and A_0 is the initial concentration of A.[5] Since eq 6 and 7 have the same denominator

$$\frac{dQ}{dP} = \frac{V/K_q}{V/K_p} \quad (10)$$

and the ratio of products formed is constant regardless of the concentration of chitose present at any time in the course of the reaction. If eq 10 is integrated from zero time (when P and Q are zero) to infinite time (at which point P and Q have their final values after all chitose has been phosphorylated), one obtains

$$\frac{Q_\infty}{P_\infty} = \frac{V/K_q}{V/K_p} \quad (11)$$

and thus the ratio of total products finally formed is also the ratio of V/K values. Since about 25% more ADP was formed with fructokinase than with hexokinase, V/K for the ATPase reaction is 25% that for the kinase reaction. This simultaneous ATPase and kinase activity may also occur to a small extent when chitose is a substrate for hexokinase (see Table II), since some inorganic phosphate was formed during phosphorylation.

Acknowledgments

We thank Dr. Marion O'Leary, Department of Chemistry, for advice and help in the preparation of liquid HCN and Mark Clark, Department of Chemistry, for running the catalytic deuteriogenation experiments.

[5] Note that when either dP/dt or dQ/dt is measured, the apparent Michaelis constant will be the same, namely, $1/(1/K_p + 1/K_q)$. The V_{max} for kinase activity will be $k_3/(1 + K_p/K_q)$, while that for the ATPase activity will be $k_7/(1 + K_q/K_p)$.

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Nuclear Magnetic Resonance Studies of Carbonic Anhydrase Catalyzed Reversible Hydration of Acetaldehyde by the Saturation Transfer Method†

Dalia Cheshnovsky and Gil Navon*

ABSTRACT: The catalysis of the chemical exchange between acetaldehyde and its hydrate by bovine carbonic anhydrase B was investigated by the NMR line broadening and saturation transfer techniques. While both chemical exchange and binding to the enzyme had an effect on the line broadening of acetaldehyde and its hydrate, the saturation transfer method enabled us to measure the exchange without the interference from the effect of the binding. *p*-Toluenesulfonamide and azide ion were found to inhibit completely the carbonic anhydrase catalyzed exchange reaction. However, there was a residual line broadening of the acetaldehyde by the enzyme in their presence. This was interpreted as binding to sites other than the active site of the enzyme. The pH profile of the catalysis of the reversible hydration of acetaldehyde by carbonic anhydrase in the absence of a buffer indicates high- and low-activity forms of the enzyme with a transition at pD 7.65. The cadmium(II) enzyme was found to be inactive, and the cobalt(II) derivative was found to have similar activity to that of the native system.

The enzyme carbonic anhydrase catalyzes the interconversion of carbon dioxide and bicarbonate at a rate which is one of the fastest known for enzyme catalysis [for recent reviews, see Wyeth & Prince (1977) and Pocker & Sarkanen (1978)]. The enzyme is capable of catalyzing other reactions such as hydrolysis of some carboxylic esters (Armstrong et al., 1966) and sulfonic and carbonic esters (Pocker & Stone, 1968a; Pocker & Guilbert, 1974) and the reversible hydration of aldehydes and pyruvic acid (Pocker & Meany, 1965, 1967a,b, 1970), although with much smaller rates as compared with that of the hydration of CO_2.

Since the process of obtaining NMR spectra is relatively slow, NMR investigations of the binding of small molecules to enzymes are limited mostly to inhibitors, activators, or very slowly reacting substrates (Dwek, 1973; Mildvan, 1974; James,

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1975; Kushnir & Navon, 1975). However, the main strength of the NMR method in investigating reaction mechanisms is for exchange reactions under conditions of equilibrium. Thus, the NMR method seemed to us very suitable for the investigation of the reversible hydration of acetaldehyde and its catalysis by carbonic anhydrase.

Experimental Section

Enzyme Preparation. Bovine carbonic anhydrase B (carbonate hydrase, EC 4.2.1.1), prepared and purified by the method of Lindskog (1960), was obtained from Miles. In order to prevent possible contamination by paramagnetic impurities, all metal ions were removed by dialysis against 0.01 M 1,10-phenanthroline in 0.1 M sodium acetate buffer, pH 5.2, at 4 °C for 3 weeks. The dialysis solutions were changed every 48 h. The enzyme was further dialyzed for 4 days against four changes of 0.05 M Tris–sulfate buffer, pH 7.5, until no absorption at 264 nm due to residual 1,10-phenanthroline was detected. Zinc was introduced by dialyzing the apoenzyme against 5×10^{-4} M $ZnSO_4$ in Tris buffer, and the excess Zn^{2+} ions was removed by dialysis against two changes of 0.05 M Tris buffer, pH 7.5. Since Tris buffer was found to react with acetaldehyde, it was replaced by phosphate buffer by dialysis against four changes of 0.02 M phosphate buffer, pH 7.5, 24 h each, and then four changes of the same buffer in D_2O solution. A precipitate of denatured material was centrifuged off. All H_2O solutions were prepared in doubly distilled water. After this treatment the metal content of the enzyme was determined by an atomic absorption spectrophotometer, Varian-Techtron, Type AA-5. One gram-atom of zinc was found per mol of enzyme.

Manganese(II) carbonic anhydrase was prepared using the same procedure except that Mn(II) was introduced by dialyzing the apoenzyme against a solution of $MnSO_4$ instead of $ZnSO_4$ and the final dialyses were done against Pipes buffer solutions, which contained 5×10^{-4} mol/L $MnSO_4$. The apoenzyme for cobalt(II) and cadmium(II) carbonic anhydrase was prepared by using pyridine-1,6-dicarboxylic acid according to the method of Hunt et al. (1977). Cobalt and cadmium were introduced into the apoenzyme by dialysis against $CoSO_4$ and $CdSO_4$, respectively. Atomic absorption measurements indicated the presence of 0.99 ± 0.02 mol of manganese, cobalt, or cadmium, respectively, and 0.01 ± 0.001 mol of zinc per mol of protein.

Protein concentrations were determined spectrophotometrically at 280 nm, with a molar absorbance of $\epsilon = 57\,000$ M^{-1} cm^{-1} and a molecular weight of 30 000 (Nyman & Lindskog, 1964).

pH values were recorded as the uncorrected glass electrode reading.

Enzymatic activity was determined by measuring the esterase activity toward the hydrolysis of *p*-nitrophenyl acetate by following the absorbance at 400 nm (Armstrong et al., 1966). The optical and kinetic measurements were recorded on Varian Techtron spectrophotometer, Model 635. No effect of phosphate buffer on the enzymatic activity was found at pH 7.5.

Acetaldehyde (puris) was obtained from Fluka. It was kept in the dark at 4 °C to avoid oxidation. Fresh solutions were prepared for each experiment. The amount of the acetaldehyde oxidation product, acetic acid, present in the stock solutions was estimated by potentiometric pH titration and was found to be smaller than 0.5%. The acetaldehyde concentrations in their aqueous solutions were determined spectrophotometrically by using the value of $\epsilon = 17.5$ M^{-1} cm^{-1} for the acetaldehyde absorbance at 278 nm. This value for the molar absorbance is based on the spectrophotometric data of Bell & Clunie (1952) in the temperature range of 18–30 °C and the equilibrium constants of the acetaldehyde hydration obtained by NMR in the present work (Figure 5). This value should be compared with the value of $\epsilon = 17.0$ M^{-1} cm^{-1} obtained by Bell and Clunie by a combination of spectrophotometric and calorimetric measurements.

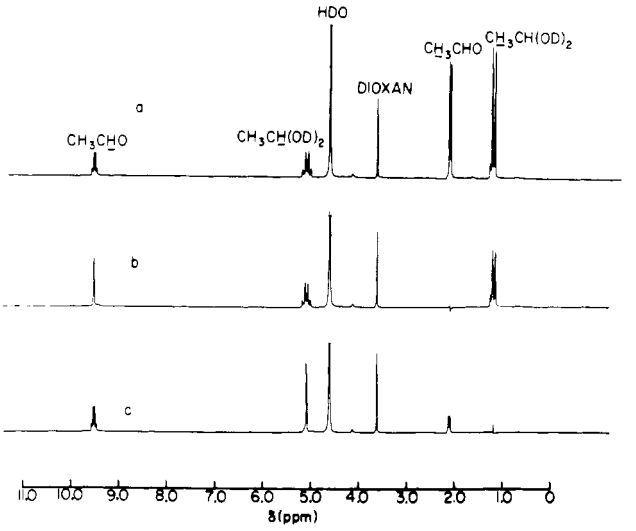

FIGURE 1: NMR spectra of the D_2O solution of acetaldehyde in the presence of 1.3×10^{-4} M bovine carbonic anhydrase B in 0.02 M phosphate buffer, with a pH meter reading of 7.5. The total concentration of acetaldehyde and its hydrate was 0.42 M. The temperature was 28 °C. The chemical shifts were calibrated by using a hexamethyldisiloxane which was taken as zero chemical shift. (a) No double irradiation. (b) Double radiation at the position of the acetaldehyde methyl protons. (c) Double irradiation at the position of acetaldehyde hydrate methyl protons.

Inhibitors. *p*-Toluenesulfonamide was obtained from Koch-Light Laboratories and sodium azide was obtained from Merck. The two chemicals were recrystallized twice from boiling water.

Nuclear magnetic resonance spectra were obtained with Varian HA 100 and Bruker WH 90 spectrometers. Each instrument was equipped with an homonuclear double-resonance unit. The probe temperatures were measured by a 2100A Fluke digital thermometer. A capillary containing hexamethyldisiloxane was used as an external lock for the CW spectra. The solvent D_2O was used for locking the Bruker WH 90 spectrometer. T_1 values were obtained by using the 180°–90° method (Carr & Purcell, 1954) with the Bruker WH 90 spectrometer. Line integral intensities were measured either by a digital integrator or, in cases where slightly overlapping lines were present, with a manual planimeter.

Results

The proton magnetic resonance spectrum of an aqueous solution of acetaldehyde is shown in Figure 1a. The spectrum appears as two doublets and two quartets which belong to the acetaldehyde and acetaldehyde hydrate as is indicated in the spectrum. The ratio of their intensities under the condition of no saturation allows the calculation of the equilibrium constant of the reaction given in eq 1.

$$CH_3CH{=}O + H_2O \underset{K_{-1}}{\overset{K_1}{\rightleftharpoons}} CH_3CH(OH)_2 \qquad (1)$$

$$K_{eq} = [\text{hydrate}]/[\text{aldehyde}] \qquad (2)$$

Preliminary experiments indicated that the catalysis of this exchange reaction has many complicating features, when investigated by the line broadening technique. If the line

Table I: Relative Magnetizations of Methyl Protons of Acetaldehyde and Its Hydrate at Low Levels of Double Irradiation[a]

double irradiation pulse width (μs)	M_z^A/M_0^A	M_z^B/M_0^B	τ_A/T_1^A
0	1.0	1.0	
0.8	0.69	0.45	0.74
1.0	0.58	0.28	0.72
1.5	0.54	0.20	0.74
2.0	0.47	0.10	0.68
extrapolated	0.415	0.0	0.71

[a] The total concentration of acetaldehyde and its hydrate was 0.42 M at a phosphate buffer concentration of 0.02 M and a pH meter reading of 7.5. Enzyme concentration was 5×10^{-4} M and the temperature was 19 °C.

broadening is determined only by chemical exchange, the ratio of the broadening of the aldehyde and the hydrate peaks is expected to be equal to the equilibrium constant of eq 1. This was found to be true in the case of catalysis by hydrogen ions. However, in experiments of enzymatic catalysis of the exchange, the broadening of the lines of the aldehyde species at room temperature was found to be much greater than that of the hydrate, while the equilibrium constant was close to unity. This can be interpreted by an effect of binding on the line width, and the separation of the contributions of exchange and binding is not straightforward. Therefore, we decided to use the saturation transfer method (Forsen & Hoffman, 1963a,b, 1964; Gupta & Redfield, 1970; Mann, 1977) that is less affected by phenomena other than chemical exchange. This method has an extra advantage that slower reaction rates can be followed, since the measurable exchange rates are comparable to T_1^{-1} rather than to the line width. Thus, the enzyme concentrations used for the saturation transfer experiments were about 10 times smaller than those used for line broadening measurements.

Measurement of Exchange by the Saturation Transfer Method. The saturation transfer technique consists of observing the decrease in the intensity of a peak belonging to one species (A), following an irradiation of the corresponding group in the second species (B). Such a decrease is a result of saturation transfer which is due to a chemical exchange between these two groups. Let us denote the irradiated group by B and the group which is being observed as A, then (Gupta & Redfield, 1970)

$$\tau_A = T_1^A \frac{M_z^A/M_0^A - M_z^B/M_0^B}{1 - M_z^A/M_0^A} \qquad (3)$$

where M_z and M_0 are the longitudinal magnetization with and without double irradiation, respectively. T_1^A is the longitudinal relaxation time of group A in the absence of exchange, and τ_A is its exchange lifetime. Since the signal intensity is proportional to M_z, the ratio M_z/M_0 is obtained from the ratio of the signal intensities in the presence and absence of the double irradiation.

In most experiments the intensity of the irradiation was chosen so that the irradiated lines were completely saturated, and a simplified form of eq 3 with $M_z^B/M_0^B = 0$ can be used. However, with the use of the time-sharing technique of the double irradiation in the WH-90 FT NMR spectrometer, the residual intensity of the irradiated line can be observed, and the validity of eq 3 can be experimentally ascertained. One example is shown in Table I where τ_A/T_1^A is calculated by using values of M_z^A and M_z^B under conditions of low irradiation intensities, so that signal B is only partially saturated. One can see from Table I that the same τ_A/T_1^A is calculated for the different irradiation intensities.

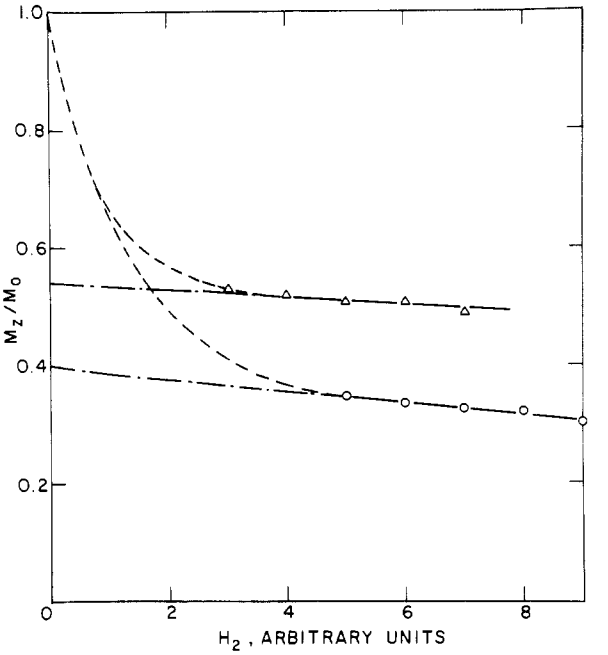

FIGURE 2: An illustration of the determination of the extrapolated M_z/M_0. (Δ) Relative intensity of the acetaldehyde hydrate methyl protons with double irradiation at the acetaldehyde methyl protons resonance. The solution contained 1.25×10^{-4} M carbonic anhydrase, a total concentration of acetaldehyde and its hydrate of 0.42 M, and 0.02 M phosphate buffer, with a pH meter reading of 7.5. The temperature was 27 °C. (O) The same as the previous curve but the methyl doublet of the acetaldehyde was observed while that of the acetaldehyde hydrate was being irradiated, and the enzyme concentration was 5.2×10^{-5} M.

A further decrease of the amplitude of signal A at higher irradiation intensities is caused by direct saturation. Therefore, for each point a series of measurements with increasing irradiation intensities were done, and the final M_z^A/M_0^A was found by extrapolating the curve to zero irradiation intensity. This is shown in Figure 2. The effect of direct saturation would be greater when the T_1 relaxation time of the observed line is longer. T_1 values of the protons of the aldehyde molecule are much longer than those of the hydrate molecule, but the chemical exchange tends to equalize them (see below). At low enzyme concentrations, when the exchange is slow and T_1 values of the aldehyde and hydrate are still different, the effect of direct saturation is more pronounced when the hydrate protons are being irradiated. Therefore, most experiments were performed by irradiating the aldehyde protons and observing the saturation transfer to the hydrate.

A comparison between the saturation transfer method and exchange broadening is possible in a system where no causes other than chemical exchange are expected to contribute to the line broadening of the exchanging species. Such a system is the acid-catalyzed exchange reaction between acetaldehyde and its hydrate. Indeed, as can be seen from Table II, the specific rate constants calculated from the two methods are the same within the experimental error. Furthermore, the ratio of the forward and reverse rate constants agrees in the two methods with K_{eq} which was measured independently from the ratio of the areas under the peaks of the two species. It is interesting to note that the values of k_{D^+} obtained by us are greater by about a factor of 2 from the rates in H_2O, k_{H^+}, obtained by Evans et al. (1965) by the line broadening technique.

Longitudinal Relaxation Times. In the calculation of the exchange rates according to eq 3, the values of T_1 are those

Table II: Rate Constants of the Acid-Catalyzed Reversible Hydration of Acetaldehyde

	rate constants		
	pD[a] 2.31, LB[b]	pD[a] 2.50, LB[b]	pD[a] 3.62, ST[b]
$1/\tau^{hyd}$ (s^{-1})	4.54	2.95	0.223
$1/\tau^{dehyd}$ (s^{-1})	3.97	2.64	0.185
$k_{D^+}^{hyd}$ (M^{-1} s^{-1})[c]	927	933	930
$k_{D^+}^{dehyd}$ (M^{-1} s^{-1})[c]	811	835	771
$k_{D^+}^{hyd}/k_{D^+}^{dehyd}$	1.14	1.12	1.20
K_{eq}	1.16	1.16	1.19

[a] Calculated by adding 0.41 to the pH meter reading. [b] LB = line broadening method. $1/\tau$ is calculated according to the relation $1/\tau = \pi[\Delta\nu(0)]$ where $\Delta\nu(0)$ is the line width at neutral pH. The temperature was 27.2 °C. ST = saturation transfer method as explained in the text. The temperature was 26.6 °C. [c] k_{D^+} is calculated by dividing $1/\tau$ by D^+ concentration.

in the absence of exchange but should include any other effect such as relaxation due to dissolved oxygen or binding to the enzyme. Oxygen was found to affect T_1 of acetaldehyde and its hydrate in quantitative agreement with Polak & Navon (1974). However, since the solutions used for the measurement of the enzymatic catalysis of the exchange rate were not degassed, T_1 values of solutions with no degassing are used for the calculation of the exchange rate constants. The effect of the enzyme is discussed below. Since we measured the relaxation times of AX_3 spin–spin multiplets by inverting all their peaks, there was a danger that cross relaxation of the coupled nuclei would affect the measured T_1 values (Campbell & Freeman, 1973). In order to find out whether we should consider this effect in our calculation, we repeated some of the T_1 measurements in the presence of double irradiation to produce spin–spin decoupled spectra. Since no effect of the double irradiation was observed on the measured T_1 values, we conclude that the cross relaxation effects can be neglected in the present system.

The reversible hydration of acetaldehyde is known to undergo general acid and general base catalysis (Bell & Darwent, 1950); therefore, it is expected to be catalyzed by the buffer present in our solutions. Thus, the dependence of T_1 values of the methyl residues in acetaldehyde and its hydrate on phosphate buffer concentration was measured. There was a very slight increase in the T_1 values of the hydrate molecule and a more pronounced decrease in the T_1 values of the aldehyde as the phosphate buffer concentration was increased from 0.01 to 0.1 M. Chemical exchange between these two species causes their T_1 values to approach each other, and the value of $\bar{T}_1^{-1}$ in the limit of fast exchange is the weighted average of their T_1^{-1} values in the absence of exchange (Strehlow & Frahm, 1975):

$$1/\bar{T}_1 = f_A/T_{1A} + f_B/T_{1B} \quad (4)$$

Since T_1 of the hydrate molecule is much shorter than that of the aldehyde, it is understandable why it is less sensitive to the exchange. The T_1 values were found to be unchanged upon the addition of EDTA, indicating no effect of impurities of paramagnetic ions. The direct saturation effect did not allow us to use the method of Mann (1977) where one can estimate the value of T_1 in the absence of exchange by measuring T_1 of one line while saturating the other. This method should be applicable, however, in cases where the exchanging lines are better separated than in the present system.

The temperature dependences of T_1^{-1} of methyl groups of the hydrate and the aldehyde are shown in Figure 3. Our

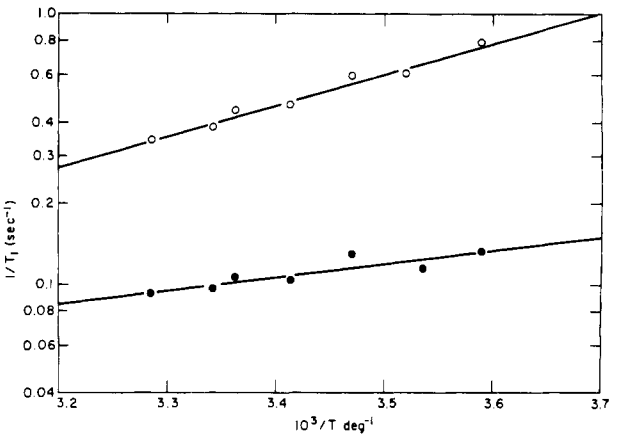

FIGURE 3: Temperature dependence of longitudinal relaxation rates of the methyl protons of acetaldehyde hydrate (○) and acetaldehyde (●) at a phosphate buffer concentration of 0.01 M and a pH meter reading of 7.5. The total concentration of acetaldehyde and its hydrate was 0.42 M.

T_1 measurements were done at a buffer concentration of 0.01 M, where the effect of the buffer on T_1 is small for the aldehyde and completely negligible for the hydrate. The activation energies of the T_1 of the methyl protons of acetaldehyde and its hydrate are −2.26 and −5.28 kcal mol^{-1}, respectively. For the calculation of the rate constants, only the values of T_1 of the hydrate were used and they were taken from the fitted straight line in Figure 3.

Enzymatic Catalysis of the Reversible Hydration Reaction. The net exchange rate caused by the enzyme τ_E^{-1} is given by

$$\tau_E^{-1} = \tau_A^{-1} - \tau_0^{-1} \quad (5)$$

where τ_A^{-1} and τ_0^{-1} are the measured exchange rates in the presence and the absence of the enzyme, respectively.

Phenomenologically, we can write

$$\tau_E^{-1} = k_{enz}[E] \quad (6)$$

where [E] is the molar concentration of the enzyme.

An Arrhenius plot of k_{enz} for the dehydration reaction, k_{enz}^{dehyd}, gave a good straight line (see Figure 4), corresponding to an activation energy of 11.8 ± 1.8 kcal mol^{-1}. Our result at 0 °C, k_{enz}^{dehyd} = 323 M^{-1} s^{-1}, can be compared with the result obtained by Pocker & Meany (1965). The value given in their paper at 0 °C and pD 7.91, k_{enz} = 800 M^{-1} s^{-1}, is the sum of k_{enz}^{hyd} and k_{enz}^{dehyd}, so that using our equilibrium constant, K_{eq} = 3.08 at 0 °C, the value of k_{enz}^{dehyd} = 196 M^{-1} s^{-1} is calculated from their results. The reason for the discrepancy can be linked to the fact that bovine carbonic anhydrase with a specific activity of 1600 units/mg [according to the method of Wilbur & Anderson (1948)] was used in their work as compared to 3000 units/mg in the present work.

For the calculation of the activation parameters of the hydration reaction catalyzed by the enzyme, the temperature dependence of K_{eq} was determined by using the measured intensity ratio of the hydrate and the aldehyde methyl peaks. The linear plot given in Figure 5 corresponds to a heat of hydration of acetaldehyde (eq 1) of $\Delta H°$ = −5.8 ± 0.2 kcal mol^{-1}. This value should be compared with the values of $\Delta H°$ = −5.1 and −4.9 kcal mol^{-1} given in the literature (Bell & Clunie, 1952; Fujiwara & Fujiwara, 1963). Thus, combining $\Delta H°$ with the activation energy of the dehydration reaction given above, we can calculate the activation energy for the hydration reaction to be 6.0 ± 2.0 kcal mol^{-1}.

In order to establish whether the catalysis is specific to the active site of carbonic anhydrase and is not due to a general

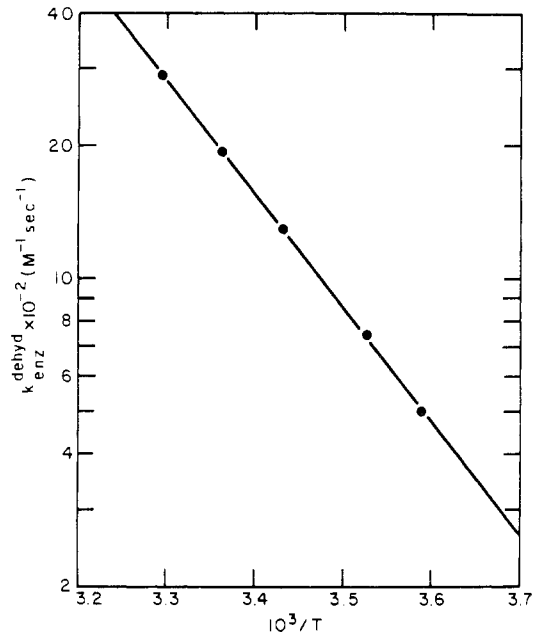

FIGURE 4: Arrhenius plot of k_{enz}^{dehyd}. Phosphate buffer concentration was 0.02 M; the pH meter reading was 7.5. The total concentration of acetaldehyde and its hydrate was 0.42 M. Concentrations of carbonic anhydrase were in the range of (1.0×10^{-4})–(1.4×10^{-3}) M.

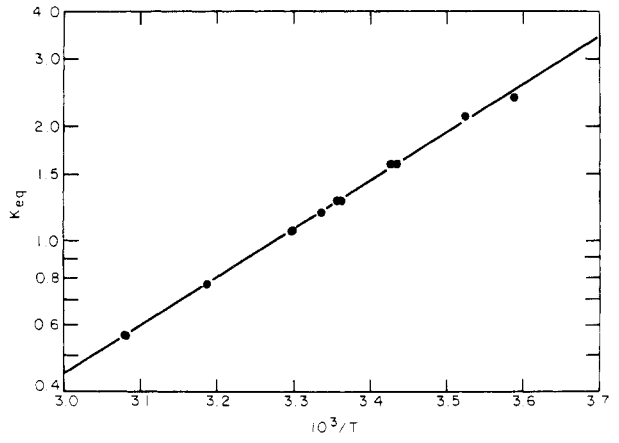

FIGURE 5: Temperature dependence of the equilibrium constant of the hydration reaction of acetaldehyde, K_{eq}, in D_2O solution containing 0.02 M phosphate buffer, with a pH meter reading 7.5, and acetaldehyde and its hydrate at a total concentration of 0.42 M.

catalysis by the protein, we measured the possible effect of bovine serum albumin on the reversible hydration reaction of acetaldehyde. No effect was detected at albumin concentrations as high as 5.5×10^{-4} M at a temperature of 31 °C and pH 6.73. As a further indication for the participation of the active site of the enzyme, we measured the effects of two of the carbonic anhydrase specific inhibitors, *p*-toluenesulfonamide and azide, on the enzyme-catalyzed exchange reaction. Both inhibitors do inhibit the exchange reaction. In the case of the strongly bound inhibitor *p*-toluenesulfonamide, the exchange rate drops to a value equal to that in the absence of the enzyme at an inhibitor to enzyme ratio slightly above unity (see Figure 6). For the azide ion, a more gradual inhibition is observed. The data of the exchange rates in the presence of various concentrations of azide ions were plotted by using a linear plot of the equation

$$[I_0]/(1 - V/V_0) = [E_0] + K_I V_0/V \qquad (7)$$

The absence of a consistent deviation from the linear plot

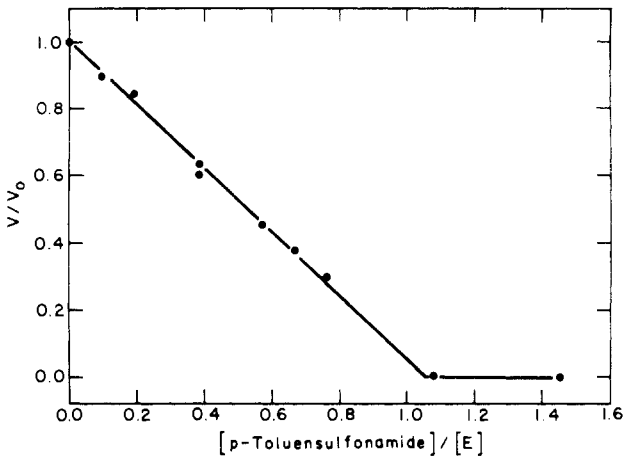

FIGURE 6: Relative dehydration rates of acetaldehyde hydrate catalyzed by carbonic anhydrase as a function of *p*-toluenesulfonamide concentration. Enzyme concentration was 2.1×10^{-4} M. The total concentration of acetaldehyde and its hydrate was 0.42 M in 0.02 M phosphate buffer, with a pH meter reading of 7.5. The temperature was 25 °C.

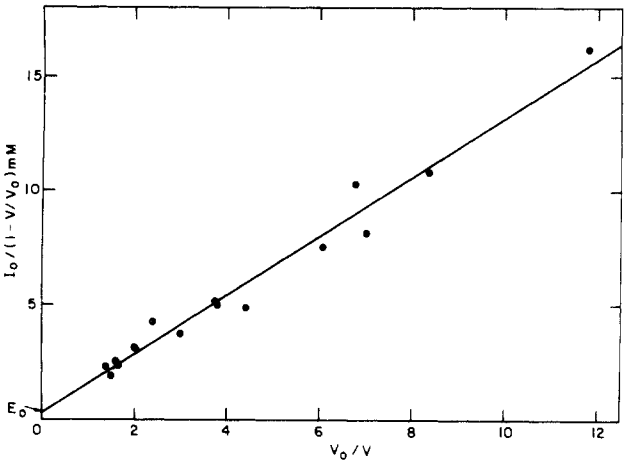

FIGURE 7: A linear plot for the determination of the inhibition constant of the enzyme-catalyzed dehydration reaction of acetaldehyde hydrate by azide ions.

(Figure 7) indicates a simple one-site inhibition pattern. The binding constant of the azide ion obtained from this plot, $K_I = (1.3 \pm 0.2) \times 10^{-3}$ M at 32 °C and pH 7.5, compares favorably with the value of $K_I = 5.9 \times 10^{-4}$ M obtained by Pocker & Stone (1968b) at 25 °C and pH 7.55 by the inhibition of *p*-nitrophenyl acetate hydrolysis.

Solutions of acetaldehyde contain traces of its oxidation product, acetic acid, which is a known inhibitor of the esterase activity of the enzyme (Pocker & Stone, 1968b; Lanir & Navon, 1974). In order to estimate the effect of a contamination by acetic acid in our experiments, we did the following measurements. An addition of 0.065 M sodium acetate to the enzyme at pD 7.6 caused the decrease of k_{enz} by 50%. This value agrees with the K_I found by Pocker & Stone (1968b) [0.085 M at pH 7.55, 25 °C, and Tris-HCl (0.009 M)] and by Lanir & Navon (1974) [ranging from 0.07 to 0.2 M at pH 7.0–8.0, respectively, 25 °C, Tris–sulfate and phosphate buffers (0.1 M)]. The acetaldehyde solutions contained less than 0.5% acetic acid (see Experimental Section), corresponding to a final concentration of about 2 mM. Since this value is much smaller than the K_I, the effect of traces of acetate ions on our results can be safely ignored.

The pH dependence of k_{enz} is shown in Figure 8. The experiment was performed in the absence of a buffer to avoid

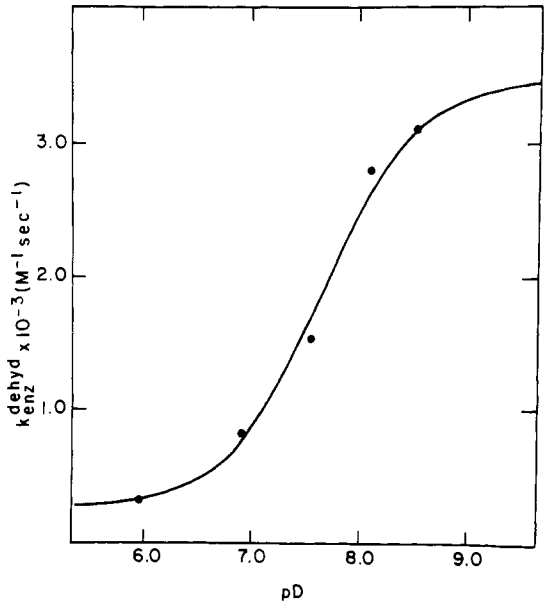

FIGURE 8: pH dependence of the enzymatic activity toward the reversible hydration of acetaldehyde. Enzyme concentration was 2.0 $\times 10^{-4}$ M. The total concentration of acetaldehyde and its hydrate was 0.48 M. The temperature was 27 °C. pD was calculated by adding 0.41 to the pH meter reading. The solid line is a calculated least-squares curve.

any possible interference by anionic inhibition. The pK of the pH profile of the activity corresponded to pD 7.65 at 27 °C. This value is similar to that found by Pocker & Meany (1965), pD 7.5 at 0 °C and with 2 mM phosphate buffer.

Effect of Binding on the Relaxation Times. The resonance lines in the NMR spectra of $CH_3CH{=}O$ and $CH_3CH(OH)_2$ are broadened by the addition of carbonic anhydrase as the combined result of the effect of binding (T_{2b}^{-1}) and the enzyme-catalyzed exchange reaction (τ_E^{-1}). In all our experiments the condition of slow exchange was satisfied by a large margin, i.e.

$$1/\tau << \Delta\nu_0$$

where $\Delta\nu_0$ is the separation between the aldehyde and the hydrate resonance lines. Therefore, the net broadening $\Delta\nu_p$ is given by the relation

$$\pi\Delta\nu_p = T_{2b}^{-1} + \tau_E^{-1} \quad (8)$$

$\Delta\nu_p$ was measured by subtracting the line widths in the presence and the absence of added carbonic anhydrase. A low concentration of dioxane was present as an internal reference in order to correct for occasional changes in field inhomogeneity. In cases where the line broadening caused some overlap between the spin–spin split lines, a comparison with a computer-simulated line shape was performed in order to determine the line broadening.

As mentioned above, the fact that the broadening of the aldehyde lines is much larger than that of the hydrate indicates a specific effect of binding. The contribution of the binding to the line width, T_{2b}^{-1}, was estimated by subtracting τ_E^{-1} calculated from eq 5 from the observed line broadening $\pi\Delta\nu_p$ according to eq 8. The line broadening and exchange lifetimes for several enzyme concentrations at a temperature of 25 °C are summarized in Table III in terms of molar line broadening $\pi\Delta\nu_p/[E]$ and exchange rate constants $k_{enz} = ([E]\tau_E)^{-1}$.

The effect of *p*-toluenesulfonamide on the line broadening of the methyl protons of acetaldehyde and its hydrate is shown in Figure 9. It is seen from Figure 9 that, when the ratio of inhibitor to enzyme exceeds unity, the broadening of the hydrate drops to a value close to zero, while there is an appreciable residual broadening for the aldehyde, which amounts to ~24% of the broadening without the added inhibitor. A summary of these results is given in the last line of Table III. Similar results were obtained also when azide ions were added to solutions containing acetaldehyde (0.42 M) and carbonic anhydrase (1.1×10^{-3} M). The reduction in the line broadening was more gradual compared to that with *p*-toluenesulfonamide because of the weaker binding, but at azide concentrations sufficient to inhibit the enzymatic activity, the broadening of the hydrate was reduced to zero and that of the aldehyde was reduced to a constant value: 25 and 24% at azide concentrations of 2.44×10^{-2} and 7.0×10^{-2} M, respectively. As was shown above, added *p*-toluenesulfonamide or azide inhibits the enzyme-catalyzed exchange reaction. According to eq 8, this inhibition should lead to a reduction in the line width. However, the line broadening due to binding to the active site is also expected to be abolished by the binding of the inhibitors. Thus, the residual broadening in the presence of excess inhibitors may be the result of binding to the enzyme in sites other than the active site. It is seen from Table III that this binding has a much larger effect on the line width of acetaldehyde as compared with that of the hydrate.

The effect of the binding to the enzyme on T_1 could not be measured for the aldehyde and the hydrate separately since the exchange between them, which is catalyzed by the enzyme, tends to equalize them. However, the effect of binding to the enzyme on the average relaxation rate of the aldehyde and the

Table III: Contributions of Exchange and Binding to the Line Broadening of the Methyl Protons of Acetaldehyde and Its Hydrate Caused by Carbonic Anhydrase[a]

	aldehyde	hydrate
$\pi\Delta\nu_p/[E]$	3.8 ± 0.2	2.3 ± 0.2
k_{enz}	2.3 ± 0.3	1.9 ± 0.2
$([E]T_{2b})^{-1}$	1.5 ± 0.5	0.4 ± 0.4
$\pi\Delta\nu_p/[E]^b$	0.9 ± 0.2	0.2 ± 0.2

[a] Data are given in units of 10^3 s^{-1} M^{-1}. Enzyme concentration was 1.1×10^{-3} M. Total concentration of acetaldehyde and its hydrate was 0.42 M, phosphate buffer concentration was 0.02 M, and the pH meter reading was 7.5. The temperature was 25 °C. [b] In the presence of excess *p*-toluenesulfonamide.

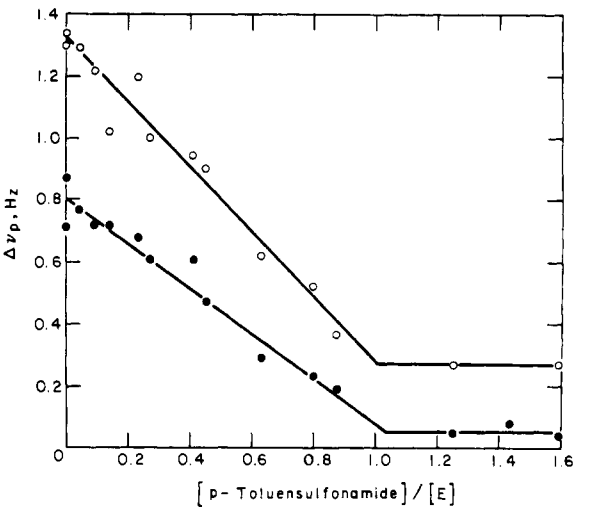

FIGURE 9: Line broadening of the methyl protons of acetaldehyde (○) and its hydrate (●) by carbonic anhydrase as a function of *p*-toluenesulfonamide concentration. Enzyme concentration was 1.1×10^{-3} M. The total concentration of acetaldehyde and its hydrate was 0.39 M in 0.02 M phosphate buffer, with a pH meter reading of 7.5. The temperature was 25 °C.

hydrate could be measured at high enzyme concentrations. The contribution of the binding to the average relaxation rate is defined as

$$1/\bar{T}_{1b} = 1/\bar{T}_{1E} - 1/\bar{T}_1(0) \quad (9)$$

where $1/\bar{T}_{1E}$ is the average relaxation rate obtained in the presence of high enzyme concentrations and $1/\bar{T}_1(0)$ was calculated according to eq 4 by using the T_1 values in the absence of exchange. $([E]\bar{T}_{1b})^{-1}$ was found to be 18 M^{-1} s^{-1} at 25 °C. This value is a weighted average of the effect of binding on T_1^{-1} of the aldehyde and the hydrate peaks.

In the calculation of the exchange lifetimes from the saturation transfer data, we used the T_1 values in the absence of added enzyme. It is seen from the present result that the effect of binding to the enzyme is indeed negligible. Even if we count the whole effect on $1/\bar{T}_{1b}$ as due to the hydrate, the effect on the calculated exchange lifetime would be smaller than 0.2%.

Enzymatic Catalysis of the Reversible Hydration of Acetaldehyde by Cadmium(II) and Cobalt(II) Carbonic Anhydrase. Saturation transfer experiments with cadmium(II) carbonic anhydrase added to acetaldehyde solutions indicated no significant catalysis of the dehydration rate. With enzyme concentrations up to 1.6×10^{-3} M, a total concentration of acetaldehyde and its hydrate of 0.30 M, a temperature of 28 °C, and a pH meter reading of 7.55, a value of $k_{enz} = 69 \pm 37$ M^{-1} s^{-1} was obtained. Atomic absorption measurements indicated the presence of 0.9% zinc(II) enzyme which accounted for $k_{enz} = 22$ M^{-1} s^{-1}. The net k_{enz} due to the cadmium(II) enzyme, 47 ± 37 M^{-1} s^{-1}, corresponds to an activity which is 2 ± 1.5% of the activity of the zinc enzyme. This small activity may be attributed to a general acid or general base catalysis by ionizable groups on the protein molecule.

Cobalt(II) carbonic anhydrase was found to have a strong catalytic effect on the reversible hydration of acetaldehyde. However, it was found that unlike the zinc(II) enzyme the cobalt(II) enzyme has a significant effect on the T_1 relaxation times of both the acetaldehyde and its hydrate. Therefore, it was necessary to find the values of T_1 without exchange but in the presence of the enzyme. This information could be obtained by studying the saturation transfer in both directions. The value of M/M_0 of the aldehyde was measured while irradiating the hydrate, and in a separate experiment, M/M_0 of the hydrate was measured while irradiating the aldehyde. From the values of $(M/M_0)^{ald}$ and $(M/M_0)^{hyd}$, the ratios τ^{ald}/T_1^{ald} and τ^{hyd}/T_1^{hyd} were calculated by using eq 3. Defining

$$\alpha = (\tau^{ald}/T_1^{ald})/(\tau^{hyd}/T_1^{hyd}) \quad (10)$$

we obtained by using eq 4

$$T_1^{hyd} = \frac{1 + \alpha}{1 + K_{eq}^{-1}} \bar{T}_1 \quad (11)$$

Substituting the experimental $\bar{T}_1$ in the presence of cobalt(II) carbonic anhydrase, we obtained T_1^{hyd} without exchange. T_1^{ald} can be obtained from T_1^{hyd} by using eq 12:

$$T_1^{ald} = T_1^{hyd}/(\alpha K_{eq}) \quad (12)$$

At a temperature of 28.5 °C, a pH meter reading of 7.7, and a total concentration of acetaldehyde and its hydrate of 0.54 M, a value of $k_{enz}^{dehyd} = 2550$ M^{-1} s^{-1} was found. Similar values were obtained at lower acetaldehyde concentrations, indicating $K_m > 1$ M. For comparison, $k_{enz}^{dehyd} = 2810$ M^{-1} s^{-1} was found at similar pH (pD 8.1) for the zinc(II) enzyme (see Figure 8). Thus, after substitution of the zinc atom by the cobalt atom, the enzyme retains most of its activity toward the reversible hydration of acetaldehyde. In experiments with manganese(II) carbonic anhydrase, T_1 relaxation times of both aldehyde and hydrate were too short to enable us to estimate the catalysis of the exchange by this enzyme.

Discussion

Two advantages of the NMR saturation transfer method for the investigation of the kinetics of enzyme reactions over the more conventional line broadening technique are apparent in the present work. Since rates comparable to T_1^{-1} are measured by the saturation transfer method while rates larger than T_2^{-1}, which include magnetic field inhomogeneity, can be followed in the line broadening technique, it is obvious that in the saturation transfer method slower reaction rates can be measured. Thus, lower concentrations of enzyme are needed for such measurements. It is seen in the present work that while millimolar concentrations of carbonic anhydrase were needed for the line broadening technique, concentrations of 0.1 mM were suitable for the observation of saturation transfer. Another advantage is that while the line broadening includes both the effect of chemical exchange and the effect of binding of the substrates to the enzyme, the separation of which cannot often be done unambiguously, the saturation transfer method measures the rate of the reaction independently from the effects of binding.

By combining the saturation transfer method and the effect of inhibitors on the line width, we have shown that there are three contributions to the line broadening of acetaldehyde induced by carbonic anhydrase: catalysis of the exchange reaction between the aldehyde and its hydrate, binding to the active site of the enzyme, and binding to other sites on the enzyme. While the two inhibitors used in this work abolished completely the catalysis of the hydration reaction, as was measured by the saturation transfer method, a residual line broadening was observed in their presence. This is explained in terms of binding sites other than the site which is active in the catalysis of the hydration reaction. It is interesting to note that the effect of the binding on the line broadening was much larger than the effect on T_1. Also, the line broadening effects were larger for the acetaldehyde than for its hydrate. We cannot offer an interpretation for these differences, but it gives some indication for different modes of binding for these two substrates. From the chemical point of view, the aldehydes are more analogous to carbon dioxide while the hydrates are more analogous to the bicarbonate ion. Different modes of binding of carbon dioxide and bicarbonate were indeed suggested by several studies (Riepe & Wang, 1968; Yeagle et al., 1975; Kannan et al., 1977).

Our result that the bovine cadmium(II) carbonic anhydrase is inactive toward the reversible hydration of acetaldehyde agrees with previous results of Nyman (1964) for the CO_2 hydration and Thorslund & Lindskog (1967) for *p*-nitrophenyl acetate hydrolysis. This was found to be true also for human cadmium(II) carbonic anhydrase B at neutral pH, though there is an increase of activity at high pH values, reaching 23% of the activity of the native enzyme, at pH 9.7 (Bauer et al., 1976). The high activity of the cobalt(II) enzyme found in the present work should be compared with that found for CO_2 hydration (50% activity) and *p*-nitrophenyl acetate hydrolysis (97%) (Nyman, 1964; Thorslund & Lindskog, 1967).

Recently, the pH profiles of the optical spectrum and the proton relaxation rates of the cobalt(II) carbonic anhydrase were reinvestigated (Bertini et al., 1977; Jacob et al., 1978a), suggesting that they were significantly influenced by anionic inhibition by either Cl^-, $H_2PO_4^-$, or HSO_4^- present in all previous measurements. It may be inferred (Jacob et al.,

1978b) that the pH profile of the enzymatic activity was also determined by the presence of those ions, and the concept of nonactive, low-pH and active, high-pH forms of the enzyme has to be revised. Unlike the hydration of CO_2 and the hydrolysis of esters, the reversible hydration of acetaldehyde does not involve participation of anions and can be followed without the presence of buffers. Also, the equilibrium constant of the reaction (eq 2) is not pH dependent, thus avoiding the complication of separating the pH profiles of the forward and reverse reactions. Our results for the pH dependence of the activity in the absence of buffers, which agree with the results of Pocker & Meany (1965), obtained in a very low concentration of buffer, indicate the presence of the two forms of the enzyme.

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