Enzyme Kinetics in Solvents of Increased Viscosity. Dynamic Aspects of Carbonic Anhydrase Catalysis†

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Received September 18, 1986; Revised Manuscript Received December 8, 1986

ABSTRACT: The dependence of enzymatic catalysis on diffusion rates in solution was examined with regard to high specific activity carbonic anhydrase (CA II) by varying the viscosity of the reaction medium with added glycerol, sucrose, and ficoll (a copolymer of sucrose and epichlorohydrin). Responses of the Michaelis–Menten parameters associated with CO_2 hydration and HCO_3^- dehydration were deduced and analyzed by utilizing a spectrophotometric stopped-flow technique. It was found that both $k_{cat}^{HCO_3^-}$ (=3.9 × 10^5 s^{-1} at pH 5.90) and $k_{cat}^{CO_2}$ (=1.2 × 10^5 s^{-1} at pH 5.90 and 8.6 × 10^5 s^{-1} at pH 8.80) steadily decreased with the addition of monomeric viscogen while both $K_m^{HCO_3^-}$ (=20 mM at pH 5.90) and $K_m^{CO_2}$ (=18 mM at pH 5.90 and 13 mM at pH 8.80) remained independent of viscosity, within experimental error. These results indicate that some kind of proton-transfer-related event is primarily responsible for the observed rate decrease. The three polyhydroxy cosolutes exhibited significant differences with regard to the magnitude of the viscosity effect on the k_{cat} of the enzyme, with glycerol affecting the largest decrease, sucrose affecting a moderate one, and ficoll having virtually no effect. The discrepancy between glycerol and sucrose could be largely reconciled by correcting for diffusion-unrelated effects as estimated from rate studies of considerably slower CA II catalyzed acetaldehyde hydration and *p*-nitrophenyl acetate hydrolysis. Ficoll, however, was found to be unsuitable as a viscogenic probe because it failed to appreciably decrease the mobilities of smaller ions (as deduced from electrolytic conductance measurements) despite its capacity to greatly increase the macroscopic viscosity of the medium. Our best estimates indicate that this reaction comes within ca. 30% of the diffusion limit at 0.890 cP and 25 °C for both CO_2 hydration and HCO_3^- dehydration reactions. However, it is reasonable to expect this value to be considerably higher in the natural environment of the enzyme because of the relatively high viscosities attained in the interior of erythrocytes.

Interconversion of CO_2–HCO_3^- in erythrocytes, perhaps one of the simplest physiological reactions requiring enzymatic catalysis (Edsall, 1967, 1984; Coleman, 1984), has long been recognized as one of the fastest biochemical processes. Although present in smaller concentrations than its lower activity isozyme (CA I),[1] the high-activity form of mammalian carbonic anhydrase (CA II) is the predominant catalyst for this reversible hydration due to its extreme catalytic efficiency. Some 50 years after its discovery, CA II, with a turnover number of about 1 million, remains one of the fastest enzymes known (Pocker & Sarkanen, 1978; Lindskog, 1983; Bertini & Luchinat, 1983). The absolute limit for the velocity of any reaction in solution is the encounter frequency between the reacting species as determined by the diffusion rates in the particular solvent. For small reactants, the limiting value of 10^{10} M^{-1} s^{-1} has been generally accepted. Because of considerably slower diffusion rates for large molecules and other geometric and orientational constraints imposed by confinement of the active site to a specific region on the polypeptide, enzymatic reactions have a somewhat lower limit of about 10^8–10^9 M^{-1} s^{-1} (Alberty & Hammes, 1958; Šolc & Stockmayer, 1973). The question that then imposes itself is how close the evolutionary process brought this reaction to the diffusion-controlled limit. With an activation energy of only 5 kcal/mol for CA II catalyzed CO_2 hydration (Pocker et al., 1981) and ~5 kcal/mol for HCO_3^- dehydration (Pocker and Tanaka, unpublished observations) and with second-order rate constants k_{cat}/K_m approaching 10^8 M^{-1} s^{-1}, this reaction is sufficiently fast to be a likely candidate for a partially diffusion-controlled process.

Influence of diffusion rates on other fast enzymatic catalyses has been inferred previously in fumarase (Alberty & Hammes, 1958; Peller & Alberty, 1959), horseradish peroxidase (Farwell & Ackermann, 1963; Dunford & Hewson, 1977; Nakatani & Dunford, 1979), chymotrypsin (Brouwer & Kirsch, 1982), β-lactamase I (Hardy & Kirsch, 1984), acetylcholinesterase (Bazelyansky et al., 1986), and many others. The diffusion-controlled character of CA II catalysis has also been frequently implied in various publications (Dunford & Hewson, 1977; Jönsson & Wennerström, 1978; Hasinoff, 1984); however, detailed quantitative analysis of the magnitude of this effect was not performed. It is the main purpose of this paper to present and analyze the dependence of individual Michaelis–Menten parameters k_{cat} and K_m for the reversible hydration of CO_2 on solution viscosity increases induced by addition of a variety of viscogenic cosolutes and to provide a reasonable estimate for the extent to which this reaction is influenced by functionaly important motions (Frauenfelder & Wolynes, 1985) and/or diffusion rates.

Experimental Procedures

Bicarbonate Solutions. Reagent-grade sodium bicarbonate was purchased from Baker and used as substrate in all kinetic

† This work was supported by grants from the National Science Foundation, the National Institutes of Health, and the Muscular Dystrophy Association.

[1] Abbreviations: CA I and II, carbonic anhydrases I and II (low- and high-activity isozymes, respectively); MES, 2-(*N*-morpholino)ethanesulfonic acid; TAPS, 3-[[tris(hydroxymethyl)methyl]amino]propanesulfonic acid, BCP, bromocresol purple; MCP, *m*-cresol purple; pNPA, *p*-nitrophenyl acetate.

0006-2960/87/0426-2597$01.50/0

Table I: Solubility of Carbon Dioxide in Glycerol–Water, Sucrose–Water, and Ficoll–Water Solutions at 30.0 °C[a]

[glycerol] (% w/w)	5.25	20.7	30.3	34.3	45.8	55.3
CO_2 solubility (mM)	26.7	22.9	19.8	19.2	15.6	14.3
[sucrose] (% w/w)	6.78	13.0	19.0	24.8	30.3	35.6
CO_2 solubility (mM)	27.9	25.7	24.1	22.4	20.8	19.0
[ficoll] (% w/w)	1.6	3.2	5.2	8.0		
CO_2 solubility (mM)	29.2	29.2	29.1	28.0		

[a] CO_2 solubility in water at 30.0 °C = 29.2 mM.

experiments. Bicarbonate solutions were prepared by dissolving a measured amount of this compound in 100 mL of distilled, deionized, CO_2-free water containing glycerol, sucrose, or ficoll. The ionic strength of all solutions was adjusted to 0.1 by addition of an appropriate amount of sodium sulfate (Baker; reagent grade). Kept in stoppered, airtight 50-mL disposable syringes, bicarbonate solutions were good for 24 h but were normally used within 3 h of preparation.

CO_2 Solutions. Saturated solutions in glycerol–water, sucrose–water, and ficoll–water were prepared by bubbling CO_2 gas (Airco, grade 4, 99.99% pure) through a fine glass frit for over 20 min into a solution contained in a glass reservoir thermostated at 30.0 ± 0.05 °C (as the solubility of CO_2 increases at lower temperatures, solutions saturated at 30 °C are slightly undersaturated at 25 °C and thus were more stable, giving improved accuracy of the measurements). The solutions were withdrawn through airtight tubing into a calibrated 20-cm^3 glass syringe containing a glass bead as a mixing weight. Dilution was accomplished by withdrawing a desired volume of CO_2-free solution with a syringe needle and shaking the glass bead. All CO_2 solutions were rapidly transferred into the stopped-flow syringe, avoiding any contact with air, thermostated at 25.0 ± 0.02 °C, and used within 15 min of preparation.

Concentrations of CO_2 at saturation in these mixed solvents at 30.0 °C were determined by adding a known volume of saturated CO_2 solution to an excess of standardized $Ba(OH)_2$ solution and then back-titrating with standardized HCl (Skoog & West, 1976). Phenolphthalein was used as indicator, and each titration was repeated at least 5 times. The value of 29.2 mM obtained for the maximum solubility of CO_2 in distilled, deionized, degassed water is in good agreement with the literature value (Harned & Davis, 1943; Hildebrand & Scott, 1964). Decreased CO_2 solubility with added sucrose and glycerol (Table I, Figure 1) is consistent with previous observations (Usher, 1910; von Kiss et al., 1937).

Enzyme Solutions. Dialyzed, lyophilized bovine carbonic anhydrase (CA II) was purchased from Sigma (2700 W-A units/mg) and dissolved in distilled, deionized water to make ~10^{-5} M stock solutions whose spectrophotometric concentration was determined from the absorbance at 280 nm (ϵ_{280} = 54 000 M^{-1} s^{-1}). Titration of enzymatic activity with acetazolamide yielded a factor by which the spectrophotometric concentration needed to be divided in order to obtain the concentration of active enzyme. A single batch of CA II was used throughout this work. In all of the enzyme-catalyzed kinetic runs, a small volume of the CA II stock solution was pipetted into the buffer solution to give concentrations of 1 × 10^{-7}–2 × 10^{-7} M upon 1/1 dilution in the stopped flow for CO_2 hydration and HCO_3^- dehydration runs and ~1 × 10^{-6} M for the hydrolysis of pNPA. Although all buffer–CA II solutions were made fresh and used within 48 h of preparation, it was found that the enzyme was quite stable at both pH 5.90 and pH 8.80, as its activity was not reduced by more than 2% even after 3 weeks of storage at <5 °C.

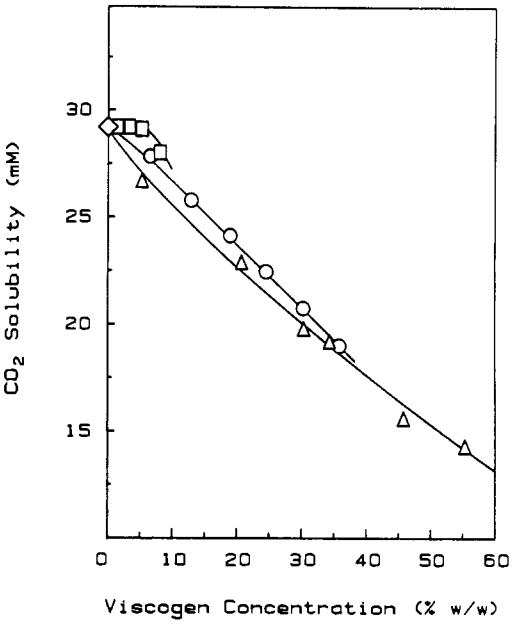

FIGURE 1: Solubility of carbon dioxide in water (◇), glycerol–water (△), sucrose–water (○), and ficoll–water (□) solutions at 30.0 ± 0.05 °C.

Buffer–Indicator System. Initial rates of proton uptake in dehydration of HCO_3^- or proton release in hydration of CO_2 were measured by the changing indicator method described in detail elsewhere (DeVoe & Kistiakowski, 1961; Gibbons & Edsall, 1964; Kalifah, 1971; Pocker & Bjorkquist, 1977). 2-(*N*-Morpholino)ethanesulfonic acid (MES) buffer, pK_a = 6.10, was found to be a monohydrate by gravimetric titration (molecular mass = 213.2 g/mol) and was used for work at pH 5.90. 3-[[Tris(hydroxymethyl)methyl]amino]propanesulfonic acid (TAPS) buffer, pK_a = 8.38, was anhydrous (M_r 243.3) and was used for the work at pH 8.80. Both buffers were purchased from Sigma and used without further purification. Twenty millimolar buffer solutions with an ionic strength of 0.1 (adjusted with Na_2SO_4) were used in all hydration–dehydration runs. Bromocresol purple (BCP; pK_a = 6.2, $\Delta\epsilon_{589}$ = 71 400) and *m*-cresol purple (MCP; pK_a = 8.4, $\Delta\epsilon_{578}$ = 32 000), obtained from Sigma, were used as indicators for work at low and high pH, respectively, and were present in the buffer solutions in concentrations of ~1 × 10^{-5} M to give initial absorbances of 0.2 in the stopped flow, after 1/1 dilution. All buffer factors Q (=$d[H^+]/dA$) were determined experimentally by measuring the absorbance changes of the buffer–indicator system at 589 and 578 nm for MES–BCP and TAPS–MCP solutions, respectively, upon addition of microliter aliquots of 1.00 N HCl.

Acetaldehyde Solutions. Acetaldehyde was obtained from Aldrich and distilled immediately prior to use under inert atmosphere (N_2) to remove oxidation products. Because of its low boiling point (21 °C at 1 atm), it was kept on dry ice in a 10-mL pear-shaped flask stoppered with a rubber septum. The kinetic run was initiated by injecting 5 μL of neat acetaldehyde into a 1-cm quartz cuvette containing 3 mL of 20 mM MES buffer adjusted to pH 5.90 and ionic strength 0.1 and monitoring the decay of the absorbance signal at 278 nm (carbonyl n → π* transition) (Pocker & Meany, 1965; Pocker & Dickerson, 1968).

p-Nitrophenyl Acetate (pNPA) Solutions. pNPA was purchased from Aldrich and recrystallized 5 times from an anhydrous diethyl ether/petroleum ether mixture (20/80 v/v). Stock solution was prepared by dissolving this reagent in spectroscopic-grade acetone dried over molecular sieves.

Hydrolysis of pNPA was initiated by the addition of 300 μL of stock solution via a hypodermic syringe to 2.7 mL of the buffer solution so that the resulting mixture was 10% v/v acetone. The presence of acetone as the cosolvent was somewhat inhibitory but considerably improved the solubility of pNPA, which is only sparingly soluble in water. Buffer concentrations were 20 mM, with ionic strength adjusted to 0.1 by addition of Na_2SO_4. Rates of hydrolysis of pNPA were monitored by observing the appearance of the *p*-nitrophenolate anion at 400 nm (Pocker & Stone, 1967).

Viscosity. Glycerol (Aldrich, spectrophotometric grade, Gold Label), sucrose (MCB, reagent grade), and ficoll 400 (Sigma, dialyzed and lyophilized) were used as viscosity-inducting cosolutes. Glycerol was distilled under reduced pressure (142 °C at 3 mmHg), while sucrose and ficoll were used without further purification. Viscosities of aqueous solutions of glycerol, sucrose, and ficoll at 25.0 ± 0.01 °C were determined on a calibrated Cannon-Ubbelohde viscometer and found to be in good agreement with values in the literature (Freier, 1978; Swindells et al., 1958; Stokes & Weeks, 1964).

In order to prevent potential uneven mixing in the stopped-flow reaction chamber as well as to overcome the difficulty with temporary turbidity caused by mixing two unlike solutions, equal concentrations of viscogen were used in both the substrate and the enzyme–buffer solutions to be mixed.

Other Reagents. DL-Glyceraldehyde used for inhibition studies was purchased from Aldrich and used without further purification.

Conductance. The conductances of $NaHCO_3$, NaMES, and NaTAPS at several concentrations were measured at 25.0 ± 0.01 °C and converted to equivalent conductances after correction for conductances of solvent viscogen–water solutions. Extrapolation to zero electrolyte concentration yielded limiting equivalent conductances.

Instrumentation. All HCO_3^- dehydration and CO_2 hydration runs were performed on an extensively modified Durrum-Gibson Model 1300 stopped-flow spectrophotometer. Both the stopped-flow apparatus and the experimental technique have been described in detail previously (Pocker & Bjorkquist, 1977; Pocker & Fong, 1980). A Cary Model 210 UV–visible double-beam spectrophotometer interphased with an Apple II/e microcomputer was used for the rate analyses of acetaldehyde hydration and pNPA hydrolysis, determination of spectrophotometric concentrations of CA II stock solutions, and evaluation of buffer factors. Circular dichroism measurements were done on a Jobin Yvon Mark III dichrograph. Electrolytic conductance measurements were carried out on a Model RC-18 conductivity bridge equipped with a custom-made platinum electrode cell. As it has been shown that the glass electrode method yields correct values of hydrogen ion activities in aqueous organic solvents (Marshall & Grunwald, 1953; Bacarella et al., 1958), a Radiometer Model PHM 84 research pH meter equipped with a Cole-Palmer Ag/AgCl glass electrode was used to obtain buffer pH readings. A Hewlett-Packard Model 5790A gas chromatograph connected to a capillary column was used for analysis of purified glycerol. All kinetic runs were done at 25 °C.

Kinetic Scheme. It has been shown previously that the reversible hydration of CO_2 (eq 1), when catalyzed by human

$$CO_2 + H_2O \rightleftharpoons HCO_3^- + H^+ \quad (1)$$

erythrocyte carbonic anhydrases (Khalifah, 1971) or their bovine analogues (Kernohan, 1965), can be formally analyzed in terms of the classical Michaelis–Menten mechanism (eq 2). An expression for the total forward rate can be derived

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P \quad (2)$$

from this mechanism, assuming steady-state conditions:

$$V_{enz} = (k_{cat}/K_m)[E][S] \quad (3)$$

where K_m $[=(k_{cat} + k_{-1})/k_1]$ is the Michaelis constant. It is relevant to point out that by employing the one-substrate, one-product mechanism, one obtains the lower limits for all the rate constants in an enzymatic catalysis regardless of the number of intermediates involved (Peller & Alberty, 1959).

Satisfactory resolution of the second-order rate constant in eq 3 into k_{cat} and K_m was accomplished by analyzing the initial rate measurements according to the formalism of Lineweaver and Burk. Experimentally determined initial slopes of absorbance vs. time plots were converted into velocity by

$$V_{tot} = (dA/dt)Q \quad (4)$$

where V_{tot} is the total forward velocity, A is the indicator absorbance, and Q $(=d[H^+]/dA)$ is the buffer factor. The component of the rate due to enzymatic catalysis was obtained by subtracting the buffer (uncatalyzed) velocity from the total velocity.

Acetaldehyde hydration and pNPA hydrolysis were more conveniently analyzed in terms of pseudo-first-order reaction kinetics. To obtain good A_∞ values, these reactions were monitored for at least 10 half-lives. Rate constants were calculated from the least-squares analysis of ln $(A - A_\infty)$ vs. time plots. Correlation coefficients of 0.999 or better were obtained for at least three half-lives for all nonenzymatic runs. Although a slight departure from linearity was observed only with enzyme-catalyzed acetaldehyde hydration with glycerol and sucrose as cosolutes, analyses of all enzymatic runs were restricted to the initial 10–15% of the reaction in order to avoid possible complications from product inhibition. The catalytic constant k_{enz} for these reactions was obtained by subtracting the buffer rate constant from the total rate constant and dividing by the stoichiometric enzyme concentration.[2]

Results

Response of the kinetic parameters of CO_2 hydration and HCO_3^- dehydration to solution viscosity increase in the 1–4-cP range was found to be significantly affected by the kind of viscogenic reagent used. In all cases, glycerol caused the largest rate deceleration, with sucrose producing a smaller effect at comparable viscosities. Ficoll, on the other hand, had no significant effect on the rate of CO_2 hydration at either low or high pH. In the case of HCO_3^- dehydration, however, there was an unexpected increase in both k_{cat} and K_m, with their ratio $(=k_{cat}/K_m)$ nevertheless remaining essentially unchanged. Values of k_{cat} and K_m for HCO_3^- dehydration at pH 5.90 (Table II) and CO_2 hydration at pH 5.90 and pH 8.80 (Tables III and IV, respectively) are plotted against solution viscosities in Figure 2. It can be seen that k_{cat} steadily decreased with the addition of monomeric viscogens (i.e., glycerol and sucrose) while K_m remained independent of viscosity increase, within experimental error, in both directions of catalysis.

In the absence of enzyme at pH 5.90, neither dehydration nor hydration rates changed significantly with the solution viscosity increase (Tables II and III, respectively), but at pH 8.80 there was a marked enhancement in the velocity of buffer (uncatalyzed) CO_2 hydration as the amounts of cosolutes were

[2] The absolute value of the slope of ln $(A - A_\infty)$ vs. time yields k_{obsd}, which is in the case of acetaldehyde hydration equal to $k_{hydration} + k_{dehydration}$. To obtain the forward rate constant $k_{hydration}$, k_{obsd} must be multiplied by the fraction of hydration $\chi = K_{eq}/(K_{eq} + 1)$.

Table II: Dependence of HCO_3^- Dehydration Rate Parameters on Solution Viscosity Increase with Glycerol, Sucrose, and Ficoll as Cosolutes[a]

	soln viscosity (cP)	$k_{buffer} \times 10^2$ (s^{-1})	$k_{cat} \times 10^{-5}$ (s^{-1})	$K_m \times 10^2$ (M)	$k_{cat}/K_m \times 10^{-7}$ (M^{-1} s^{-1})
no cosolutes	0.890	6.61	3.90	2.05	1.90
[glycerol] (% w/w)					
7.46	1.08	6.52	4.14	2.22	1.86
14.6	1.28	6.64	3.09	1.78	1.74
23.9	1.76	6.42	3.01	2.06	1.46
34.9	2.66	6.63	2.55	2.39	1.07
45.3	4.05	6.71	1.77	2.27	0.78
[sucrose] (% w/w)					
16.1	1.45	7.10	3.61	2.20	1.64
24.8	2.10	6.97	3.23	2.15	1.50
30.3	2.78	6.91	3.24	2.17	1.49
35.6	3.79	6.92	2.36	1.94	1.22
[ficoll] (% w/w)					
1.6	1.20	6.80	3.99	2.16	1.85
3.2	1.60	6.93	4.63	2.73	1.70
5.2	2.21	7.05	5.07	2.81	1.80
6.4	2.67	6.88	5.31	3.13	1.70
8.0	3.47	6.96	6.32	3.66	1.73

[a] Kinetic runs were done in 20 mM MES buffer, pH 5.90, ionic strength 0.1, and 25.0 °C.

Table III: Dependence of CO_2 Hydration Rate Parameters on Solution Viscosity Increase with Glycerol, Sucrose, and Ficoll as Cosolutes[a]

	soln viscosity (cP)	$k_{buffer} \times 10^2$ (s^{-1})	$k_{cat} \times 10^{-5}$ (s^{-1})	$K_m \times 10^2$ (M)	$k_{cat}/K_m \times 10^{-6}$ (M^{-1} s^{-1})
no cosolutes	0.890	3.15	1.23	1.85	6.65
[glycerol] (% w/w)					
7.46	1.08	3.62	1.17	1.75	6.68
23.9	1.76	3.84	1.00	1.79	5.59
34.9	2.66	3.76	0.726	1.80	4.03
45.3	4.05	4.05	0.503	1.80	2.78
[sucrose] (% w/w)					
16.1	1.45	3.19	1.16	1.89	6.15
24.8	2.10	3.40	1.14	2.06	5.53
30.3	2.78	3.75	1.04	1.88	5.53
35.6	3.79	3.85	0.815	1.63	5.01
[ficoll] (% w/w)					
1.6	1.20	3.11	1.09	1.60	6.83
3.2	1.60	3.00	1.09	1.62	6.71
5.2	2.21	3.29	1.22	1.92	6.38
8.0	3.47	3.37	1.16	1.75	6.62

[a] Kinetic runs were done in 20 mM MES buffer, pH 5.90, ionic strength 0.1, and 25.0 °C.

increased (Table IV), probably resulting from the involvement of alkoxy anions of these polyhydroxy compounds in the kinetic mechanism.

As a true catalyst, the enzyme does not affect the equilibrium constant for the reversible hydration of CO_2 (eq 5). The

$$pK_{eq} = -\log (k_{cat}^{CO_2} K_m^{HCO_3^-} / K_m^{CO_2} k_{cat}^{HCO_3^-}) + pH \quad (5)$$

value of 6.35 for pK_{eq} calculated in this way from enzyme-catalyzed CO_2 hydration and HCO_3^- dehydration rate parameters at pH 5.90[3] does not change with added glycerol, sucrose, and ficoll (Table V) and agrees well both with previous estimates (Pocker & Bjorkquist, 1977a) and with the value determined in the absence of the enzyme at ionic strength 0.1 (Harned & Davis, 1943; Pocker & Bjorkquist, 1977b).

Acidity constants of the zwitterionic MES and TAPS buffers used in this study were remarkably resistant to medium perturbations induced by the viscogenic cosolutes (Table VI).

[3] In order to evaluate pK_{eq}, the kinetic parameters for both the forward and the reverse reactions must be evaluated at the same pH. Since dehydration becomes increasingly difficult to monitor at pH values above 7.6, we present here the considerably more reliable values associated with CO_2 hydration and HCO_3^- dehydration at low pH (5.90).

Table IV: Dependence of CO_2 Hydration Rate Parameters on Solution Viscosity Increase with Glycerol, Sucrose, and Ficoll as Cosolutes[a]

	soln viscosity (cP)	$k_{buffer} \times 10^2$ (s^{-1})	$k_{cat} \times 10^{-5}$ (s^{-1})	$K_m \times 10^2$ (M)	$k_{cat}/K_m \times 10^{-7}$ (M^{-1} s^{-1})
no cosolutes	0.890	0.101	8.63	1.26	6.84
[glycerol] (% w/w)					
14.6	1.28	0.473	7.74	1.49	5.19
23.9	1.76	0.788	5.80	1.06	5.46
34.9	2.66	1.36	4.69	1.13	4.16
45.3	4.05	2.04	2.85	0.99	2.88
[sucrose] (% w/w)					
6.69	1.06	0.589	8.21	1.42	5.80
16.1	1.45	1.72	7.56	1.44	5.27
24.8	2.10	2.11	7.31	1.51	4.83
30.3	2.78	3.04	6.28	1.33	4.72
35.6	3.79	4.04	6.22	1.31	4.74
[ficoll] (% w/w)					
1.6	1.20	0.110	8.79	1.24	7.07
3.2	1.60	0.148	8.09	1.11	7.28
5.2	2.21	0.185	8.88	1.24	7.16
8.0	3.47	0.262	8.52	1.14	7.46

[a] Kinetic runs were done in 20 mM TAPS buffer, pH 8.80, ionic strength 0.1, and 25.0 °C.

Table V: Dependence of pK_{eq}[a] for the Reversible Hydration of CO_2 on Glycerol, Sucrose, and Ficoll Concentrations at pH 5.90

	pK_{eq}		pK_{eq}
no cosolutes	6.35	[sucrose] (% w/w)	
[glycerol] (% w/w)		30.3	6.34
7.46	6.34	35.6	6.36
23.9	6.32	[ficoll] (% w/w)	
34.9	6.32	1.6	6.33
45.3	6.35	3.2	6.30
[sucrose] (% w/w)		5.2	6.35
16.1	6.33	8.0	6.32
24.8	6.34		

[a] Calculated according to the Haldane relation (eq 5) using the Michaelis–Menten parameters for HCO_3^- dehydration and CO_2 hydration in Tables II and III.

Table VI: Effect of Viscogenic Cosolutes on Acidity Constants of Buffers[a] at Ionic Strength 0.1 and 25.0 °C

	pK_a^{MES}	pK_a^{TAPS}
no cosolutes	6.10	8.38
[glycerol] (% w/w)		
12.6	6.10	8.39
24.6	6.10	8.45
35.8	6.12	8.54
46.6	6.15	8.65
[sucrose] (% w/w)		
9.90	6.12	8.39
19.1	6.13	8.41
27.6	6.13	8.46
35.6	6.15	8.51
[ficoll] (% w/w)		
2.0	6.10	8.39
4.0	6.10	8.41
6.0	6.10	8.42
8.0	6.11	8.43

[a] Determined by the glass electrode method.

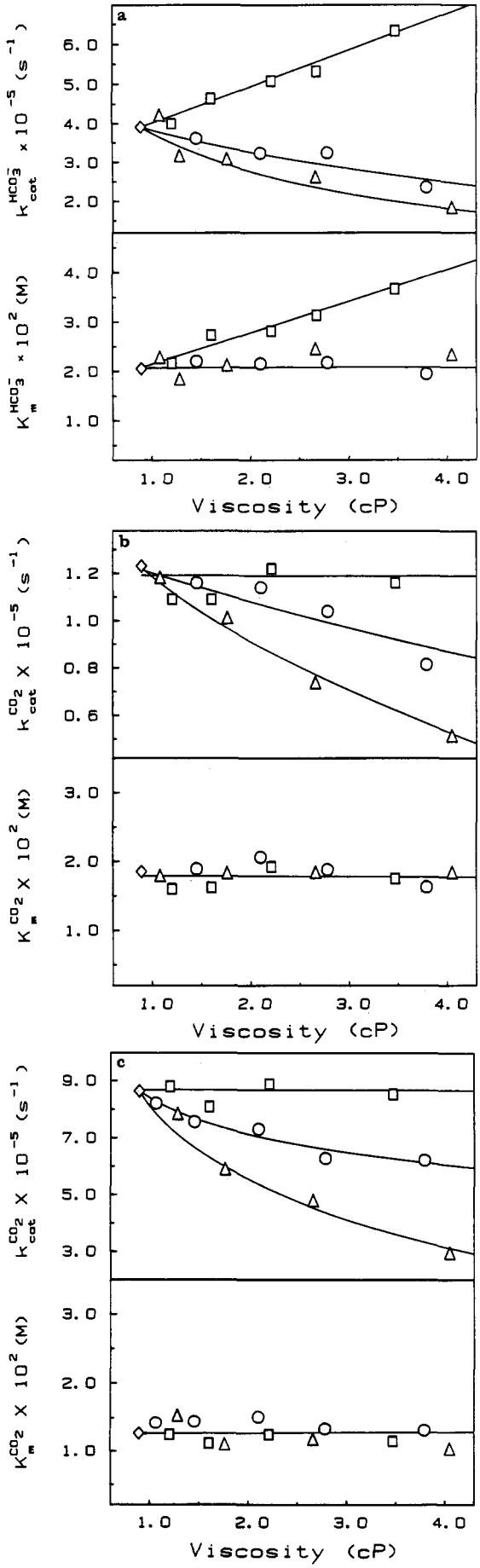

FIGURE 2: Dependence of k_{cat} and K_m on viscosity in solutions with no cosolutes (◇) and solutions with viscosity adjusted by addition of glycerol (△), sucrose (○), and ficoll (□), at 25.0 ± 0.02 °C and ionic strength 0.1 for (a) HCO_3^- dehydration at pH 5.90 in 20 mM MES buffer and for (b and c) CO_2 hydration at pH 5.90 in 20 mM MES buffer and at pH 8.80 in 20 mM TAPS buffer, respectively.

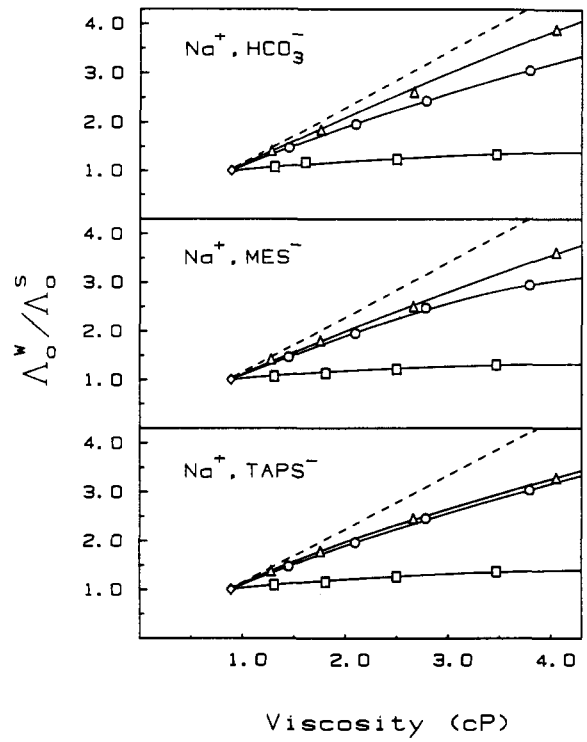

FIGURE 3: Plots of limiting equivalent conductance ratios (Λ_0^w/Λ_0^s) vs. solution viscosity for electrolytes $Na^+HCO_3^-$, Na^+MES^-, and Na^+TAPS^- in water (◇), glycerol–water (△), sucrose–water (○), and ficoll–water (□) solutions at 25.0 °C: $(\Lambda_0^w)_{Na^+HCO_3^-}$ = 94.9 cm² ohm⁻¹ equiv⁻¹, $(\Lambda_0^w)_{Na^+MES^-}$ = 77.0 cm² ohm⁻¹ equiv⁻¹, and $(\Lambda_0^w)_{Na^+TAPS^-}$ = 76.3 cm² ohm⁻¹ equiv⁻¹. Dashed lines indicate behavior predicted by the Stokes–Einstein equation.

This observation is relevant in light of the finding that the rate-limiting proton transfer between CA II and external buffer is a function of the pK difference between donor and acceptor species in a manner consistent with theory (Eigen, 1964; Hammes, 1982). Furthermore, the kinetic analyses were done in the pH plateau regions of HCO_3^- dehydration and CO_2 hydration sufficiently away from the pK_a (=7.0) of the activity-linked group of CA II so that the effect on the rate of possible deviations from this value induced by the viscogenic cosolutes would be negligible.

The ability of ions to transfer electrical charge through a solution can be used to directly determine their diffusion coefficients, and this conveniently bypasses the need for relating the mobility of a particle to the macroscopic solution viscosity. Our measurements of electrolytic conductances at 25.0 °C for HCO_3^-, MES^-, and $TAPS^-$ (substrate and buffer molecules with Na^+ as the counterion) in solvents of increased viscosity (Figure 3) confirm previous observations that smaller ions are less retarded by the viscous medium than would be expected from the Stokes–Einstein relation as well as that glycerol is somewhat better than sucrose in reducing ionic mobility at equal viscosities (Steel et al., 1958; Hoshino & Sato, 1967). A considerably larger departure from Stokes–Einstein behavior resulted when ficoll was used as cosolute. Since this phenomenon was also detected previously in connection with solutions of ficoll (Stokes & Weeks, 1964) and other hydrophilic polymers (Hoshino & Sato, 1967), it is clear that polymeric viscogens do not significantly reduce diffusion coefficients of small solutes.

Apart from inducing the desired solvent viscosity increase, large amounts of polyhydroxy compounds can produce other perturbations in the solvent structure that may affect the enzymatic rate. In order to test for cosolvent effects unrelated to diffusion, we also examined two other reactions catalyzed by CA II in glycerol–water, sucrose–water, and ficoll–water

Table VII: Dependence of $k_{enz,f}^{CH_3CHO}$[a] on Glycerol, Sucrose, and Ficoll Concentrations in 20 mM MES Buffer, pH 5.90, Ionic Strength 0.1, and 25.0 °C

	viscosity (cP)	χ	$k_{enz,f}^{CH_3CHO} \times 10^{-3}$ (M^{-1} s^{-1})
no cosolutes	0.890	0.512	1.20
[glycerol] (% w/w)			
14.6	1.28	0.585	1.11
23.9	1.76	0.642	0.966
34.9	2.66	0.693	0.807
45.3	4.05	0.756	0.657
[sucrose] (% w/w)			
16.1	1.45	0.532	1.35
24.8	2.10	0.559	1.44
30.3	2.78	0.565	1.49
35.6	3.79	0.574	1.68
[ficoll] (% w/w)			
2	1.31	0.513	1.21
4	1.81	0.511	1.20
6	2.50	0.512	1.21
8	3.47	0.510	1.20

[a] $k_{enz,f}^{CH_3CHO} = \chi k_{enz,obsd}^{CH_3CHO}$; $\chi = K_{eq}/(K_{eq} + 1)$; $K_{eq} = [CH_3CH(OH)_2]/[CH_3CHO]$.

Table VIII: Dependence of k_{enz}^{pNPA} on Glycerol, Sucrose, and Ficoll Concentrations at 25.0 °C

	viscosity (cP)[a]	$k_{enz}^{pNPA} \times 10^{-2}$ (M^{-1} s^{-1})[b] at pH 5.90	$k_{enz}^{pNPA} \times 10^{-3}$ (M^{-1} s^{-1})[c] at pH 8.80
no cosolutes	1.07	1.48	1.45
[glycerol] (% w/w)			
13.2	1.48	1.48	1.50
21.7	1.87	1.40	1.48
31.7	2.57	1.08	1.47
41.2	3.76	0.837	1.45
[sucrose] (% w/w)			
14.6	1.61	1.83	1.55
22.5	2.21	2.00	1.65
27.6	2.79	2.12	1.80
32.4	3.68	2.27	1.85
[ficoll] (% w/w)			
2.0	1.54	1.49	1.49
4.0	2.20	1.47	1.50
6.0	3.06	1.45	1.52
8.0	4.02	1.45	1.51

[a] Viscosities are those in 10% v/v acetone solutions at 25 °C. [b] Done in 20 mM MES buffer and ionic strength 0.1. [c] Done in 20 mM TAPS buffer and ionic strength 0.1.

solutions: hydration of acetaldehyde and hydrolysis of pNPA. With second-order rate constants k_{enz} of 10^2–10^4 M^{-1} s^{-1}, these reactions are clearly far enough from the diffusion-controlled limit not to be affected by viscosity and consequently provide a control that can be used to explain and correct, in part, for the differences observed among the cosolvent effects. Dependence of k_{enz} for acetaldehyde hydration at pH 5.90 and pNPA hydrolysis at pH 5.90 and pH 8.80 on viscogen concentration is summarized in Tables VII and VIII, respectively, and the behavior of the inverse of k_{enz} is plotted against viscosity in Figure 4. It is evident that both the hydration and the hydrolysis reactions are affected similarly by the cosolvents at low pH, with glycerol causing a significant rate reduction, sucrose causing a rate increase, and ficoll having virtually no effect. At high pH, the effects are similar except that the inhibitory effect of glycerol is abolished.

Discussion

The degree of dependence of a chemical reaction on diffusion rates has been traditionally studied by varying the viscosity

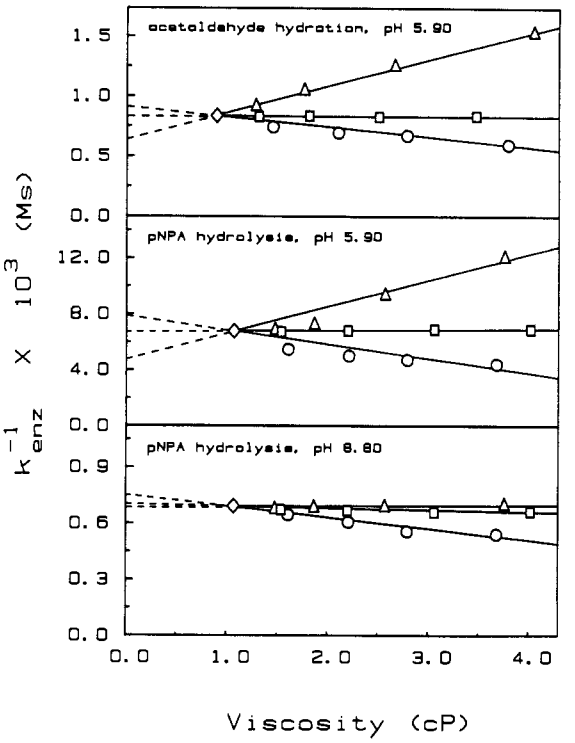

FIGURE 4: Dependence of $1/k_{enz}$ on solution viscosity for acetaldehyde hydration at pH 5.90 and pNPA hydrolysis at pH 5.90 and pH 8.80. Reaction conditions and symbols are the same as in Figure 2 except that pNPA hydrolysis was done in 10% v/v acetone solutions.

of the medium with suitable, chemically inert reagents and measuring the changes in the observed velocity. A key assumption made in such studies is that the solvent perturbation induced as a side effect of the presence of viscogenic cosolute is small and generally insignificant in comparison with the larger viscosity change. Although this assumption is usually valid for most ordinary chemical processes, one should exercise great caution when applying this technique to enzymatic systems where such phenomena as inhibition, surface adsorption, conformational changes, and other nonspecific diffusion-unrelated effects may be partially responsible for the observed rate changes. Conclusions drawn from such studies can be made substantially safer by utilizing more than one viscogen and extending the study of cosolute effects to include the enzymatic reactions with substrates that have catalytic rates well below the diffusion-controlled limit. With these precautions, the likelihood of overlooking the possible cosolute-specific perturbations imposed on the enzymatic system is substantially reduced. Here we utilized three viscogens belonging to a family of polyhydroxy compounds but of widely different molecular weights and structure. In addition to studying the rates of reversible CO_2 hydration, we also examined the effects of these cosolutes on CA II catalyzed acetaldehyde hydration and pNPA hydrolysis.

One of the most consistent observations throughout this work has been the viscosity independence of the Michaelis constant, K_m [$=(k_{-1} + k_{cat})/k_1$], noted in both CO_2 hydration and HCO_3^- dehydration reactions (the only exceptional case was HCO_3^- dehydration with ficoll as the cosolute; this and other anomalies of this viscogen will be addressed later). Constancy of this kinetic parameter with various physical perturbations is indeed striking, in view of the previous findings that this quantity is also pH and temperature independent. Because the value of K_m is predominantly determined by k_1 and k_{-1} terms, these rate constants either are not significantly affected by the viscosity increase or, more likely, are reduced by a similar factor. Regardless of which of these possibilities

is true, the observed second-order rate constant, k_{cat}/K_m, is apparently not influenced by the diffusion of the substrate molecules.

In order to complete a full round of catalysis, the enzyme has to abstract or release a proton, depending on the direction of the hydration reaction. The importance of external buffer species in aiding this process has been widely recognized (Khalifah, 1973; Prince & Wooley, 1973; Lindskog & Coleman, 1973; Pocker et al., 1981, 1985, 1986; Rowlett & Silverman, 1982). The possibility that an internally located group B_i or B_iH^+ may be involved in the dynamics of proton transfer between the zinc-coordinated H_2O or OH^- and the buffer system of the medium (eq 6) has been considered earlier in

$$ZnOH_2|B_i|B \underset{k_{-a}}{\overset{k_a}{\rightleftharpoons}} ZnOH|B_iH^+|B \underset{k_{-b}}{\overset{k_b}{\rightleftharpoons}} ZnOH|B_i|BH^+ \quad (6)$$

papers from our laboratory (Pocker & Meany, 1965; Pocker & Bjorkquist, 1977a; Pocker et al., 1986). The proton relay depicted in eq 6 is envisioned as occurring in two distinct stages: an intramolecular stage a and an intermolecular stage b. Retarded diffusion of the buffer molecules toward or away from the active site would then have an effect on stage b and the overall rate, provided that this step is partially rate determining. Stage a, on the other hand, could be affected through viscosity-induced changes in intramolecular isomerization rates involving functionally motions (Frauenfelder & Wolynes, 1985). Our results strongly suggest that the viscosity effect resides primarily in the k_{cat} term for both directions of catalysis, indicating that some kind of proton-transfer-related process is primarily responsible for the observed rate decrease.

The possibility that the protein may be altering its tertiary structure in response to added viscogen needs to be considered. The absence of detectable differences in the circular dichroic spectra of CA II in water and mixed solvents (45% w/w glycerol, 35% sucrose, and 8% ficoll) is a good indication that the enzyme is sturdy enough to withstand changes of this kind in its environment and that the observed rate reduction is not due to gross conformational changes induced by these cosolutes. This is not surprising in light of the fact that a number of polyhydroxy compounds including glycerol and sucrose have been shown to stabilize native conformations of many polypeptides in aqueous solution (Lee & Timasheff, 1981; Gekko & Timasheff, 1981). However, more subtle perturbations in the enzymatic structure affecting interplay of catalytically related conformational states would not be detected by circular dichroism and thus cannot be ruled out. Resting enzymes perform significant fluctuations in solution, and an understanding of these fluctuations is important for gaining insights into relationships between dynamics and function. Even more important, however, are motions performed by an enzyme during turnover.

Evaluation of the degree to which CA II catalysis is subject to changes in diffusion rates requires analysis of relations describing the limiting situations of such processes. The original Smoluchowski expression for the second-order rate constant in solution (Smoluchowski, 1917)

$$k_d = 4\pi N_a R_e D/1000 \qquad M^{-1}\ s^{-1} \quad (7)$$

where N_a is Avogadro's number, R_e is the encounter radius (for small reacting species this is generally assumed to be equal to the sum of their hydrodynamic radii), and D is the sum of the translational diffusion coefficients of the reactants, has been shown to be a limiting case of a more general expression (Collins & Kimball, 1949; Noyes, 1961)

$$k_{obsd} = k_d/(1 + k_d/k_0) \quad (8)$$

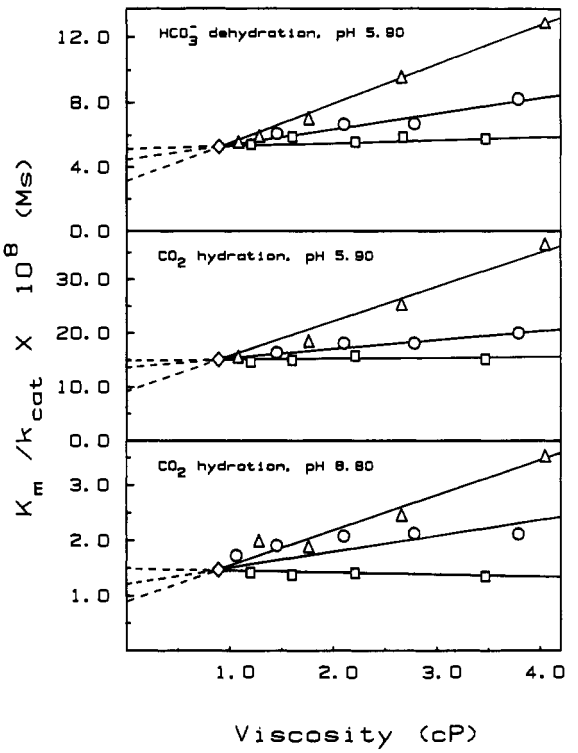

FIGURE 5: Dependence of K_m/k_{cat} on solution viscosity for HCO_3^- dehydration at pH 5.90 and CO_2 hydration at pH 5.90 and pH 8.80. Conditions and symbols are the same as in Figure 2.

where k_{obsd} is the observed second-order rate constant and k_0 ($=\lim_{\eta\to 0} k_{obsd}$) is the intrinsic rate constant for the reaction.

Assuming the validity of the Stokes–Einstein expression, the diffusion constant for a spherically symmetric particle in a liquid can be related to solution viscosity (η) by

$$D = kT/6\pi\eta r \quad (9)$$

where k is the Boltzmann constant, T is the absolute temperature, and r is the hydrodynamic radius of the diffusing species. Substitution in eq 7 and 8 yields upon rearranging

$$1/k_{obsd} = K_m/k_{cat} = 1/k_0 + K_r\eta \quad (10)$$

where $K_r = 3000r_E r_X/[2RT(r_E + r_X)R_e]$, with r_E and r_X being the hydrodynamic radii of the enzyme and the catalytically important small molecule (CO_2, HCO_3^-, or the buffer molecule), respectively. The distinction between $r_E + r_X$ and R_e is deliberately maintained because we felt that, due to the fact that the active site of CA II is buried (Vaara, 1974), the encounter radius is in all probability significantly smaller than the sum of the hydrodynamic radii of the reactants.[4]

The form of eq 10 predicts the linear relationship between $1/k_{obsd}$ and viscosity with the slope equal to K_r and y intercept equal to $1/k_0$. Furthermore, when $k_0 \ll k_d$, the diffusion rates would have little or no effect on the observed reaction rate and the plot of K_m/k_{cat} ($=1/k_{obsd}$) vs. viscosity would have zero slope with the y intercept equal to $1/k_{obsd}$. At the other extreme, when $k_0 \gg k_d$, the observed rate would be limited only by the frequency of encounter of the reacting species and the plot of K_m/k_{cat} vs. viscosity would have the largest allowed slope and pass through the origin. The existence of a sig-

[4] Using ^{13}C NMR spectroscopy, Williams and Henkens (1985) have further investigated the binding of HCO_3^- in the active site of Co(II) human carbonic anhydrase I. They conclude that HCO_3^- is associated with the tetracoordinated metalloenzyme, that the ^{13}C of HCO_3^- is located 3.22 ± 0.02 Å from the Co(II), and that the enzyme functions as a catalyst by direct coordination of the HCO_3^- to the metal ion.

Table IX: Apparent Degrees of Diffusion Control, δ, for CA II Catalyzed HCO_3^- Dehydration, CO_2 Hydration, Acetaldehyde Hydration, and pNPA Hydrolysis at 0.890 cP and 25.0 °C

CA II reaction	pH	cosolute	δ^a	δ^b	δ^c	δ^d
HCO_3^- dehydration	5.90	glycerol	0.40	0.50	0.55	
HCO_3^- dehydration	5.90	sucrose	0.16	0.25	0.26	
CO_2 hydration	5.90	glycerol	0.38		0.50	
CO_2 hydration	5.90	sucrose	0.11		0.20	
CO_2 hydration	8.80	glycerol	0.36			0.55
CO_2 hydration	8.80	sucrose	0.18			0.27
acetaldehyde hydration	5.90	glycerol	0.24			
acetaldehyde hydration	5.90	sucrose	−0.10			
pNPA hydrolysis	5.90	glycerol	0.28			
pNPA hydrolysis	5.90	sucrose	−0.17			
pNPA hydrolysis	8.80	glycerol	0			
pNPA hydrolysis	8.80	sucrose	−0.10			

[a] Calculated from K_m/k_{cat} vs. viscosity plots. [b] Calculated from K_m/k_{cat} vs. $1/D_{HCO_3^-}$ plots. [c] Calculated from K_m/k_{cat} vs. $1/D_{MES^-}$ plots. [d] Calculated from K_m/k_{cat} vs. $1/D_{TAPS^-}$ plots.

nificant y intercept and a finite slope of the above-mentioned plots (Figure 5) indicates a process in between the two extremes, or a partially diffusion-controlled reaction.

The Stokes–Einstein relation, eq 9, was originally derived for diffusion of molecules whose hydrodynamic radii are significantly bigger than those of the solvent and whose motion in solution could be described as flow through a viscous continuum. Large deviations from this rule have been observed for smaller molecules and ions whose sizes are of the same order of magnitude as the solvent (Steel et al., 1958; Evans et al., 1979, 1981). Diffusion coefficients of such species were not only found to be less responsive to increased solution viscosity than would be predicted by eq 9 but were also dependent on the particular cosolute used. It was because of these limitations that we experimentally determined the mobilities of HCO_3^- and the conjugate bases of MES and TAPS buffers in the water–viscogen solvent systems used in this study. By use of the Nernst–Einstein equation, an expression can be derived relating the diffusion coefficient of an ion D_i to the ratio of limiting equivalent conductances in the viscous solvent and water:

$$D_i^s = D_i^w \lambda_0^s / \lambda_0^w \quad (11)$$

where superscripts s and w refer to viscous solvents and water, respectively. Limiting equivalent conductances for individual ions (λ_0) can be calculated from the limiting equivalent conductances of the entire electrolyte (Λ_0) and the limiting transference numbers for separate ions (t_0) by the Kohlrausch principle, $\lambda_0 = t_0\Lambda_0$. However, the value of the ratio λ_0^s/λ_0^w is nearly identical with Λ_0^s/Λ_0^w since it has been shown that the limiting transference numbers for similar electrolytes differed by no more than 1% in water and 20% aqueous glycerol and sucrose solutions, i.e., $t_0^s \simeq t_0^w$ (Steel et al., 1958). Evaluation of electrolytic conductances provides an accurate and convenient measure of the actual diffusion coefficients for the above-mentioned anions in viscous solutions and therefore eliminates the necessity for use of the Stokes–Einstein equation in a situation for which its assumptions are not strictly valid. Analogously with the treatment used to derive eq 10, substituting D_i in eq 7 and 8 and solving for $1/k_{obsd}$, we obtain

$$1/k_{obsd} = K_m/k_{cat} = 1/k_0 + K_\lambda/D_i^s \quad (12)$$

where $K_\lambda = 1000/4\pi N_a R_e$.

The apparent degree of diffusion-controlled character for a given reaction δ at a given viscosity can be obtained by comparing the slope of the experimental K_m/k_{cat} vs. viscosity plot (if the Stokes–Einstein relation is assumed valid) or K_m/k_{cat} vs. $1/D_i^s$ plot (if the Nernst–Einstein equation is used to obtain values for ionic diffusion coefficients experimentally) to the slope of the line representing the theoretical diffusion-controlled limit (eq 13). By use of the values of k_0 and k_{obsd}

$$\delta = 1 - k_{obsd}/k_0 \quad (13)$$

obtained from the linear least-squares analysis of the above-mentioned plots, the apparent degree of diffusion control can be calculated for any solution viscosity. Values for HCO_3^- dehydration and CO_2 hydration at viscosity equal to 0.890 cP (or $\lambda_0^w/\lambda_0^s = 1$) are presented in Table IX. Included for comparative purposes are also values for acetaldehyde hydration and pNPA hydrolysis, although these estimates are obviously not related to diffusion.

Of the two monomeric cosolutes used for this work, glycerol caused a larger reduction of the CA II catalyzed CO_2 hydration and HCO_3^- dehydration rates at comparable viscosities. The magnitude of this effect is considerably bigger than could be accounted for by the relatively small discrepancy between the extents to which the two cosolutes retarded ionic mobilities (Figure 3). Large quantities of glycerol necessary to significantly raise the viscosity of the medium and the fact that small primary and secondary alcohols were shown to inhibit the hydratase activity of CA II with K_i values ranging from 0.08 to 1.2 M (Pocker & Dickerson, 1968) render it probable that the observed large rate reduction is partially due to the inhibitory effect of this cosolute.

It is also important to mention that, even after vacuum distillation of the best commercially available glycerol, a trace impurity of glyceraldehyde (0.022% w/w) was detected by gas chromatography. This implies that the amount of this oxidation product in our kinetic assays did not exceed 0.010% w/w. When added in this concentration to aqueous buffers containing CA II, glyceraldehyde was not measurably inhibitory toward CO_2 hydration or HCO_3^- dehydration for at least 48 h.[5]

Although it may be anticipated that sucrose and ficoll should have similar effects on the rate at equal viscosities (ficoll being a polymer of sucrose and epichlorohydrin), this was found not to be the case. A similar discrepancy in the magnitude of effects induced by these cosolutes was recently observed in the reaction of β-lactamase I with a variety of penicillins and cephalosporins (Hardy & Kirsch, 1984). The origin of this difference is most likely due to the fact that the mobility of smaller ions (Figure 3) and neutral molecules (Hoshino & Sato, 1967) is only moderately impaired by the solution viscosity increase and is mostly dependent on the volume fraction of the cosolute (Biancheria & Kegeles, 1957; Steel et al., 1958;

[5] Significantly larger amounts of glyceraldehyde (0.3% w/w), however, caused time-dependent inhibition of CA II, resulting in 8% and 20% reduction in activity in 4 and 32 h, respectively.

Stokes & Weeks, 1964). Obviously, considerably less polymer than monomer is necessary to produce a comparable viscosity increase, and the volume fraction of solution occupied by ficoll will be substantially smaller than that taken up by glycerol or sucrose. In such an environment, although the macroscopic solution viscosity increase may be considerable, smaller solutes are able to effectively move around the hydrated polymer without significant compromise in their diffusion rates. Consequently, estimates of the degree of diffusion control from $1/k_{obsd}$ vs. viscosity plots would be meaningless in ficoll solutions. Alternatively, the use of $1/k_{obsd}$ vs. $1/D_i^s$ relations does have a real physical significance; however, the relatively small change in the mobility of small solutes produced by ficoll (Figure 3) makes the estimates of slopes of such plots unacceptably inaccurate. It is also possible that ficoll may be partially removing the inhibitory effect of certain anions (i.e., SO_4^{2-}) by acting as a nonspecific scavenger for these species. In view of these complications, we consider ficoll to be an unsuitable viscogen for reducing diffusion rates of small solutes.

If it is assumed that the nonspecific inhibition induced by glycerol and the nonspecific activation induced by sucrose evident in acetaldehyde hydration and pNPA hydrolysis reactions are a good reflection of the diffusion-unrelated effects of these cosolutes, estimates of the apparent degree of diffusion control for the reaction of CA II with the natural substrates can be corrected accordingly. This is accomplished by subtracting the values of δ for acetaldehyde hydration or pNPA hydrolysis from the values for HCO_3^- dehydration and CO_2 hydration (at corresponding pH) in Table IX. This correction lowers the values of δ for glycerol solutions to 0.12 or 0.16 (depending on whether acetaldehyde hydration or pNPA hydrolysis is used as the control reaction, respectively) for HCO_3^- dehydration and 0.10 or 0.14 for CO_2 hydration at low pH, but the value for CO_2 hydration at high pH of 0.36 remains unchanged as a consequence of the disappearance of the inhibitory effect of this cosolute (only estimates of δ from $1/k_{obsd}$ vs. viscosity plots were used for simplicity; obviously, somewhat higher corrected values of δ would be calculated from $1/k_{obsd}$ vs. $1/D_i^s$ plots). The same treatment raises the estimates of δ obtained in sucrose solutions to 0.26 or 0.32 for HCO_3^- dehydration, 0.21 or 0.27 for CO_2 hydration at low pH, and 0.28 for CO_2 hydration at high pH. Because of smaller and more consistent absolute rate changes associated with slower reactions of CA II and because of the lack of any inhibitory tendencies, we prefer to regard the results obtained in sucrose solutions as a true index of the viscosity effect.

In addition to being of interest from a purely mechanistic standpoint, catalysis of CA II in solutions of increased viscosity has some important physiological ramifications. The viscosity of the erythrocyte interior at physiological temperature (i.e., 37 °C) is approximately 6 cP in normal cells, with this condition being primarily regulated by intracellular hemoglobin concentration (Cokelet & Meiselmann, 1968; Chien et al., 1970). As the viscosity of hemoglobin solutions rises rapidly as a function of concentration at values near or above those physiologically normal (Cokelet & Meiselmann, 1968), it is expected that the reduction of the mean red blood cel volume due to dehydration, which has been observed in connection with processes such as erythrocyte aging (Williams & Morris, 1980; Nash & Meiselmann, 1983) and sickling (Chien et al., 1970; Glader & Nathan, 1978; Nash et al., 1984), will produce a significant increase in internal viscosity. It can be estimated that maximally shrunk red blood cells have an internal viscosity about 5 times that of normal cells (Ross & Minton, 1977). In a solution of such thickness, it is highly likely that the CA II catalyzed reversible hydration of CO_2 is considerably closer to the diffusion-controlled limit than was estimated here for aqueous solutions with no added viscogens.

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Anomeric Specificity of L-Fucose Dehydrogenase: A Stereochemical Imperative in Aldopyranose Dehydrogenases?†

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ABSTRACT: A set of hypotheses is proposed that explains the anomeric specificity of aldopyranose dehydrogenases in terms of an evolutionarily selected function. The first hypothesis, based on stereoelectronic theory, argues that, in the "allowed" transition state for oxidation at the anomeric carbon, the two oxygens attached to the anomeric carbon each bear a lone pair of electrons antiperiplanar to the departing "hydride". The second hypothesis is that the dehydrogenase is functionally constrained to bind the anomer that has this arrangement of lone pairs in its lowest energy chair conformer. The anomeric specificity of L-fucose dehydrogenase is experimentally examined. The enzyme oxidizes preferentially the β-anomer, consistent with the prediction made by these hypotheses. Available experimental data for other enzymes (D-glucose-6-phosphate dehydrogenase, D-glucose dehydrogenase, D-galactose dehydrogenase, D-abequose dehydrogenase, and D-arabinose dehydrogenase) are found to be also consistent with the proposed hypotheses.

For more than a quarter of a century, it has been known that certain aldose dehydrogenases act on only one anomer of their sugar substrates. In 1952, Strecker and Korkes (Strecker & Korkes, 1952) showed that an NAD^+-dependent[1] D-glucose dehydrogenase selectively catalyzes the oxidation of the β-anomer,[2] and a half-dozen other enzymes have been examined

† This investigation was supported by the National Science Foundation and the National Institutes of Health. D.B.B. was partially supported by an Eastman Kodak fellowship during the course of this work.
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[1] Abbreviations: NAD^+, nicotinamide adenine dinucleotide, oxidized form; NADH, nicotinamide adenine dinucleotide, reduced form; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/87/0426-2606$01.50/0