

Thermodynamics of Carbonic Anhydrase Catalysis

A COMPARISON BETWEEN HUMAN ISOENZYMES B AND C*

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The CO₂ hydration and HCO₃⁻ dehydration activities of human red cell carbonic anhydrase isozymes B and C (HCAB and HCAC) have been studied as a function of temperature from 0° to 37°C. The Arrhenius plots of $\ln k_{\text{cat}}$ versus $1/T$ are linear for both isozymes in both hydration and dehydration reactions, indicating that the rate-determining steps remain unchanged over this temperature range. The 37°C hydration k_{cat} at pH 7.5, is $13 \times 10^5 \text{ s}^{-1}$ for isozyme C and $0.71 \times 10^5 \text{ s}^{-1}$ for isozyme B. K_m , for hydration, is 10 mM for C and 5 mM for B, and invariant with temperature. The uncatalyzed reactions are significantly affected by temperature, 30- to 40-fold rate enhancements being observed from 0° to 37°C. The enzyme-catalyzed processes are much less sensitive to temperature, the rate enhancements being 2- to 3-fold for HCAB and 5- to 6-fold for HCAC in this temperature range. These observations are consistent with a significant lowering of the free energy of activation by both isozymes. This effect is greater for C accounting for its higher catalytic power. The enthalpy of activation, at pH 7.5 and 8.2, in the rate-limiting step is considerably less for the B enzyme compared to C. This is, however, more than offset by a large negative entropy of activation in the case of HCAB. This observation indicates either a mechanistic difference in the rate-limiting events or a difference in the structural organizations of the active sites of the two isozymes, or both.

Carbonic anhydrase (carbonate hydro-lyase, EC 4.2.1.1) catalyzes the reversible hydration of CO₂ to HCO₃⁻. The enzyme in human red cell occurs in two isozymes, designated B and C. Both isozymes are structurally well characterized, and their tertiary structures show considerable similarity (1-3). The catalytically essential unit in both isozymes is generally believed to be zinc-bound water, the metal being also liganded to three histidines. In spite of this critical similarity in the active sites, significant differences between the two isozymes exist. The most obvious and important of these is the kinetic difference, the C enzyme being a faster or catalytically more efficient enzyme than B. The Michaelis-Menten parameters, namely k_{cat} and K_m , for CO₂ hydration and HCO₃⁻ dehydration of these isozymes and their pH dependence have been extensively studied and reviewed (4-10). We now report work on the effect of temperature on CO₂ hydration and

HCO₃⁻ dehydration of HCAB and HCAC isozymes and attempt to provide a thermodynamic basis for the kinetic difference between the two enzymes. The 37°C data, reported fully for the first time, gives an important base for physiological calculations.

EXPERIMENTAL PROCEDURES

Enzymes—Human erythrocyte carbonic anhydrase isoenzymes B and C were commercially obtained (Worthington Biochemicals, Freehold, NJ) or isolated in pure forms using an affinity chromatography technique (11). Concentrations of active enzymes were determined as described by Maren and Couto (12).

Kinetic Methods—The uncatalyzed and enzyme-catalyzed initial rates for both CO₂ hydration from 0° to 25°C and HCO₃⁻ dehydration from 0° to 37°C were determined using the methods of Maren and Couto (12). In hydration reactions, 7 mM barbital buffer was used and the mean pH (i.e. the pH at which the buffer was half-titrated) was 7.53. For dehydration, the buffer was 7.5 mM or 9 mM phosphate, and the mean pH was 7.20. Phosphate was found not to have either inhibitory or activating effect on the carbonic anhydrase-catalyzed dehydration reaction at these concentrations. No external ion was added in reaction media to adjust the ionic strength. In dehydration reaction, ionic strength varied with substrate (HCO₃⁻) concentration. Earlier work in this laboratory showed that the initial rates are insensitive to ionic strength up to at least 0.25 M (12). The buffer factor at each temperature was determined by titrating barbital and phosphate buffers with standard H₂SO₄ and NaOH, respectively, over our experimental pH ranges. A few CO₂ hydration experiments were carried out in the pH range 8.5 to 8.0, the mean pH being 8.22. In these runs, 21 mM and 42 mM barbital buffers were used respectively for experiments at 1.5° and 21°C. The higher barbital concentrations did not affect the enzymes, but made the reaction times longer. The pH of the buffer solution was adjusted at each experimental temperature.

CO₂ hydration kinetics at 37°C were studied in a Durrum-Gibson D-110 stopped flow spectrophotometer coupled with a digital oscilloscope (Nicolet Instrument Corporation, Madison, WI). The buffer and the indicator used were barbital and phenol red in final concentrations (after mixing) of 0.025 M and 2×10^{-6} M, respectively. Initial rates of absorbance changes at 557 nm were measured and then converted to initial rates of CO₂ hydration by multiplication by a buffer factor, determined as described by Khalifah (4).

Calculation of K_m and k_{cat} —At each temperature, initial rates were obtained for a set of four to six different substrate concentrations. Fig. 1 shows typical double reciprocal plots of rate versus concentration for HCAB and HCAC.¹ The solid lines represent weighted least square analysis of data, assuming each initial rate measurement has the same standard error (13). K_m and k_{cat} were usually obtained at each temperature for a set of three to six different experiments. The limits of standard deviations from the means were 12% for k_{cat} and 20% for K_m .

Calculation of Activation Parameters—The apparent activation energy ($E^\ddagger$) value for a particular pH was obtained from the slope of the Arrhenius plot of $\ln k$ versus $1/T$. The apparent enthalpy of activation $\Delta H^\ddagger = E^\ddagger - RT$. Knowing $\Delta H^\ddagger$, the apparent entropy of activation ($\Delta S^\ddagger$) was calculated by using the equation $k = (k_B \cdot T/h) \cdot$

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¹ The abbreviations used are: HCAB and HCAC, human erythrocyte carbonic anhydrase isozymes B and C.

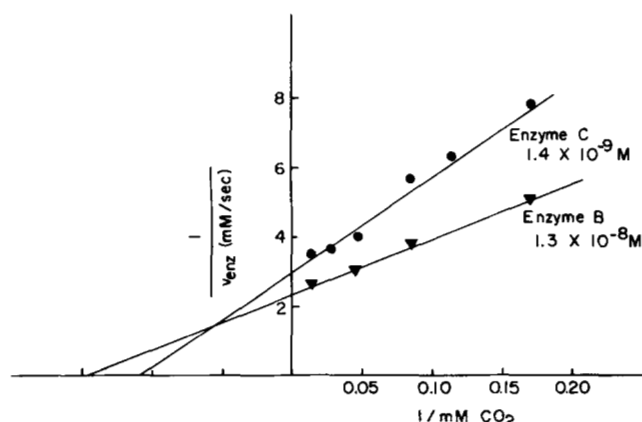


FIG. 1. Double reciprocal plots of initial CO_2 hydration rates by carbonic anhydrase B and C versus CO_2 concentration. $T = 1^\circ\text{C}$. Mean pH = 7.5. —, weighted least squares (see text). Concentrations are those of the pure enzymes.

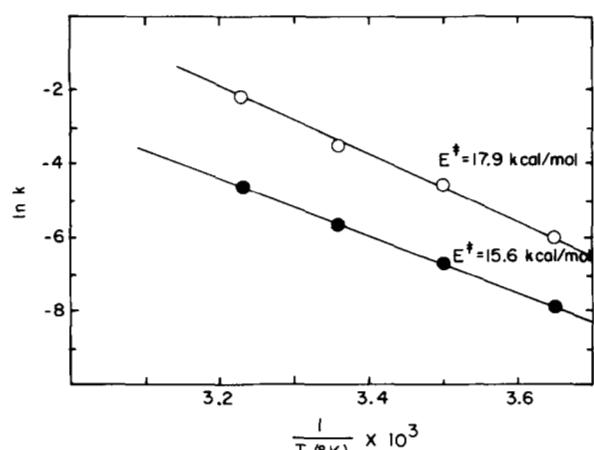


FIG. 2. Arrhenius plots for uncatalyzed rate constant (k) in s^{-1} of $\text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{H}^+ + \text{HCO}_3^-$ at pH 7.2. $\circ$, hydration (k_1); $\bullet$, dehydration (k_{-2}). Lines are drawn by unweighted least squares, correlation coefficients being 1.000 for hydration and 0.999 for dehydration. Standard deviations are less than the heights of the symbols.

$e^{-\Delta H^\ddagger/RT} \cdot e^{\Delta S^\ddagger/R}$, where k_B is Boltzmann's constant and h is Planck's constant.

RESULTS

Uncatalyzed Reactions

For the uncatalyzed reversible $\text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{H}^+ + \text{HCO}_3^-$ process, the rate constants are defined as k_1 in the forward direction and k_{-2} (at a fixed pH) in the reverse direction. Fig. 2 shows the Arrhenius plots for the k_1 and k_{-2} (for pH 7.2). Each point represents several (at least six) determinations, agreeing within 5%. The plots are linear. For k_1 , we obtain an activation energy of 17.9 kcal/mol. This agrees with earlier data (14, 15). For k_{-2} , an activation energy of 15.6 kcal/mol is obtained at pH 7.2. Table I gives the corresponding $\Delta H^\ddagger$, $\Delta S^\ddagger$, and $\Delta G^\ddagger$ values. Our k_1 data from 0° to 25°C agree with that reported and reviewed earlier (4, 14, 15). At 37°C , our k_1 is 0.110 s^{-1} . This is intermediate among data reported by several workers (5, 16, 17). The rate constant, k_{-1} , for $\text{H}_2\text{CO}_3 \rightarrow \text{H}_2\text{O} + \text{CO}_2$ is calculated from k_{-2} , using the relation $k_{-1} = (k_{-2}/\text{H}^+)$, K_1 , where $K_1 = [\text{H}^+][\text{HCO}_3^-]/[\text{H}_2\text{CO}_3]$ (see Table II). At 25°C , K_1 equals $2.5 \times 10^{-4} \text{ M}$ (18). This yields $k_{-1} = 13.2 \text{ s}^{-1}$ which agrees well with the literature. Calculation of k_{-1} at other temperatures, however, remains an outstanding problem, since the temperature dependence of K_1 has not

TABLE I

Apparent activation parameters (25°C) for uncatalyzed $\text{H}_2\text{O} + \text{CO}_2 \xrightleftharpoons[k_{-2}]{k_1} \text{H}^+ + \text{HCO}_3^-$ at pH 7.2

	$\Delta G^\ddagger$	$\Delta H^\ddagger$	$-\Delta S^\ddagger$ ^a
	kcal/mol		
	e.u.		
Hydration (k_1)	19.45	17.30	7.43 ± 0.11
Dehydration (k_{-2})	20.80	15.00	19.35 ± 0.15

^a Standard deviations represent deviations from the mean of $\Delta S^\ddagger$ values calculated from data at four temperatures. e.u. = $\text{cal deg}^{-1} \text{ mol}^{-1}$.

TABLE II

Calculation of $K = [\text{CO}_2]/[\text{H}_2\text{CO}_3]$ at 25°C from activation energy data: uncatalyzed

$$\text{H}_2\text{O} + \text{CO}_2 \rightleftharpoons \text{H}_2\text{CO}_3, K = \frac{[\text{CO}_2]}{[\text{H}_2\text{CO}_3]} = \frac{k_{-1}}{k_1} \quad (1)$$

$$\text{H}_2\text{CO}_3 \rightleftharpoons \text{H}^+ + \text{HCO}_3^-, K_1 = \frac{[\text{H}^+][\text{HCO}_3^-]}{[\text{H}_2\text{CO}_3]} \quad (2)$$

$$\text{H}_2\text{O} + \text{CO}_2 \rightleftharpoons \text{H}^+ + \text{HCO}_3^-, K'_1 = \frac{[\text{HCO}_3^-][\text{H}^+]}{[\text{CO}_2]} \quad (3)$$

or k_{-2} at fixed $[\text{H}^+]$

$$G^\ddagger(k_1) - G^\ddagger(k_{-2}) = 1.35 \text{ kcal/mol} = RT \ln \frac{k_1}{k_{-2}} \quad (4)$$

Equation 4 yields, for 25°C and pH 7.2,

$$\frac{k_1}{k_{-2}} = 9.78 \quad (5)$$

k_{-2} and k_{-1} are related as

$$k_{-2} = k_{-1} \cdot \frac{[\text{H}^+]}{K_1} \quad (6)$$

Substituting Equation 6 for k_{-2} in Equation 5, we obtain

$$\frac{k_{-1}}{k_1} = \frac{K_1}{[\text{H}^+]} \cdot \frac{1}{9.78} \quad (7)$$

Using $K_1 = 2.5 \times 10^{-4} \text{ M}$ at 25°C and pH = 7.2, Equation 7 yields

$$K = \frac{k_{-1}}{k_1} = 405$$

been established. This can only be done if one assumes the temperature effect on K_1 to be proportional to that on $K'_1 = [\text{HCO}_3^-][\text{H}^+]/[\text{CO}_2]$, which is from $5 \times 10^{-7} \text{ M}$ at 37°C to $2.6 \times 10^{-7} \text{ M}$ at 0°C (19).

The physiologically important equilibrium ratio (K) for $\text{CO}_2/\text{H}_2\text{CO}_3$ can be calculated by using the $\Delta G^\ddagger$ values for k_1 and k_{-2} . This is shown in Steps 4 to 7 of Table II and yields $K = 405$ for 25°C . This is in agreement with values reported in earlier studies (12, 14).

Catalyzed Reactions

Temperature Effect on k_{cat} —Fig. 3 shows Arrhenius plots from 0° to 37°C for both isozymes, for hydration and dehydration. This is the first complete study of the temperature problem, so we can only make comparisons with certain of the literature values. This will be attempted as follows. We exclude reference to work using 25 mM phosphate which inhibits hydration by B (20) and activates dehydration by B (5). The only previous work across the entire temperature range was by Kernohan, who plotted V_{max}/K_m for CO_2 hydration by the bovine enzyme (21). Between 5° and 50°C , only a

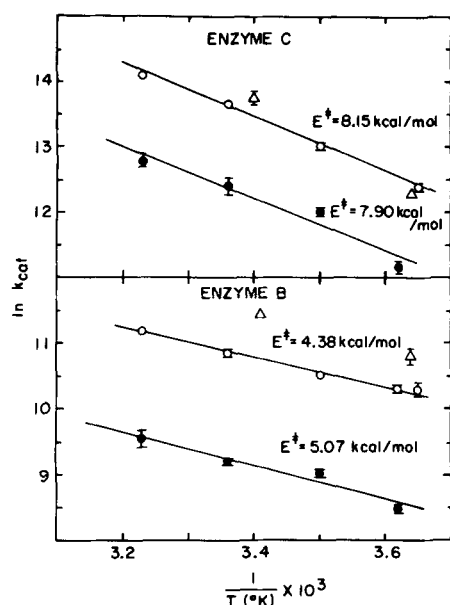


FIG. 3. Arrhenius plots for CO_2 hydration (pH 7.5) and HCO_3^- dehydration (pH 7.2) kinetics of carbonic anhydrases B and C. $\circ$, hydration; $\bullet$, dehydration. Δ , hydration at pH 8.2. —, unweighted least square lines. Correlation coefficients are between 0.970 and 0.997. The bars represent standard deviations of the mean $\ln k_{\text{cat}}$, where deviations are more than symbol heights.

2-fold increase was noted. Individual kinetic factors were not determined.

The bulk of reliable data in literature are for hydration at 25°C (4, 8, 22, 23). Our values for $k_{\text{cat}} = 860,000 \text{ s}^{-1}$ and $K_m = 12 \text{ mM}$ for enzyme C, and $k_{\text{cat}} = 51,000 \text{ s}^{-1}$ and $K_m = 4 \text{ mM}$ for B, agree well with these studies, usually within 25%. For dehydration at 25° using C, we are some 35% lower than earlier studies (22, 23); our values are $k_{\text{cat}} = 234,000 \text{ s}^{-1}$ and $K_m = 47 \text{ mM}$.

For 0°C , we agree fairly well with Christiansen and Magid (7), who studied both isozymes for hydration and dehydration in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid or low (<10 mM) phosphate buffer. For 37°C , we are in fair agreement with the only other study.²

The Arrhenius plots for both B and C isozymes are linear in either hydration at pH 7.5 or dehydration at pH 7.2. This indicates that the rate-determining steps do not change in the catalytic pathways of HCAB and HCAC enzymes. Both isozymes cause a significant lowering of the activation energy barrier for $\text{CO}_2 \rightleftharpoons \text{HCO}_3^-$ interconversion. This is reflected in the relative lack of temperature sensitivity of the enzyme-catalyzed reactions compared to the uncatalyzed reaction. While the uncatalyzed hydration and dehydration rate constants at 37°C are, respectively, 44-fold and 27-fold higher relative to the rate constants at 0°C , the turnover numbers of HCAB are increased by only 2.4-fold in hydration at pH 7.5 and 2.9-fold in dehydration at pH 7.2. Over the same temperature range for HCAC, these ratios are 5.5-fold in hydration and 5-fold in dehydration. The apparent enthalpy of activation of HCAC is higher than that of HCAB in the rate-limiting step. These differences are significant, HCAC being 3.8 kcal/mol higher for $\Delta H^\ddagger$ in the hydration reaction at pH 7.5, and 2.8 kcal/mol higher in dehydration at pH 7.2. When the apparent entropies of activation for the rate-limiting steps are calculated, it is found that the entropy of activation, e.g. in

² Data presented by P. J. Wistrand and S. Lindahl (Department of Medical Pharmacology, University of Uppsala, Sweden) at the Sixth International Congress of Pharmacology, July 20 to 25, 1975, in Helsinki.

hydration reaction, is about the same for HCAC as for the uncatalyzed reaction. The $\Delta S^\ddagger$ for HCAB catalysis is, on the other hand, about 18 e.u. more negative at pH 7.5 than for HCAC. This more than offsets the lower enthalpy barrier in HCAB catalysis, as reflected in the higher apparent free energy of activation ($\Delta G^\ddagger$) at pH 7.5 in the rate-limiting step of HCAB compared to that of HCAC. In HCO_3^- dehydration at pH 7.2, catalysis by HCAC is entropically favorable compared to the uncatalyzed reaction. Catalysis by HCAB is, however, again associated with a larger negative entropy of activation. In sum, $\Delta G^\ddagger$ values for HCAC at 25°C are 1.6 kcal/mol lower in hydration at pH 7.5 and 1.8 kcal/mol lower in dehydration at pH 7.2 than those for HCAB. Table III summarizes these thermodynamic data for 25°C . These differences are consistent with those of the turnover numbers of the two isozymes, in accordance with the equation $k = (k_B \cdot T/h) \cdot e^{-G^\ddagger/RT}$, for a particular temperature.

The CO_2 hydration kinetics of HCAB and HCAC were also studied at pH 8.2 and at two temperatures, namely, 1.5° and 21°C . The k_{cat} values are indicated in Fig. 3. Calculation of the activation parameters from the high pH data again show that the apparent enthalpy of activation is 2.4-fold lower for HCAB than for HCAC. The apparent free energy of activation for the isozyme is, however, higher due to an unfavorable entropy contribution. These data, calculated for 25°C , are given in Table IV. The apparent enthalpies of activation for both isozymes are different at pH 8.2 from those at pH 7.5, indicating a pH-dependent contribution to $\Delta H^\ddagger$.

Temperature Effect on K_m —The temperature insensitivity of K_m for the substrates CO_2 and HCO_3^- against HCAB and HCAC is shown in Fig. 4. It is difficult to explain this observation, unless the meaning of K_m is precisely known. One possibility is that K_m is not a true equilibrium constant (K_s) representing enzyme-substrate dissociation, but a composite

TABLE III
Effects of human carbonic anhydrases B and C on apparent activation parameters (25°C) for CO_2 hydration (pH 7.5) and HCO_3^- dehydration (pH 7.2)

Enzyme	Substrate	$\Delta G^\ddagger$ ^a	$\Delta H^\ddagger \pm \text{S.D.}^b$	$-\Delta S^\ddagger \pm \text{S.D.}^c$
		kcal/mol		e.u.
None	CO_2	19.45	17.30	7.43 ± 0.11
	HCO_3^-	20.80	15.00	19.35 ± 0.15
HCAC	CO_2	9.37	7.56 ± 0.14	6.07 ± 0.16
	HCO_3^-	10.13	7.30 ± 0.18	9.46 ± 0.27
HCAB	CO_2	10.97	3.79 ± 0.21	24.10 ± 0.14
	HCO_3^-	11.96	4.48 ± 0.35	25.10 ± 0.21

^a $\Delta G^\ddagger$ is calculated by using mean values of $\Delta H^\ddagger$ and $\Delta S^\ddagger$.

^b Standard deviations in $\Delta H^\ddagger$ reflect limits of variation when standard deviations in $\ln k$ at each temperature are considered. For uncatalyzed reactions, these were insignificantly small.

^c Standard deviations are deviations, from the mean, of $\Delta S^\ddagger$ values calculated from data at four temperatures, using the mean values of $\Delta H^\ddagger$. e.u. = $\text{cal deg}^{-1} \text{ mol}^{-1}$.

TABLE IV
Apparent activation parameters (25°C) for CO_2 hydration catalyzed by human red cell carbonic anhydrases B and C at pH 8.2

Calculations are based on data at two temperatures only, 1.5° and 21°C .

Enzyme	$\Delta G^\ddagger$	$\Delta H^\ddagger$	$\Delta S^\ddagger$
	kcal/mol		e.u. ^a
HCAC	9.07	11.7	8.81
HCAB	10.58	4.87	-19.15

^a e.u. = $\text{cal deg}^{-1} \text{ mol}^{-1}$.

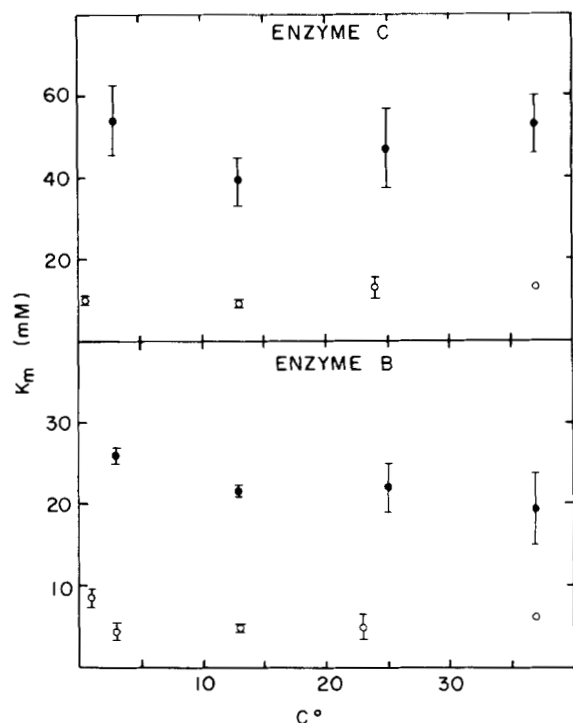


FIG. 4. Lack of temperature effect on K_m for carbonic anhydrases B and C. ○, hydration; ●, dehydration. The bars represent standard deviations of the mean.

TABLE V

Haldane constants (H) for human red cell carbonic anhydrases B and C at pH 7.2

Temperature °C	Enzyme	H^a	$\frac{\text{HCO}_3^-}{\text{CO}_2} = \frac{k_1^b}{k_{-2}}$
1-3	HCAC	12	6.6
	HCAB	11	
13	HCAC	9.9	8.9
	HCAB	12	
25	HCAC	11	10
	HCAB	11	
37	HCAC	12	11
	HCAB	11	

$$^a H = \frac{k_{\text{cat}}(\text{hyd}) \cdot K_m(\text{dehyd})}{k_{\text{cat}}(\text{dehyd}) \cdot K_m(\text{hyd})}$$

^b k_1 and k_{-2} are uncatalyzed CO_2 hydration and HCO_3^- dehydration rate constants at pH 7.2.

constant containing kinetic parameters. Alternatively, if K_m is equal to K_s , then the enthalpy of substrate binding must be close to zero and cannot be determined beyond the experimental error of our method.

Haldane Relationship—Table V gives a comparison between our kinetically determined Haldane ratios at different temperatures and the calculated equilibrium constant for $\text{HCO}_3^-/\text{CO}_2$ at pH 7.2, based on our direct measurements of uncatalyzed hydration and dehydration rate constants k_1 and k_{-2} . The k_1/k_{-2} ratios agree with the $[\text{HCO}_3^-]/[\text{CO}_2]$ equilibrium values of Harned and Davis (19). For calculating Haldane ratios, we corrected our hydration k_{cat} data at pH 7.5 to pH 7.2. This correction is based on Khalifah's pH rate profile (4), which yields pK_a values of 7.0 and 7.6 at 25°C, respectively, in the C and B enzymes for ionizations of the catalytic groups determining rates. At 25°C, the agreement between the Haldane constants for the two isozymes and the equilibrium $\text{HCO}_3^-/\text{CO}_2$ ratio is good. At other temperatures, pH correc-

tion of our hydration k_{cat} data included the change in pK_a with temperature, -0.02 per degree, determined for the bovine enzyme using the esterase activity (24). This was used in our calculation in absence of equivalent data for CO_2 hydration by HCAB and HCAC. No correction in K_m values was made since these are pH independent (4).

DISCUSSION

Our data show that at physiological pH human red cell carbonic anhydrase B has a lower activation enthalpy than the C isozyme. However, a significantly unfavorable entropy contribution is responsible for a higher apparent free energy of activation and lower catalytic efficiency of the B isozyme. A similar conclusion follows from our pH 8.2 hydration data.

A comparison of the pH 7.5 and 8.2 data (Tables III and IV) indicates that our activation enthalpy and entropy values are pH dependent. The pH-dependent contributions probably come from the temperature effect on the pK_a for the active site ionizations of the two isozymes. It is widely believed that both B and C isozymes have a zinc-bound water molecule essential for catalytic activity (see Ref. 33 for a recent discussion). The deprotonated form or the so-called zinc hydroxide form is responsible for CO_2 hydration, while the protonated form of the same group causes HCO_3^- dehydration activity. The very low pK_a of ~ 7 for this group in HCAC reflects a special, possibly hydrophobic, environment at the active site. The pK_a of this catalytically active group is at least 0.6 unit higher at 25°C in the B enzyme (4), indicating a difference in the environments of zinc-water in the two isozymes. To obtain activation enthalpy and entropy values independent of any contribution from the temperature effect on pK_a , one has to consider the effect of temperature on the pH-independent turnover number (k_{cat}^0). In hydration, k_{cat}^0 may be considered to be equivalent to the k_{cat} when the enzyme is in fully basic form and should be related to the apparent k_{cat} at a particular pH by the relation $k_{\text{cat}} = k_{\text{cat}}^0 / (1 + \text{H}^+/\text{K}_a)$. We are unable, at this point, to calculate k_{cat}^0 at different temperatures since the temperature dependence of the K_a values of the two isozymes are not known for CO_2 hydration. Calculation of k_{cat}^0 at 25°C from our pH 7.5 data, using $\text{pK}_a = 7.0$ for HCAC and 7.6 for HCAB (4), yield $1.1 \times 10^6 \text{ s}^{-1}$ and $1.2 \times 10^5 \text{ s}^{-1}$, respectively, for the C and B isozymes. These numbers are only 9% higher for HCAC and 20% higher for HCAB than our experimental values at pH 8.2 and 21°C. Our data are, then, consistent with the expected 94% ionization of HCAC and 80% ionization of HCAB at pH 8.2 and 25°C. The pK_a dependent contributions to $\Delta H^\ddagger$ must then be substantially reduced at this pH, unless the temperature effects on the active site ionizations are unusually large. Consequently, our pH 8.2 data indicate that the lower $\Delta H^\ddagger$ and more negative $\Delta S^\ddagger$ situation for HCAB, compared to HCAC, may be valid for the completely basic forms of the two isozymes in CO_2 hydration, that is to say when the data would be independent of temperature perturbation of pK_a .

It is conceivable that the large negative $\Delta S^\ddagger$ for HCAB may be due to freezing out of water molecules at the active site in the transition state. In comparison, the active site of HCAC may be more organized in the ground state, so that the loss in entropy is smaller in forming the transition state. This situation would be consistent with a looser active site structure of HCAB, accessible to more water molecules, in analogy to the proposal of Roberts and Elmore (25) for α -trypsin compared with β -trypsin. The presence of water molecules in the enzyme-active site would be of mechanistic significance if the rate-determining step is an internal proton transfer mechanism (8, 26–30) mediated through water molecules (34).

We are not certain, however, whether the activation en-

thalpies and entropies represent the same reaction steps in the two isozymes. While a proton transfer step is thought to be rate-limiting in catalysis by bovine and human C isozymes (8, 26–30), chemical identity of the rate-limiting step for the human B enzyme has not been established. There are indications that, in the presence of buffer, the proton exchange mechanism in the B enzyme is different from the mechanism in human C and bovine enzymes. This is based on proton NMR relaxation studies by Fabry (31) and an oxygen-18 exchange investigation by Tu and Silverman (32).

The kinetic meaning of K_m is not clear from our data. For human C and bovine carbonic anhydrase, k_{cat} and K_m show similar magnitudes of isotope effects, so there is no isotope effect on k_{cat}/K_m (8, 26). This is consistent with k_{cat} and K_m being composed of the same kinetic steps (8). However, since we now find that K_m is temperature independent and k_{cat} is not, k_{cat}/K_m (which is the lower limit for the second order enzyme-substrate association rate constant) varies with temperature in the same way as k_{cat} . K_m and k_{cat} do not, then, appear to be measurements of identical pathways.

Finally, we have established the 37°C kinetic parameters which may be used in physiological work. Since C is the chiefly active isozyme in red cells and is (or is very closely akin to) the carbonic anhydrase in most secretory tissues, these values are summarized. For hydration, $k_{cat} = 13 \times 10^5 \text{ s}^{-1}$ and $K_m = 13 \text{ mM}$. For dehydration, $k_{cat} = 3.5 \times 10^5 \text{ s}^{-1}$ and $K_m = 53 \text{ mM}$.

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