THE KINETIC PARAMETERS OF CARBONIC ANHYDRASE BY ^{13}C NMR*

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ABSTRACT

Carbonic anhydrase, an enzyme found in red blood cells, catalyses the reversible hydration of carbon dioxide: $CO_2 + H_2O \rightleftarrows H^+ + HCO_3^-$. We have used measurements of the ^{13}C n.m.r. linewidths of $^{13}CO_2$ and $H^{13}CO_3^-$ in solutions of the native human B enzyme to obtain the kinetic parameters that describe the enzymatic activity. The measurements, made under thermal equilibrium conditions and with no added buffer, were taken as a function of pH and substrate concentration at 25°C, and at pH 7.7 over a range of temperatures. The kinetic results, which compare well with published results obtained by stopped-flow methods in buffered solutions, are discussed in terms of the several models that have been suggested to describe the enzymatic mechanism.

INTRODUCTION

The carbonic anhydrases are a class of enzymes that very effectively catalyse the interconversion of CO_2 and HCO_3^- in solution[1]. They are found in plants and in a variety of animal tissues. The mammalian forms are similar, each enzyme molecule of molecular weight 30000 containing (as the only metal) one Zn atom, which is necessary for enzymatic activity. In humans the B enzyme (the subject of this report) constitutes $\sim$1 per cent of the protein of red blood cells; a more active form, the C enzyme (similar in activity and sequence to the predominant bovine enzyme), is present in much lower concentrations. Though the enzymes have been known for half a century, it is only during the past decade that the various structural and functional properties have become known in any detail. The amino acid sequences and x-ray structures are now available for the human B and C isozymes[2,3], and other catalytic capabilities (e.g. the hydrolysis of esters and the hydration of aldehydes) have been studied extensively[4]. However, the physiological role of carbonic anhydrase is not yet understood. More significantly, the reasons for the observed pH dependence (with a pK near

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neutrality) of the activity[5,6] and, indeed, the nature of the enzymatic mechanism itself is a subject of considerable current controversy[7–10].

What is intriguing about carbonic anhydrase, and what has motivated the present work, is the extreme rapidity of the enzymatic interconversion of CO_2 and HCO_3^-. Whereas the lifetime of CO_2 against attack by solvent water to form H^+ and HCO_3^- is about half a minute, and is the dominant interconversion mechanism below pH 10, the addition of 2×10^{-5} M of enzyme ($\sim$0.06% by weight) reduces the lifetime for the conversion of CO_2 to HCO_3^- to $\sim 10^{-3}$ s at pH 8. This rapid action leads to an assortment of paradoxes when one attempts to model the system[7–10]. If, for example, it is assumed that the enzyme catalyses the attack of CO_2 by water and that the products of the reaction, H^+ and HCO_3^-, are released independently, then in order for the protons to diffuse away from the enzyme molecules at a rate commensurate with the observed CO_2 hydration rate, the concentration of H^+ within a $\sim$20 Å neighbourhood of the enzyme would have to build up excessively, and lower the pH to $\sim$4–5, in order to produce the concentration gradients necessary to drive the diffusion. Moreover, the enzyme is known to be inactive, and in fact unstable, at so low a pH. Conversely, the dehydration reaction ($HCO_3^- + H^+ \rightarrow H_2O + CO_2$) is observed to proceed at a rate which requires that protons be supplied to an enzyme molecule at a rate 100-fold faster than they can be transported by diffusion. If, on the other hand, it is assumed that the enzyme catalyses the attack of CO_2 by OH^-, analogous arguments can be made regarding the limitations set by diffusion of OH^-. Additionally, it is difficult to reconcile the latter assumption with the observed n.m.r. relaxation of solvent protons[11] and Cl^- anions[12] by the Co^{2+}-substituted enzyme[7], which is essentially identical in activity with the native zinc enzyme.

Because of the pH dependence and the high rate of the enzymatic activity of carbonic anhydrase, the traditional method for measuring the kinetic parameters of the enzyme has been transient, stopped-flow measurements[5,6]. In such experiments a heavily buffered enzyme solution containing a small amount of pH-sensitive dye is mixed rapidly with a similarly buffered, enzyme-free solution containing the enzyme substrate: CO_2 or HCO_3^-. The buffering is sufficient to maintain the pH reasonably constant as the substrate is converted to product after mixing (with time constant $\sim$10 ms, typically), but not so great as to prevent the dye from showing a colour change as the reaction progresses and the proton concentration changes. It has been pointed out recently[8–10] that the buffer itself could act as a reservoir of protons that can absorb, supply and transfer protons, as needed, at a rate far in excess of the rate possible in unbuffered solvent. Indeed, we note here that the enzyme–buffer–dye system can transport protons internally, and achieve an internal pH equilibrium without contact with the solvent at all. The situation is analogous to a spin system with a short T_2 caused by spin diffusion among several components (enzyme–buffer–dye) of the system, and a long T_1 to describe the contact of this system with the lattice (solvent).

It thus became important to measure the kinetics of the enzyme in the absence of buffer, yet under circumstances in which the system parameters did not change significantly during the enzymatic reaction. In water solutions

of enzyme, $^{13}CO_2$ and $H^{13}CO_3^-$, the two ^{13}C lines are separated by $\sim$900 Hz at 25 MHz, the T_1s are long (many seconds) and the linewidths are due almost entirely to the lifetime broadening caused by interchange of ^{13}C between CO_2 and HCO_3^-[13, 14]. By measuring linewidths, one observes the effects of fluctuations of the system at *thermal equilibrium*, from which the enzyme kinetics can then be deduced.

In this paper we present results obtained at thermal equilibrium for the kinetic parameters (mainly dehydration) of human carbonic anhydrase B, and derive values for the rate constants and dissociation constants of substrate and product within the framework of a Michaelis–Menten approach. This also supplements the few data in the literature for the dehydration reaction[15]; moreover, for the pH range over which data were desired, the equilibrium concentration of HCO_3^- is greater than that of CO_2, giving better signal-to-noise ratio for a given running time for the HCO_3^- signal.

KINETIC BACKGROUND

Reversible one-substrate ($\vec{S}$), one-product ($\overleftarrow{S}$), enzymatically catalysed reactions generally proceed with the formation of an enzyme–substrate complex as an initial intermediate; there may then be a chain of successive intermediates until an enzyme–product complex is formed, which dissociates into product and free enzyme. Under very general conditions, such reactions may be represented by the scheme

$$E + \vec{S} \rightleftarrows E\cdot\vec{S} \rightleftarrows E\cdot\overleftarrow{S} \rightleftarrows E + \overleftarrow{S} \tag{1}$$

and the kinetic parameters that describe the binding and turnover of substrate and product may be expressed in terms of four so-called Michaelis–Menten parameters, $\vec{k}_{cat}$, $\overleftarrow{k}_{cat}$, $\vec{K}_M$ and $\overleftarrow{K}_M$[16]. These are related to v, the net interconversion velocity of $\vec{S}$ to $\overleftarrow{S}$, by

$$v = \left[\frac{\vec{k}_{cat}[\vec{S}]}{\vec{K}_M} - \frac{\overleftarrow{k}_{cat}[\overleftarrow{S}]}{\overleftarrow{K}_M}\right][E] \tag{2}$$

and

$$[E] = [E_0]\left[1 + \frac{[\vec{S}]}{\vec{K}_M} + \frac{[\overleftarrow{S}]}{\overleftarrow{K}_M}\right]^{-1} \tag{3}$$

Here $[E]$ is the free enzyme concentration and $[E_0]$ is the total enzyme concentration. Equation (3) takes into account the binding, under dynamic conditions, of $\vec{S}$ and $\overleftarrow{S}$ to the enzyme.

These expressions are valid regardless of the number of additional intermediates. The intermediates may exist in several states of ionization (protonation) so long as only one of these states is in the enzymatic pathway. If the enzyme itself can exist in more than one protonation state (e.g. a high-pH and a low-pH form) then equation (2) will hold if there is no change of protonation of the enzyme as $\vec{S}$ and $\overleftarrow{S}$ interconvert; if there is a protonation

change, then equation (2) will still be valid if the rate of establishment of the equilibrium among the various states of the enzyme is rapid compared with the rate of turnover of substrate. In these cases [E] becomes the total concentration of all forms of free enzyme.

In stopped-flow (non-equilibrium) experiments, one tries to maintain either $[\vec{S}]$ or $[\overleftarrow{S}]$ equal to zero; then v reduces to a 'one-way' velocity $\vec{v}$, say, for the conversion of $\vec{S}$ to $\overleftarrow{S}$ when $[\overleftarrow{S}] = 0$:

$$\vec{v} = \frac{(\vec{k}_{cat}/\vec{K}_M)[E_0][\vec{S}]}{1 + ([\vec{S}]/\vec{K}_M)} \tag{4}$$

Two of the Michaelis–Menten parameters, $\vec{k}_{cat}$ and $\vec{K}_M$, can then be obtained at a given enzyme concentration, temperature, pH, etc., by measuring $\vec{v}$ as a function of $[\vec{S}]$; $\overleftarrow{k}_{cat}$ and $\overleftarrow{K}_M$ can be determined from the converse experiment.

When dealing with the CO_2–HCO_3^- system in the pH range 6–9, we can usually disregard the equilibrium concentrations of H_2CO_3 and CO_3^{2-} and regard carbonic anhydrase, *empirically*, as interconverting CO_2 and HCO_3^-. It is well established that for stopped-flow experiments, considering CO_2 as $\vec{S}$ and HCO_3^- as $\overleftarrow{S}$, equations (1–4) provide an adequate description of the experimental results[5, 6, 15, 17, 18]. This does not mean that HCO_3^- is the product of hydration of CO_2, and indeed it has been argued[7] that H_2CO_3 could well be the product with subsequent rapid dissociation ($\sim 10^{-7}$ s) into H^+ and HCO_3^-. Rather, the Michaelis–Menten language is a simple and efficient way to catalogue the experimental data. The constants k_{cat} and K_M can of course depend on pH, and even for the simplest models of the enzyme mechanism are complex algebraic combinations of the many more than four parameters that these models contain.

At thermal equilibrium $v = 0$ by definition, and $[\overleftarrow{S}]$ and $[\vec{S}]$ have equilibrium values $[\overleftarrow{S}_e]$ and $[\vec{S}_e]$ such that the ratio $[\vec{S}]/[\overleftarrow{S}]$ is determined by an equilibrium constant K_e. Then, by equation (2), there must exist a relation among the four Michaelis–Menten parameters and K_e, known as the Haldane relation[19], so that only three independent parameters, all properties of the enzyme, are needed to characterize the kinetics of the enzymatic processes considered here. Though $v = 0$, it is still possible to measure one-way velocities if the interconverting molecules can be tagged in some way. This can readily be done by ^{13}C n.m.r. for the CO_2–HCO_3^- system. In the slow exchange limit (which applies under the conditions of the experiments reported here) the time for the round trip $\overleftarrow{S} \rightarrow \vec{S} \rightarrow \overleftarrow{S}$ is much greater than T_2 for the ^{13}C resonance in $H^{13}CO_3^-$, and the lifetime broadening due to the enzymatic interconversion becomes the major source of the linewidth. This width, $\Delta\overleftarrow{\nu}$, is related to the one-way velocity per unit $\overleftarrow{S}$, and, from equation (2), is given by

$$\Delta\overleftarrow{\nu} = \overleftarrow{k}_{cat}[E]/\pi\overleftarrow{K}_M \tag{5}$$

where, from equation (3),

$$[E] = [E_0]\left[1 + \frac{[\vec{S}_e]}{\vec{K}_M} + \frac{[\overleftarrow{S}_e]}{\overleftarrow{K}_M}\right]^{-1} \equiv [E_0]\left[1 + \frac{[\overleftarrow{S}_e]}{\overleftarrow{K}_{eff}}\right]^{-1} \quad (6)$$

Here we have defined an effective dissociation constant $\overleftarrow{K}_{eff}$ which accounts for the binding of both CO_2 and HCO_3^- (and, in fact, H_2CO_3 and CO_3^{2-} as well), at equilibrium, to all forms of the enzyme present. This is the only enzyme parameter needed (and measurable) to describe the dependence of the linewidths on $[\vec{S}_e]$ or $[\overleftarrow{S}_e]$.

It should be noted (cf. equations 5 and 6) that an equilibrium experiment that measures $\Delta\overleftarrow{\nu}$ as a function of substrate concentration $[\overleftarrow{S}_e]$ yields the ratio $\overleftarrow{k}_{cat}/\overleftarrow{K}_M$, the *same quantity* obtained from a one-way stopped-flow measurement, and $\overleftarrow{K}_{eff}$, which can only be related to the results of stopped-flow measurements for both directions of the catalysed reaction.

In what follows, we present experimental results derived from linewidth measurements as a function of pH and substrate concentration at 25°C, and discuss the results in terms of current models for the enzymatic mechanism. Some limited results on the temperature dependence of the enzymatic activity also will be presented.

EXPERIMENTAL PROCEDURES

Samples

Both native and apo- (i.e. zinc-free) human carbonic anhydrases were prepared by Pesando[20] from whole blood and supplied as a powder obtained from lyophilized distilled water solutions of the highly purified enzyme. A redissolved aliquot of the native powder was tested for esterase activity and was essentially 100 per cent active, as was the supernatant of an aliquot of reconstituted apo-enzyme obtained after spinning down suspended material that invariably appears when the apo-enzyme is redissolved.

Values of $\overleftarrow{K}_{eff}$ were determined by measuring $\Delta\overleftarrow{\nu}$ as a function of $[\overleftarrow{S}]$ at fixed pH. For such a series of runs the initial sample was prepared with the maximum $[HCO_3^-]$ to be used. NaOH was first added to distilled water in amounts such that $[Na^+]$ equalled the desired $[HCO_3^-]$ at the chosen pH. The equilibrium $[CO_2]$ in solution at this pH (and temperature) was computed from the known equilibrium constant[21] of the reaction $CO_2 + H_2O \rightleftarrows H^+ + HCO_3^-$, and a mixture of $^{13}CO_2$ and dry N_2 was then bubbled through the solution. The $^{13}CO_2$ concentration in the gaseous mixture was chosen so that, at atmospheric pressure (570 mm at Los Alamos), the solution would come to the desired pH and $[HCO_3^-]$. Enzyme (either already dissolved in distilled water or as powder) was then added to yield the starting sample, typically $[E_0] \sim 1$ mM. Subsequent dilutions were made by adding distilled water. This would lower all concentrations in proportion (keeping $[E_0]/[\overleftarrow{S}]$ constant) and, in principle, not alter the pH. As a precaution against CO_2 loss upon dilution, samples were rebubbled with an appropriately altered CO_2–N_2 mixture and sealed. For representative samples the enzyme and Na concentrations were measured after the dilutions, as a check. The samples contained no buffer, but the ionic strength varied with dilution. (For future

runs, it will be held constant.) In the analysis of the data the consequences of the slight but significant dependence of the CO_2–HCO_3^- equilibrium constant on ionic strength[21] are corrected for.

NMR measurements

Data were taken on a modified XL-100 Varian Associates spectrometer operating in the Fourier transform mode, using D_2O in an inner tube as a lock. The ^{13}C linewidths were obtained either by direct measurement of the chart tracings of the resonance or from a computer fit of a Lorentzian line shape to the data; the two methods gave no systematic differences except for a few of the very dilute samples with low signal-to-noise ratio. In this case the computer fits were used, since they account better for the broad 'wings' of a Lorentzian line. Contributions to the linewidth not due to the active enzyme were obtained both from measurements of the $H^{13}CO_3^-$ linewidths in apo-enzyme solutions and in a solution of denatured native enzyme. The results were $\sim$1.25 Hz, which value was subtracted from all linewidth data obtained from solutions of active enzyme. This width is somewhat wider than the very narrowest lines observed under some conditions, and there do appear to be several contributions to the linewidth of the order of a few tenths of a hertz each. Though for the present these are somewhat elusive (micromolar concentrations of paramagnetic ion impurities cause broadenings of this order), they do appear consistently and reproducibly, so that there should be no appreciable systematic errors in the present results from these sources. For the data reported here typical linewidths were of the order of 5 to 10 Hz, with over-all uncertainty (due to resolution, measurement error and uncertainties in the non-enzyme contribution) of $\sim$0.3 Hz.

EXPERIMENTAL RESULTS

For our experimental procedure, in which the ratio $[HCO_3^-]/[E_0]$ is kept constant as $[HCO_3^-]$ is reduced, by use of equation (6), equation (5) can be written as

$$\frac{1}{\Delta\overleftarrow{\nu}} = \frac{\pi\overleftarrow{K}_M[\overleftarrow{S}]}{\overleftarrow{k}_{cat}[E_0]}\left[\frac{1}{[\overleftarrow{S}]} + \frac{1}{\overleftarrow{K}_{eff}}\right] \tag{7}$$

Thus a plot of $[E_0]/[\overleftarrow{S}]\pi\Delta\overleftarrow{\nu}$ vs $1/[\overleftarrow{S}]$ should give a straight line, the slope of

Table 1.

pH	$(\overleftarrow{k}_{cat}/\overleftarrow{K}_M) \times 10^{-6}$ $M^{-1}s^{-1}$	$\overleftarrow{K}_{eff}$ mM	pH	$(\overleftarrow{k}_{cat}/\overleftarrow{K}_M) \times 10^{-6}$ $M^{-1}s^{-1}$	$\overleftarrow{K}_{eff}$ mM
6.4	3.8	5.6*	7.2	1.4	24†
6.7	2.5	8.9*	7.45	1.15	21*
6.9	2.7	11†	7.55	1.5	17†
7.0	1.45	15*	7.75	1.0	24*
7.2	1.3	19*	7.9	1.2	26†

* $\overleftarrow{K}_{eff}$ values obtained from *Figure 3*.

† $\overleftarrow{K}_{eff}$ values obtained from data of *Figure 1*.

which gives $\vec{K}_M/\vec{k}_{cat}$, and the intercept on the abscissa equals $1/\vec{K}_{eff}$. Data of this type, for four pH values, are shown in *Figure 1*, and derived values for the Michaelis–Menten parameters are given in *Table 1*. These results for $\vec{k}_{cat}/\vec{K}_M$ are shown in *Figure 2*, along with results derived from other linewidth data taken for a single $[HCO_3^-]$ at a given pH. These latter data were

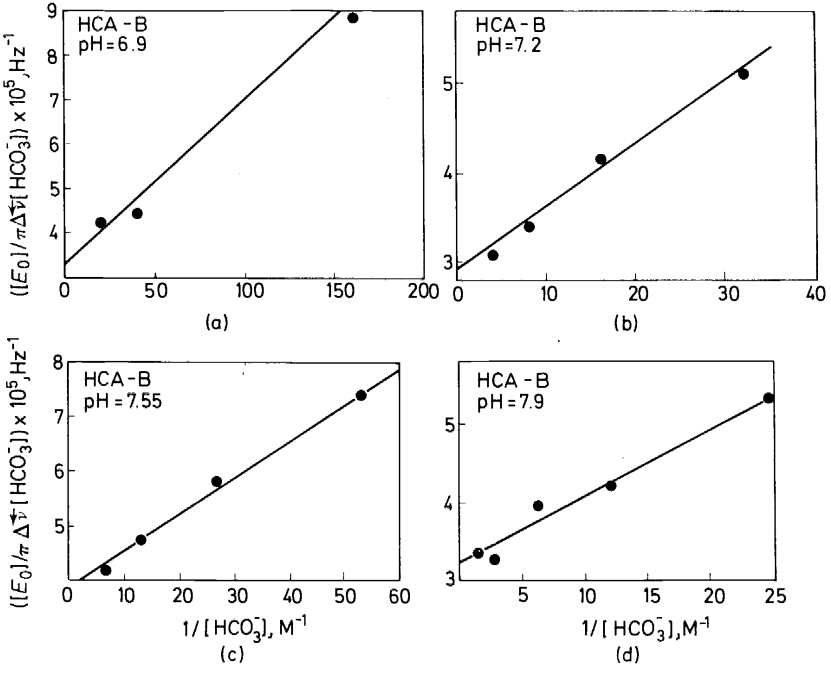

Figure 1. Reciprocal linewidth (corrected as in text) of the ^{13}C resonances in $H^{13}CO_3^-$ plotted against reciprocal HCO_3^- concentration, at 25°C, for solutions of human carbonic anhydrase B and $NaHCO_3$ in distilled water. The reciprocal linewidths are normalized, as per equation (7), by multiplying by $[E_0]/\pi[HCO_3^-]$. For (a), (b), (c) and (d), respectively, pH = 6.9, 7.2, 7.55 and 7.9, and $[E_0]/[HCO_3^-] = 8.6 \times 10^{-3}$, 5.9×10^{-4}, 3.0×10^{-3} and 8.4×10^{-4}

reduced by use of values of $\vec{K}_{eff}$ obtained as described below. The dashed hydration line (*Figure 2*) is from the stopped-flow hydration data of Khalifah[6] and Edsall and Khalifah[22], and the dashed dehydration line is calculated from these data by invoking the Haldane relation. The solid line through our dehydration data points was drawn to represent an average of the data. The solid hydration line was obtained by similarly drawing a smooth line through the hydration rates calculated (but not shown) from our dehydration data by use of the Haldane relation. The agreement is impressive, in our view, though our rates are systematically greater than the stopped-flow results, the more so at the higher pHs. *Figure 3* shows the results (*Table 1*) for $\vec{K}_{eff}$, including a low pH value taken equal to Khalifah's result[6] for $\vec{K}_M$ of 4 ± 2 mM and a line giving the theoretical pH dependence expected (to be discussed elsewhere) on the basis of the models (equation 8 or equation 10). A pK for the enzyme activity of 8.1 was assumed and $\vec{K}_M$ was treated as an adjustable parameter

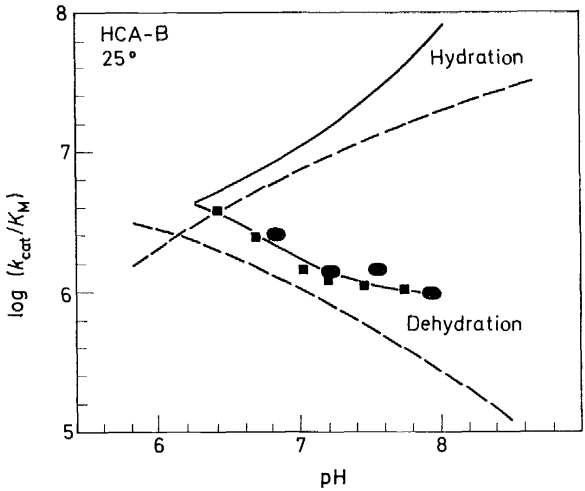

Figure 2. The pH dependence of $\overleftarrow{k}_{cat}/\overleftarrow{K}_M$, the activity of human carbonic anhydrase B for the for the dehydration of HCO_3^- at 25 °C. The ovals indicate the results obtained by measuring the activity as a function of $[HCO_3^-]$, so that $\overleftarrow{k}_{cat}/\overleftarrow{K}_M$ and $\overleftarrow{K}_{eff}$ were independently obtained (cf. equation 7). The size of the ovals indicates the spread in pH values during a run due to changing ionic strength upon successive dilutions. The squares are from linewidth data taken for only a single value of $[HCO_3^-]$ and reduced using values of $\overleftarrow{K}_{eff}$ interpolated from the graph in *Figure 3*. The lower solid line is a smooth curve drawn through the data points, from which a hydration rate curve, the upper solid line, was calculated by use of the Haldane relation and an appropriate pK value for the reaction: $CO_2 + H_2O \rightleftarrows H^+ + HCO_3^-$. The upper dashed line is from the stopped-flow CO_2 hydration results of Khalifah and Edsall[6, 22]; the lower dashed line is the corresponding rate for dehydration of HCO_3^-, computed as above. The stopped-flow data were taken at an ionic strength of 0.2, well above that for the bulk of the n.m.r. data; hence the differing pK values for the dashed and solid lines. The units of (k_{cat}/K_M) are $M^{-1}\,s^{-1}$.

to give the indicated fit to the limited data for $\overleftarrow{K}_{eff}$. The values of $\overleftarrow{K}_{eff}$ were read from this curve in reducing the data at a single $[HCO_3^-]$ (*Figure 2*).

Figure 4 shows HCO_3^- linewidth data as a function of temperature, for a sample with $[HCO_3^-] = 0.71$ M and with the pH maintained at 7.7 by adjusting the CO_2 partial pressure to compensate for its temperature-dependent solubility. Though small effects such as the temperature dependences of $\overleftarrow{K}_{eff}$ and the pK of the enzyme are folded into the measurements, the major temperature dependence (corresponding to an activation energy of 0.36 eV or 8.2 kcal/mol) must be due to temperature activation of the fundamental catalytic step. The increase in dehydration rate with temperature is comparable to, but greater than, the results of Kernohan[23] for the hydration rate of the bovine enzyme.

Magid[15] gives values for $\overleftarrow{k}_{cat}$ and $\overleftarrow{K}_M$ at 1.6°C. Assuming that $\overleftarrow{K}_M$ can be equated with $\overleftarrow{K}_{eff}$ at pH 7.7, we calculate a value of $\Delta\overleftarrow{\nu}$ less by a factor of 2 (or 0.3 log units) than the value extrapolated from the data (*Figure 4*). This disparity is similar to that between the n.m.r. and stopped-flow results at 25°C for this pH (*Figure 2*).

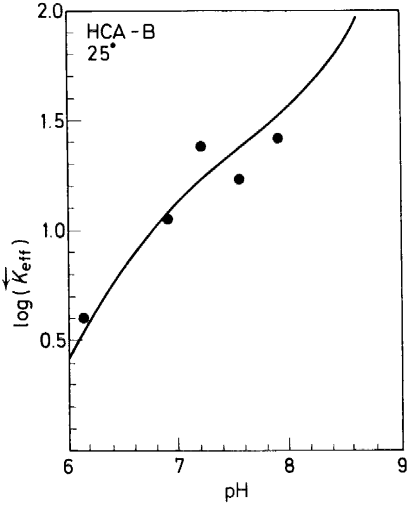

Figure 3. The pH dependence of $\overleftarrow{K}_{eff}$ of human carbonic anhydrase B at 25 °C. The point at lowest pH is computed from Khalifah and Edsall's[6, 22] value for $\vec{K}_M$ of 4 ± 2 mM, on the assumption that only the binding of CO_2 is important at low pH. The other points are from the data in *Table 1*. The solid curve is the pH variation anticipated, assuming that HCO_3^- binds only to the protonated enzyme (or, equivalently, H_2CO_3 binds only to the high-pH form of the enzyme) and that CO_2 binds independently of pH. A pK of 8.1 was assumed for the activity of the enzyme. The units of K_{eff} are mM.

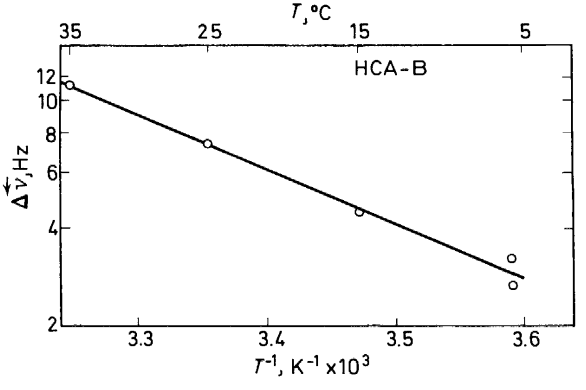

Figure 4. The temperature dependence of $\Delta\overleftarrow{\nu}$, the corrected ^{13}C linewidth of $H^{13}CO_3^-$ in a solution of human carbonic anhydrase B, at pH = 7.7, $[E_0]$ = 590 μM and $[HCO_3^-]$ = 0.71 M

DISCUSSION

We first consider the results in terms of what is perhaps the simplest general reaction scheme that describes the empirics of the situation: i.e. the interconversion of CO_2 and HCO_3^- irrespective of whether or not H_2CO_3 is an intermediate in the enzymatically catalysed interconversion. This scheme has been considered by Khalifah, Haldane and others[6, 8, 19] in some detail:

$$E + CO_2 \rightleftarrows E \cdot CO_2 \rightleftarrows EH^+ \cdot HCO_3^- \rightleftarrows EH^+ + HCO_3^- \quad (8)$$

$$EH^+ + CO_2 \rightleftarrows EH^+ \cdot CO_2$$

$$E + H^+ \rightleftarrows EH^+ \quad (9)$$

It should be quite clear that equation (8) can provide a consistent description of the hydration and dehydration rates only if the equilibrium (equation 9) can be maintained in the face of net turnover of substrate. Otherwise the available enzyme will be 'used up' to an extent that depends on the turnover rate (equation 8) relative to the rate of establishment of the equilibrium (equation 9). It is known that for typical values of substrate concentration the turnover is 100-fold faster than the rate at which the equilibrium (equation 9) can be established by proton diffusion, yet the results of the stopped-flow experiments indicate that, paradoxically, this equilibrium is in no way rate limiting. The point has been made[8, 9, 10], however, that the presence of buffer will increase the rate of establishment of this equilibrium so that equation (8) affords adequate description of the observed kinetics. (We made the point in the Introduction that the presence of buffer would allow protons to bypass this equilibrium in reaching the indicator dye.)

For the experiments reported here (and also for isotope exchange experiments[24]) the various components of the reaction are always in thermal equilibrium, the net substrate turnover is zero, and the kinetics of equation (9) become irrelevant. Rather, every ^{13}C nucleus fluctuates between being in a CO_2 or an HCO_3^- environment, *and* every enzyme fluctuates between the high- and low-pH forms. Under conditions where equation (9) would be rate limiting for transient experiments (presumably in the absence of buffer), the two fluctuations would be coupled if equation (8) were the correct description of the enzymatic mechanism. That is, if the hydration of CO_2 leaves a proton on the enzyme, then while the enzyme catalyses the CO_2–HCO_3^- interconversion (at equilibrium and with no buffer), the CO_2–HCO_3^- system must also be regarded as catalysing the E–EH^+ interconversion.

In the present experiment we measure the dynamics of the fluctuations of the CO_2–HCO_3^- interconversion and deduce the kinetics parameters. The experiment to measure the dynamics of the E–EH^+ interconversion, so that it can be determined whether the fluctuations in equations (8) and (9) are coupled, remains to be done. That it can be done is suggested by the conclusions that the chemical shifts of two histidine protons titrate with the activity of the protein[20]. An experiment similar to the present one on these two protons or one similar to those done on cytochrome-c[25], in which the fluctuation rate of a protein molecule between two forms (oxidized and reduced) was measured, is possible in principle, and should give the kinetics of the E–EH^+ interconversion as a function of $[CO_2]$.

A definitive choice could then be made between the scheme of equations (8) and similar ones in which the state of protonation of the enzyme changes during catalysis and the scheme

$$E + CO_2 \rightleftarrows E \cdot CO_2 \rightleftarrows E \cdot H_2CO_3 \rightleftarrows E + H_2CO_3 \rightleftarrows E + H^+ + HCO_3^- \quad (10)$$

for which fluctuations in the E–EH^+ system are uncoupled from and unrelated

to enzymatic action. Until then, less satisfying arguments will have to be made from comparisons of the quantitative predictions of various models for the enzymatic mechanism with experimentally derived values for the Michaelis–Menten parameters.

In summary, we have shown that it is possible to measure the rapid enzyme kinetics of carbonic anhydrase by ^{13}C n.m.r. under equilibrium conditions. The preliminary results indicate both sufficiently good agreement with the results of transient methods to validate the procedure and systematic differences which appear real and warrant further study. In particular, the n.m.r. results, at thermal equilibrium, give consistently higher kinetic velocities than the transient measurements taken under conditions of continual enzyme turnover. The method can be extended to other fast enzymatic reactions normally accessible to study only by transient techniques.

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