$) with HCO_3^- the product of hydration and the substrate for dehydration (3), or whether the catalyzed reaction is the left-hand step of pathway B, with H_2CO_3 the hydration product and dehydration substrate (2). If A, according to Khalifah and Edsall, the enzyme changes its charge state during catalysis, and must be protonated or deprotonated as needed, to become reactivated as an enzyme molecule (3). The problem derives from the facts that 1) the rates of substrate turnover are pH dependent and exceedingly fast, so that to date only stopped-flow methods have been used to measure the kinetic parameters of the enzyme (4-6), and 2) in the pH range 5-9, over which the enzyme activity is usually studied, $[H_2CO_3] \ll [HCO_3^-]$ and is generally undetectably small, so that any interaction of carbonic anhydrase with H_2CO_3 must be inferred from data on other components of the system.

DeVoe and Kistiakowsky (4) have argued that $[H_2CO_3]$ is so small that H_2CO_3 could not be supplied to the enzyme, because of diffusion limitations, at the rate necessary to explain the rate of production of CO_2 observed. Subsequent workers have accepted this argument (5,6,7). However, under typical experimental conditions, $[H^+] \ll [H_2CO_3]$, so that if diffusion limitation prevents acceptance of the concept of H_2CO_3 as the substrate for dehydration, then it should be an even stronger argument against HCO_3^- as substrate for dehydration, since one proton must be supplied to the enzyme for each CO_2 molecule produced. Nonetheless, the belief persists that some mechanism other than diffusion will be found that can supply protons at the necessary rate (7). In principle, buffer could act as a reservoir of protons, thereby obviating the limitations of diffusion (8).

In this case, the enzyme kinetic parameters would depend strongly upon buffer concentration.

To date, measurements of the kinetic parameters have involved stopped flow, nonequilibrium methods in which pH changes are monitored optically, using an indicator dye. Because of the pH dependence of the activity of the enzyme, the system must be strongly buffered with a non-interacting buffer (6), but not so strongly as to suppress below noise the optical changes in the dye.

We have measured the hydration and dehydration kinetics of human carbonic anhydrase B, under equilibrium conditions and in the absence of buffer. The method is an extension and refinement of the early work of Patterson and Ettinger (9), in which the linewidths of the ^{13}C NMR signals from $^{13}CO_2$ and $H^{13}CO_3^-$ are used as measures of the enzyme-induced rates of interconversion of CO_2 and HCO_3^-. Preliminary results are reported below.

Materials and Methods: Human carbonic anhydrase B lyophilized from distilled water solution was obtained from John Pesando.* Small amounts redissolved in distilled water were added to several cc of a solution of NaOH in distilled water equilibrated with a gaseous mixture of $^{13}CO_2$ (95% ^{13}C) and N_2. The [NaOH] was chosen such that the $[Na^+]$ would balance the equilibrium $[HCO_3^-]$ at the desired pH and $[CO_2]$ (9). The NMR data were obtained at 25° on a Varian XL-100 spectrometer, run with a deuterium lock in the fourier transform mode, with proton noise decoupling.

Theory: It is straightforward to show that the relations between the linewidths $\Delta\nu$, the enzyme turnover velocities v, and the usual Michaelis-Menten kinetic parameters are:

*The details of the preparation are described in a PhD dissertation submitted to the Dept. of Molecular Biology, Albert College of Medicine, Yeshiva University, Bronx, N.Y. (1973).

$$v_{hyd} = \pi(\Delta\nu)_{CO_2}[CO_2] = \frac{R_{max}^{CO_2}}{1 + [H^+]/K_{ENZ}} \times \frac{[CO_2]}{D},$$

$$D = 1 + ([CO_2]/K_M^{CO_2}) + ([HCO_3^-]/K_M^{HCO_3}) \quad (2)$$

$$v_{dehyd} = \pi(\Delta\nu)_{HCO_3}[HCO_3^-] = \frac{R_{max}^{HCO_3}[E]}{1 + [H^+]/K_{ENZ}} \times \frac{[HCO_3^-]}{D} \quad (3)$$

$$= \frac{R_{max}^{H_2CO_3}[E]}{1 + [H^+]/K_{ENZ}} \times \frac{[H_2CO_3] \times 10^{-3.53}}{D} \quad (4)$$

where we have defined $R_{max} = k_{cat}/K_M$, the ratio of the usual Michaelis-Menten parameters in the limit of high pH, and we have invoked the mass action relation

$$[H^+][HCO_3^-] = 10^{-3.53} \times [H_2CO_3] \quad (5)$$

appropriate for the temperature and ionicity of our samples (10). [E] is the total enzyme concentration. We have assumed the pH dependent activity of the enzyme to be due to a single ionization with equilibrium-constant K_{ENZ} (which may be only an approximation (6)). At equilibrium, at every pH, one must have $v_{hyd} = v_{dehyd}$, and as a consequence

$$(\Delta\nu)_{CO_2}[CO_2] = (\Delta\nu)_{HCO_3}[HCO_3^-] \quad (6)$$

(after corrections for other sources of broadening);

$$R_{max}^{HCO_3}/R_{max}^{CO_2} = [H^+][HCO_3^-]/[CO_2] \equiv K_A \quad (7)$$

where pK_A for the uncatalyzed reaction is 6.1 (10); and

$$R_{max}^{H_2CO_3}/R_{max}^{CO_2} = [H_2CO_3]/[CO_2] \equiv K \quad (8)$$

where we have used K = 1/400 (10). Eqs. 7 and 8 are statements of the Haldane relationship, which must hold for any true enzyme.

Results: The observed values of $\Delta\nu$ for 7 samples with differing values of $[HCO_3^-]$, $[CO_2]$ and pH, are in Table I. Runs on several

Table I

Sample Parameters and ^{13}C Linewidth Data*

pH	$[CO_2]$**	$[HCO_3]$***	[E]	$(\Delta\nu)_{CO_2}$	$(\Delta\nu)_{HCO_3}$
	(mM)	(mM)	(μM)	(Hz)	(Hz)
5.9	24	20	24.5	4.8	7.8
6.21	12	9.7	21.7	5	4.5
6.39	4.8	10	22.5	21	6.9
7.13	24	220	85	28	2.9
7.18	12	140	39	24	1.8
7.64	24	800	180		1.6
7.85	12	350	115		1.3

*The linewidth data have been corrected by subtracting 2.0 Hz, the average value obtained for enzyme free samples, from the raw data.

**One atmosphere of 100% CO_2 at 25°C at the altitude of Los Alamos corresponds to 24 mM CO_2 in solution.

***Taken as equal to $[Na^+]$ in the solution.

solutions free of enzyme gave an average residual linewidth of 2 Hz, which amount has been subtracted from the raw data to give the tabulated $\Delta\nu$ values. Values for the five, independent, unknown parameters $R_{max}^{CO_2}$ (5.0×10^6 $M^{-1}sec^{-1}$), $R_{max}^{HCO_3}$ (6.0×10^{12} M^{-1} sec^{-1}), K_{ENZ} ($10^{-6.01}$), $K_M^{CO_2}$ (7.5 mM) and K^{HCO_3} (~ 4M) were obtained by a simultaneous least-squares fit of Eqs. 2 and 3 to the 12 values for $\Delta\nu$. $R_{max}^{H_2CO_3}$ (2.0×10^9 M^{-1} sec^{-1}) and pK_A (6.08) were computed from these paramters. The results of the fit are shown in Fig. 1, where the solid line shows $\log_{10}(R^{CO_2})$ and $\log_{10}(R^{H_2CO_3})$ as a functional pH, and the R's are defined as $R_{max}/(1 + [H^+]/K_{ENZ})$. The points are the measured $\Delta\nu$ values converted to the ordinate scales, using Eqs. 2 and 4.

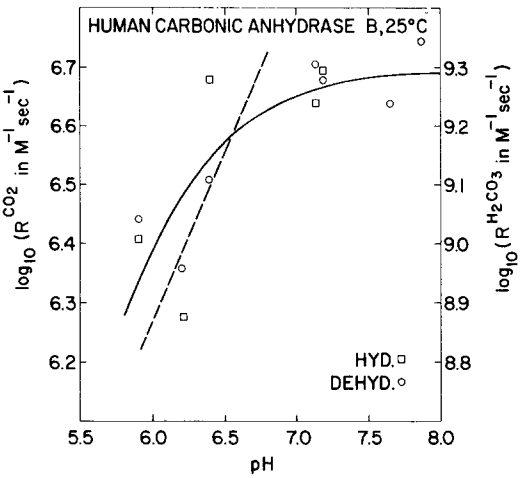

Fig. 1 The pH variation of the activity of human carbonic anhydrase B at 25°C, as determined from measurements of ^{13}C NMR linewidths in CO_2 and HCO_3^- in unbuffered solutions of the enzyme. R is the substrate turnover velocity, per mole of substrate and per mole of total enzyme present, in the limit of zero substrate concentration. The squares are derived from data on CO_2, the circles, from data on HCO_3^-. The solid line is a least-squares fit of the theory to these points. The dashed line is from the from the hydration data of Khalifah (6).

The dashed line, Fig. 1, is from the hydration data of Khalifah (6), obtained at ionic strength 0.2. His values continue to rise at higher pH, reaching ∿7.3 at pH 7.8 and 7.5 at pH 9, and do not fit a single ionization. Thus, though the present data and those of Khalifah are in excellent agreement below pH 7.0, there does appear to be a real difference between the two sets of data at high pH and comparable ionicity, but one taken in the presence, the other in the absence, of buffer. Khalifah gives $K_M^{CO_2} = 4 \pm 2$ mM, to be compared with our value of $K_M^{CO_2} =$ 7.5 mM, with a somewhat larger uncertainty which depends upon how the data are weighted in the fitting procedure.

Discussion: We summarize our results by the simple kinetic formulas which hold for $[CO_2] \ll K_M^{CO_2}$. If $[E_{ACT}]$ is the concentration of active enzyme, then for any pH:

$$v_{hyd} = 5.0 \times 10^6 \times [E_{ACT}][CO_2] \text{ M/sec} \quad (9)$$

$$v_{dehyd} = 6.0 \times 10^{12} \times [E_{ACT}][HCO_3^-][H^+] \text{ M/sec} \quad (10)$$

$$= 2.0 \times 10^{9} \times [E_{ACT}][H_2CO_3] \text{ M/sec} \quad (11)$$

The value 2.0×10^9 $(M\ sec)^{-1}$ describing the dehydration kinetics, assuming H_2CO_3 as substrate, is somewhat less than the estimated upper limit of 2.5×10^9 set by diffusion, assuming a 5 Å radius target site (2). On the other hand the value 6.0×10^{12} $(M\ sec)^{-1}$, describing the rate of approach of $[H^+]$ to the complex $E_{ACT} \cdot HCO_3^-$ is about 100-fold greater than the maximum diffusion rate possible in the absence of buffer. Thus, there can be little question, for human carbonic anhydrase B, that H_2CO_3 is the product of hydration and the substrate for dehydration, as has been argued previously on more general grounds (2).

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