Molecular Basis of Ionic Strength Effects: Interaction of Enzyme and Sulfate Ion in CO_2 Hydration and HCO_3^- Dehydration Reactions Catalyzed by Carbonic Anhydrase II†

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ABSTRACT: CO_2 hydration and HCO_3^- dehydration reactions catalyzed by carbonic anhydrase II have been examined at various concentrations of sodium sulfate with a stopped-flow technique. We find that at low ionic strength CO_2 hydration and HCO_3^- dehydration reaction rates remain unaffected by varying the salt concentration at pH higher than 7.0, while the reaction rates decrease with increasing ionic strength at low pH. For CO_2 hydration reactions, salt effects reside only in the k_{cat} term, not in the K_m term, whereas for HCO_3^- dehydration reactions, salt effects reside only in the K_m term, not in the k_{cat} term. In this regime, the salt concentration dependence of the turnover rate for CO_2 hydration at low pH is attributed to an electrostatic effect on the ionization constants of the enzyme and/or enzyme–substrate complex, which in turn affect the pH profile of k_{cat}. The rates of the bimolecular interaction between the uncharged CO_2 molecule and carbonic anhydrase II at high pH are unaffected by low salt concentration while the rates of the bimolecular interaction of HCO_3^- with enzyme at low pH decrease with increasing salt concentration, consistent with a negative salt effect on an electrostatically enhanced diffusion of the negatively charged substrate to the positively charged active site. These bimolecular reactions between enzyme and substrate at low ionic strength obey rate equations derived from the Debye–Hückel limiting law and the transition-state theory. Simple linear relationships between the logarithm of the catalytic constant, log k^d_{enz}, and the square root of the ionic strength were established. Catalytic constants at zero ionic strength, $(k_{enz})_0$, and the charge at the active site of the enzyme, Z_{enz}, were obtained for HCO_3^- dehydration reactions. Z_{enz} values at various pHs have important implications in regard to the proton inventory and the possible structure of the active site. There appear to be at least two active site groups ionizing in the pH range studied. These two groups are likely to be the $Zn–OH_2$ complex and an imidazolium group of His-64. A Z_{enz} value of ca. 1.70 at pH 5.20 and zero ionic strength indicates that the two groups are essentially protonated at lower pH ($Z_{enz} \rightarrow$ 2.0 at approximately pH 4.5). The nature of the electrostatic influence of sulfate ion on HCO_3^- dehydration reactions was further analyzed with the pH profiles of K_m at zero ionic strength and ionic strength of 0.1. At zero ionic strength, the binding of HCO_3^- depends on an ionizing group of $pK_a \sim 5.85$, while in the presence of sulfate, added in order to maintain the ionic strength of the medium, the pK_a of $Zn–OH_2$ and His-64 are coupled through electrostatic interactions and exhibit a common pK_a of ca. 7.0. We emphasize here that at low ionic strength the kinetic behavior of carbonic anhydrase catalyzed CO_2 hydration and HCO_3^- dehydration can be fully described and simulated in terms of electrostatic effects on enzyme–substrate reactions. At higher salt concentrations, however, sulfate binds to the enzyme in a mode that inhibits both hydrase and hydrolase activities of carbonic anhydrase II. In this different regime, an inner-sphere enzyme–sulfate complex is formed so that the effect of sulfate ion on molecular dynamics, recognition, and reactivity can no longer be described exclusively by simple electrostatic theories.

In general, enzymes are only stable and active under physiological conditions in which both pH and ionic strength are under control. In fact, some enzymes denature at zero ionic strength. In the kinetic study of erythrocyte carbonic anhydrase, CA, ionic strength is kept constant by the addition of sodium sulfate, a salt considered to contain the least inhibitory anion SO_4^{2-} (Roughton & Booth, 1946; Kernohan, 1965). Recently, important questions were raised concerning such reagents especially in regard to the role they play in the enzymatic process.

It was reported that Co(II) carbonic anhydrase shows different and puzzling results when its NMR relaxation spectra of solvent water protons are studied in the presence and absence of Na_2SO_4 (Fabry et al., 1970; Bertini et al., 1977). In the presence of SO_4^{2-}, there is no water proton relaxivity observed at low pH and one rapidly exchanging, metal-bound water at high pH, while with no added electrolytes there is metal-bound water observed for Co(II)-CA over the pH range 5.9–9.0. It was concluded that SO_4^{2-} may bind to Co(II) at low pH displacing water. Koenig et al. (1978, 1980) reinvestigated the problem and proposed that HSO_4^- is the inhibitory anion. However, such an assumption would require an extremely strong binding between HSO_4^- and the enzyme. Later, it was shown that the pH–rate profiles of *p*-nitrophenyl acetate hydrolase activity of bovine CAII (Y. Pocker and C. T. O. Fong, unpublished results; Simonsson & Lindskog, 1982) and the absorption spectra at 640 nm of bovine Co(II)-CA (Bertini et al., 1980) are all complex in the absence of SO_4^{2-} and appear to involve two electrostatically interacting groups within the enzyme with different pK_as. Results from both

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laboratories indicate that at high concentrations and acidic pH sulfate actually binds to the enzyme and inhibits the *p*-nitrophenyl acetate hydrolysis reaction. Clearly, the various inhibitory effects of SO_4^{2-} ion are strongly dependent upon the ionic strength of the solution.

It is not uncommon to find that when electrolytes, including even buffers, bind to a protein, the property of the protein often changes (Alberty & Bock, 1953; Massey, 1953). Isoelectric points of some proteins shift to higher, others to lower, pH by the binding of ions (Alberty, 1949; Alberty & Marvin, 1951). Interestingly, it has also been shown for human globulins that there is a linear relationship between the shift of isoelectric pH and the ionic strength (Smith, 1936). Ionic strength effects on enzyme activity have been described for urease by Kistiakowsky and Shaw (1953), who found that under limiting conditions the enzymatic hydrolysis of urea can be fitted by the Debye–Hückel equation for ionic activity coefficients. Nonetheless, it proved difficult to treat quantitatively ionic strength effects associated with enzymatic activity in more concentrated solutions. A wide variety of observations on denaturation rates of proteins has shown that they are affected by added electrolytes in the solution (Steinhardt, 1937; Steinhardt & Zeiser, 1951, 1953, 1955; Ruegamer, 1954). Here we comment only on the fact that at high salt concentrations the effects are more complex than those predicted by the simple Debye–Hückel rate equation and require some explanation based on specific interactions between ion and protein.

Besides the complicated influence of ionic strength on enzymatic reactions, it is well established for many chemical reactions in solution that reaction rates between two charged molecules are greatly affected by ionic strength (La Mer, 1932, 1938), while those between a neutral molecule and an ion are hardly influenced by ionic strength at low salt concentrations (Moelwyn-Hughes, 1971). These interesting generalizations on the influence of the ionic environment at a constant temperature were derived from transition-state theory and Debye–Hückel rate-limiting law by Brönsted (1922), and their verification is also due chiefly to him and his collaborators.

In the present study we examine the effects of sulfate ion on the enzymatic reactions of carbonic anhydrase with its two natural substrates, CO_2 and HCO_3^-. We demonstrate that under limiting conditions the kinetic behavior of carbonic anhydrase catalyzed CO_2 hydration and HCO_3^- dehydration reactions can be fully described in terms of electrostatic effects on these reactions, that in problems of enzyme mechanisms illumination often follows from the kinetic effect of salts which do not stoichiometrically participate in the catalytic process, and that a kinetic model can be constructed which is consistent with the extant data.

Experimental Procedures

Materials. Reagent-grade MES (4-morpholineethanesulfonic acid), TAPS [3-[[tris(hydroxymethyl)methyl]-amino]propanesulfonic acid], TES [2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid], malonic acid, sodium sulfate, bromocresol purple, and metacresol purple were purchased from Sigma Chemical Co. and used as obtained. *p*-Nitrophenol (Aldrich) was purified by sublimation. Research-grade (99.99% pure) carbon dioxide was obtained from Airco Co. Sodium bicarbonate (Baker) was of reagent grade and used without further purification. Bovine carbonic anhydrase (BCAII) was purchased from Sigma Chemical Co., prepared and purified from bovine erythrocytes. The concentration of the enzyme was calculated from absorbance measurement in the ultraviolet region (ϵ = 54 000 at λ_{max} = 280 nm). Active enzyme concentration was further determined by the activity test of acetazolamide inhibition of BCA activity.

Apparatus. All pH measurements were performed on a PHM84 Cole-Palmer all-glass electrode (No. 5991,50). The pH meter was frequently standardized with commercially available standard buffer solutions. We used the stopped-flow system to study the kinetics of the reversible CO_2 hydration and HCO_3^- dehydration reactions catalyzed by carbonic anhydrase. Reaction rates were monitored on a modified Durrum-Gibson stopped-flow spectrophotometer (Model 1300), which is a complete system for rapidly mixing two liquid reactant solutions and measuring the absorbance change as a function of time.

Methods. Saturated CO_2 solutions were made by bubbling CO_2 gas into a degassed distilled water solution, which was maintained in a thermostated jacketed reservoir. Bicarbonate solutions were prepared by dissolving the sodium bicarbonate salt in degassed deionized distilled water. Buffers were titrated at different ionic strengths against corresponding normality of standard NaOH solutions to obtain dissociation constant at the corresponding ionic strength for each buffer. Indicators used were bromocresol purple ($\epsilon = 6.35 \times 10^4$ M^{-1} cm^{-1} at λ_{max} = 589 nm) with MES and malonate buffer, *p*-nitrophenol ($\epsilon = 1.79 \times 10^4$ M^{-1} cm^{-1} at λ_{max} = 400 nm) with TES buffer, and metacresol purple ($\epsilon = 3.2 \times 10^4$ M^{-1} cm^{-1} at λ_{max} = 578 nm) with TAPS buffer.

All the measurements were performed at 0.02 M buffer concentrations.[1] Na_2SO_4 was added to maintain the desired ionic strength (the ionic strength contributed from bicarbonate was also included in the total ionic strength of the solution, and the ionic strength of the CO_2 solution was taken as zero in these initial rate measurements). Ionic strength was generally varied from 0.0075 to 0.1. Very small contributions arising from the presence of indicators were neglected.

Both CO_2 hydration and HCO_3^- dehydration reactions were performed on the Durrum-Gibson stopped-flow system with the changing pH-indicator method. Change of hydronium ion concentration was measured at which only 5% of the reaction was completed by monitoring the absorbance change of the indicator in the solution. The actual velocity term was obtained by multipling averaged dA/dt (absorbance change vs time) with a buffer factor (eq 1). For each substrate concentration,

$$v = (dA/dt)Q \quad (1)$$

10–20 runs were usually repeated and averaged. Lineweaver–Burk plots were employed to calculate the desired catalytic constants.

Results

Lineweaver–Burk plots were employed to analyze the salt effects in the carbonic anhydrase catalyzed carbon dioxide hydration and bicarbonate dehydration reactions with various concentrations of sodium sulfate added to the solution. It is clearly shown by the experimental data that when the ionic strength is maintained low so that the Debye–Hückel limiting law is valid, CO_2 hydration and HCO_3^- dehydration rates remain almost the same at different concentrations of sodium sulfate at pH higher than 7.0, while the rates decrease as the salt concentration is increased at low pH (Tables I and II). The effect of SO_4^{2-} on CO_2 hydration at low pH resembles the pattern of noncompetitive inhibition (Figure 1a), whereas that on HCO_3^- dehydration reaction resembles the pattern of

[1] The zwitterionic buffers used in these studies are of interest, particularly since their contribution to the ionic strength of the solution is minimized.

Table I: Effect of SO_4^{2-} on CAII-Catalyzed CO_2 Hydration Reactions

	pH 6.0[b]		pH 7.0[c]		pH 8.8[d]	
I[a]	k_0 (s^{-1})	k^h_{enz} (M^{-1} s^{-1})	k_0 (s^{-1})	k^h_{enz} (M^{-1} s^{-1})	k_0 (s^{-1})	k^h_{enz} (M^{-1} s^{-1})
0.0075	0.039	9.27×10^6	0.039	3.60×10^7	0.128	7.08×10^7
0.010	0.038	9.22×10^6	0.039	3.51×10^7	0.129	6.46×10^7
0.015	0.039	9.15×10^6	0.037	3.86×10^7	0.126	6.76×10^7
0.020	0.037	9.03×10^6	0.038	3.74×10^7	0.125	7.59×10^7
0.025	0.039	8.96×10^6	0.039	3.42×10^7	0.130	7.08×10^7
0.030	0.037	8.82×10^6	0.037	3.65×10^7	0.126	7.10×10^7
0.050	0.038	8.61×10^6	0.038	3.72×10^7	0.128	7.24×10^7
0.100	0.037	8.08×10^6	0.036	3.61×10^7	0.122	6.92×10^7

[a] I = ionic strength. [b] Kinetic runs in 20 mM MES buffer at 25.0 °C. [c] Kinetic runs in 20 mM TES buffer at 25.0 °C. [d] Kinetic runs in 20 mM TAPS buffer at 25.0 °C.

Table II: Effect of SO_4^{2-} on CAII-Catalyzed HCO_3^- Dehydration Reactions

	pH 5.20[b]		pH 5.55[b]		pH 6.05[b]		pH 6.60[b]		pH 7.00[c]		pH 7.50[c]	
I[a]	k_0 (s^{-1})	k^d_{enz} (M^{-1} s^{-1})	k_0 (s^{-1})	k^d_{enz} (M^{-1} s^{-1})	k_0 (s^{-1})	k^d_{enz} (M^{-1} s^{-1})	k_0 (s^{-1})	k^d_{enz} (M^{-1} s^{-1})	k_0 (s^{-1})	k^d_{enz} (M^{-1} s^{-1})	k_0 (s^{-1})	k^d_{enz} (M^{-1} s^{-1})
0.0075	0.292	7.16×10^7	0.144	3.808×10^7								
0.010	0.285	6.14×10^7	0.146	3.617×10^7					0.0060	8.81×10^6	0.0028	5.28×10^6
0.0124					0.063	1.828×10^7						
0.015	0.289	5.75×10^7	0.143	3.460×10^7	0.064	1.807×10^7			0.0061	8.75×10^6	0.0026	5.42×10^6
0.0177							0.022	1.226×10^7				
0.0200	0.286	5.24×10^7	0.141	3.378×10^7	0.061	1.770×10^7	0.023	1.238×10^7	0.0059	8.69×10^6	0.0027	5.36×10^6
0.0225							0.022	1.216×10^7				
0.025	0.284	4.73×10^7	0.140	3.137×10^7	0.066	1.733×10^7	0.020	1.198×10^7	0.0060	8.73×10^6	0.0026	5.35×10^6
0.030	0.283	4.57×10^7	0.143	3.041×10^7	0.062	1.733×10^7	0.020	1.194×10^7	0.0060	8.60×10^6	0.0026	5.48×10^6
0.050	0.285	3.83×10^7	0.140	2.688×10^7	0.063	1.623×10^7	0.021	1.103×10^7	0.0058	8.71×10^6	0.0027	5.37×10^6
0.100	0.275	2.99×10^7	0.138	2.286×10^7	0.058	1.530×10^7	0.019	1.028×10^7	0.0059	8.54×10^6	0.0026	5.47×10^6

[a] I = ionic strength. [b] Kinetic runs in 20 mM MES buffer at 25.0 °C. [c] Kinetic runs in 20 mM TES buffer at 25.0 °C.

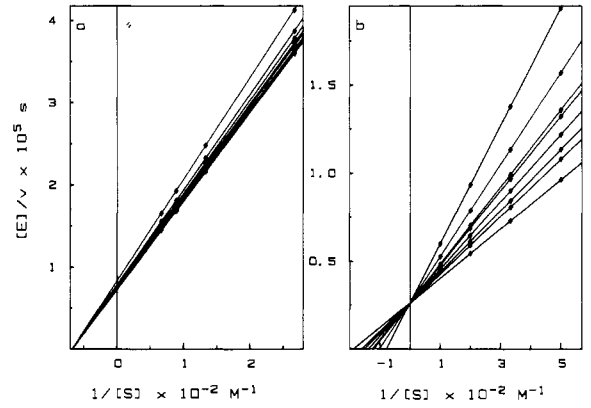


FIGURE 1: Lineweaver–Burk plot of CAII-catalyzed reactions in the presence of various concentrations of SO_4^{2-} at 25.0 °C: (a) CO_2 hydration at pH 6.00 in MES buffer; (b) HCO_3^- dehydration at pH 5.20 in MES buffer. Lines from bottom to top correspond to reactions at ionic strength 0.0075, 0.01, 0.015, 0.02, 0.025, 0.03, 0.05, and 0.1.

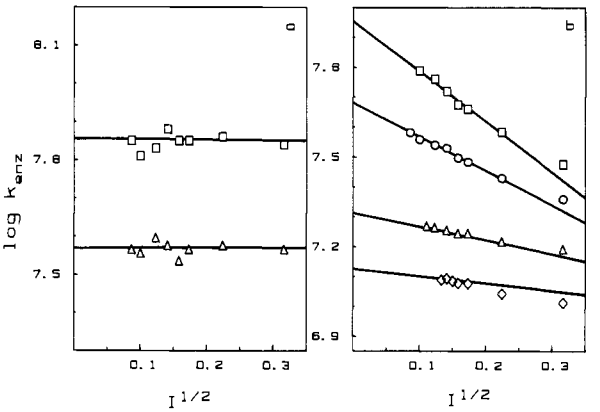


FIGURE 2: Effect of ionic strength on carbonic anhydrase II activity: (a) CO_2 hydration at (□) pH 8.8 in TAPS buffer and (△) pH 7.0 in TES buffer; (b) HCO_3^- dehydration in MES buffer at (□) pH 5.20, (○) pH 5.55, (△) pH 6.05, and (◇) pH 6.60. Ionic strength was maintained by adding Na_2SO_4.

competitive inhibition (Figure 1b). That is to say, the salt effects reside only in the k_{cat} term, not in the K_m term, for CO_2 hydration reactions, whereas the salt effects reside only in the K_m term, not in the k_{cat} term, for HCO_3^- dehydration reactions. Nonetheless, since SO_4^{2-} ion is used as an ionic strength maintaining agent, the reactions performed at different SO_4^{2-} concentrations correspond to conditions where the ionic strengths differ. In regard to the ionic strength dependencies at very low sulfate concentrations, we find that salt effect studies on carbonic anhydrase catalyzed CO_2 hydration and HCO_3^- dehydration give results that can be easily interpreted.

For CO_2 hydration at low pH where the sulfate effect resides only in the k_{cat} term, the salt concentration dependency is attributed to the electrostatic effect of sulfate ion on the ionization constants of enzyme and/or enzyme–substrate complex, which affect the turnover rate. For CO_2 hydration at high pH and for HCO_3^- dehydration where the turnover numbers are unaffected by salt, the results were analyzed as follows. Plotting the logarithm of the enzymatic rate constant of CO_2 hydration (log k^h_{enz}) vs the square root of ionic strength at pH 7.0 and 8.8, we get two straight lines that parallel the x axis (with slope zero) (Figure 2a). Plotting the logarithm of the enzymatic rate constant of HCO_3^- dehydration (log k^d_{enz}) vs the square root of ionic strength at different pHs, we get straight lines with different slopes (Figure 2b). Simple, linear relations between the logarithm of these catalytic constants and the square root of the ionic strength can be established at low sulfate concentrations. The reaction rates for both hydration and dehydration follow the Debye–Hückel rate expression (eq 2) where $(k_{enz})_0$ is the rate constant at zero ionic

$$\log k_{enz} = \log (k_{enz})_0 + 1.02 Z_{enz} Z_s \sqrt{I} \qquad (2)$$

strength, which can be obtained by extrapolation, Z_S is the charge of the substrate, which is 0 for CO_2 and −1 for HCO_3^-, and Z_{enz} is the summation of the charges of the catalytically important residues in the active site of the enzyme, which can

Table III: Ionic Strength Effects for CAII-Catalyzed HCO_3^- Dehydration Reactions in 20 mM MES Buffer at 25.0 °C

pH	charge[a]	$(k_{enz})_0$ (M^{-1} s^{-1})	log $(k_{enz})_0$	$(\Delta S^*)_{es}$ (eu)
5.20	1.68	8.59×10^7	7.939	6.25
5.55	1.07	4.68×10^7	7.670	3.98
6.05	0.44	2.05×10^7	7.312	1.64
6.60	0.29	1.35×10^7	7.130	1.07

[a] Charge at the active site of the enzyme.

vary from 0 to +2 at the pH range studied.

Total charges at the active site of the enzyme, Z_{enz}, and catalytic constants at zero ionic strength, $(k_{enz})_0$, at various pHs were calculated from the slopes and intercepts of the plot of log k^d_{enz} vs $\sqrt{I}$ for bicarbonate dehydration reactions (Table III). Clearly, these enzymatic reactions obey the simple rule that is followed by many simple bimolecular reactions in solution. The same HCO_3^- dehydration reactions were performed at pH 5.50 in malonate buffer instead of MES buffer. The same Z_{enz} and $(k_{enz})_0$ values were obtained, indicating that these reactions were not perturbed by different buffers. In addition, background rates (k_0) for CO_2 hydration in TAPS and TES buffer and HCO_3^- dehydration reactions in MES buffer remain almost constant when the ionic strength of the solution is varied from 0.0075 to 0.1 (Tables I and II), while k_0 for HCO_3^- dehydration reaction in malonate buffer increases with increasing salt concentration. These chemically catalyzed reactions clearly obey the Debye–Hückel rate equation. In the CO_2 hydration reaction, the substrate is an uncharged molecule, while in the HCO_3^- dehydration reaction the charge of the acidic form of zwitterionic buffer is zero whereas monoanionic malonate is negatively charged.

Discussion

The results of this study provide evidence that the rates of the bimolecular interaction of the uncharged CO_2 molecule with carbonic anhydrase II were unaffected by varying the salt concentration, while the rates of the bimolecular interaction of HCO_3^- with enzyme decrease with increasing salt concentration in media where the positively charged active center reacts with the negatively charged bicarbonate ion. The Debye–Hückel rate equation is obeyed by the relatively large enzyme molecule (Figure 3), and the charge values, Z_{enz}, derived from ionic strength effects at various pH values have important implications in regard to the proton inventory and the possible structure of the active site. There appear to be at least two active site groups ionizing in the pH range studied, and in principle, four microscopic pK_a values can be deduced[2] (eq 3). These two groups are suspected to be the Zn–OH_2

$$HEH^{2+} \underset{K_2}{\overset{K_1}{\rightleftharpoons}} EH^+ + H^+ \underset{K_4}{\overset{K_3}{\rightleftharpoons}} E + 2H^+ \qquad (3)$$

complex and the imidazolium group of His-64 (Pocker & Dickerson, 1968; Pocker & Bjorkquist, 1977; Simonsson & Lindskog, 1982; Rowlett & Silverman, 1982). A Z_{enz} of ca. 1.70 at pH 5.20, which is in good agreement with the value reported by Simonsson and Lindskog (1982), indicates that the two groups are essentially protonated at low pH (Z_{enz} approaches 2.0 at pH 4.5).

[2] The enzyme can be represented as a dibasic acid, HEH^{2+}, with two nonidentical acidic groups. The proton on the right indicates that the major catalytic group is protonated, EH^+, and the proton on the left represents another group, HE^+, that is involved in regulating the catalytic activity.

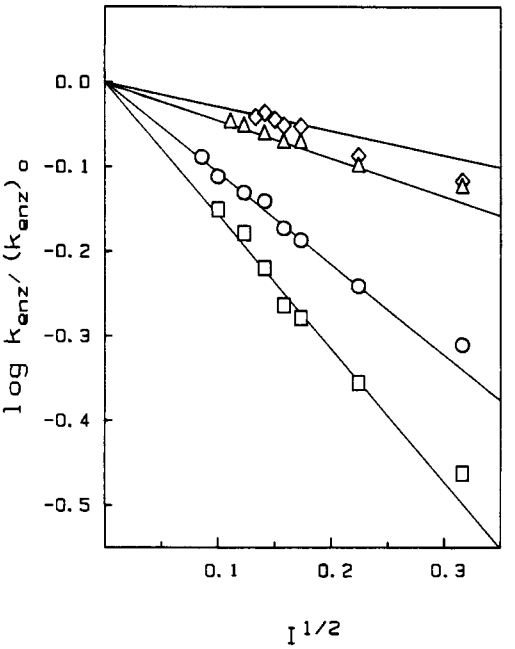


FIGURE 3: Plot of Debye–Hückel rate equation for the CAII-catalyzed HCO_3^- dehydration reactions in MES buffer, 25.0 °C: (□) pH 5.20; (○) pH 5.55; (△) pH 6.05; (◇) pH 6.60. Ionic strength was maintained by adding Na_2SO_4.

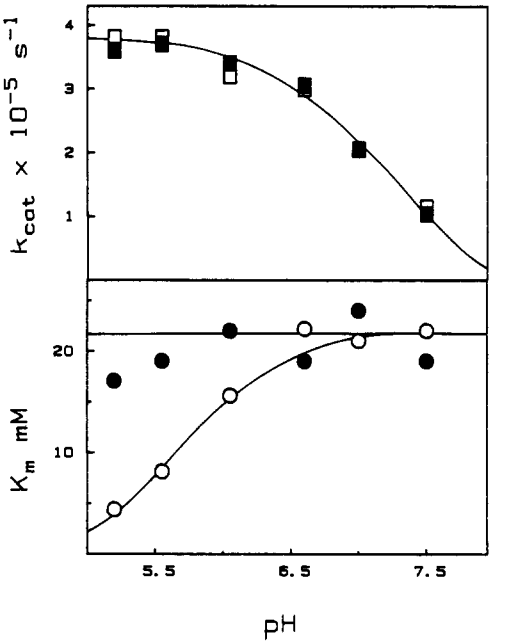


FIGURE 4: pH dependence of k_{cat} (upper graph) and K_m (lower graph) for the CAII-catalyzed HCO_3^- dehydration reactions in MES buffer: open symbols for zero ionic strength; filled symbols for ionic strength 0.1.

In order to further understand the nature of the electrostatic influence of sulfate ion on HCO_3^- dehydration reactions, the pH profiles of the separate k_{cat} and K_m terms at ionic strength 0.1 and zero ionic strength are compared in Figure 4. The pH independence of K_m for both hydration and dehydration reactions at ionic strength 0.1 has long been difficult to explain (Khalifah & Edsall, 1972; Pocker & Bjorkquist, 1975, 1977; Steiner et al., 1975). A scheme derived with two ionizing groups in the enzyme (Y. Pocker and C. H. Miao, unpublished results; Miao, 1987), which provides a refined and expanded model of the one proposed earlier (Pocker & Deits, 1982), can interpret this phenomenon in a simplified fashion assuming that CO_2 and HCO_3^- bind to both acidic and basic forms of

the enzyme.[3] At zero ionic strength, the binding of HCO_3^- seems to depend on an ionizing group with a $pK_a \sim 5.85$. While this group is protonated, the binding of HCO_3^- is much enhanced. This enhanced binding is apparently abolished when sulfate is present in solution. At zero ionic strength, the binding of HCO_3^- to one of the acidic forms of the enzyme with a $pK_a \sim 5.85$ (association of two oppositely charged entities) is much enhanced in the absence of other ions in the solution while the binding of HCO_3^- to the corresponding basic forms of this catalytically important residue of the enzyme (association of an anion with a neutral site) remains unchanged. This treatment yields a pH-dependent K_m, increasing sigmoidally from low pH to high pH and reaching a plateau corresponding to the same K_m value as that obtained at ionic strength 0.1 (Figure 4). For CO_2 hydration, the electrostatic effect is reflected only in the k_{cat} term, not in the K_m term, since the binding is essentially independent of SO_4^{2-} concentration due to the fact that CO_2 is a neutral molecule. It is difficult to predict which of the two groups is responsible for the enhanced binding of HCO_3^- at zero ionic strength. It is most commonly considered that $Zn–OH_2$ is the major catalytic group in carbonic anhydrase and His-64 serves as a proton shuttle group. These two groups interact electrostatically with one another, and both have pK_as close to 7 at ionic strength 0.1. We think that sulfate, as an ionic strength maintaining agent, may interact with both groups electrostatically. It is not unreasonable to postulate that the pK_a of one group shifts to 5.85 in the absence of sulfate and other ions in the solution. These pK_a values are also consistent with values obtained from the titration of CAII at zero ionic strength (Y. Pocker and C. T. O. Fong, unpublished results). Furthermore, we also demonstrate that the binding of substrate is enhanced by carboxymethylating His-64 or substituting Zn(II) with Co(II) (Y. Pocker and C. H. Miao, unpublished results; Miao, 1987). Clearly, the binding of HCO_3^- is affected by both groups. Nonetheless, we cannot exclude more complicated influences by some other groups in the active site that may also ionize at this pH.

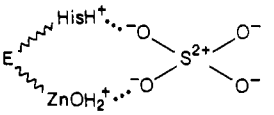
It is sometimes useful to consider the entropy of activation of such systems in a manner similar to that developed by Barnard and Laidler (1952) with reference to some proteolytic enzymes. From the electrostatic contribution to the free energy increase in forming enzyme–substrate complex (EA^*) from E + A, the enzymatic rate constant can be written as eq 4

$$\ln k = \ln k^0 - \frac{Z_A Z_B e^2 N}{\epsilon_r R T r^*} \qquad (4)$$

(Laidler & Bunting, 1973), where k^0 is the value of k when the electrostatic contribution can be neglected. This equation predicts a negative slope if the charges of the ions are of the same sign and a positive slope if the charges are of the opposite sign. By use of the rate constant at zero ionic strength, a linear plot can be constructed. From the slope we calculate the radius of the activated complex to be ca. 5.4 Å. This value is in good agreement with the suggested participation of His-64, a residue that is located at this distance from zinc–aquo complex in the enzyme. Electrostatic contribution to the entropy of activation is calculated by eq 5 [when $r^* = 5.4$ Å in water solution ($\epsilon_r = 78.3$ at 25 °C)] and given in Table III. Attention should be drawn to the small positive $(\Delta S^*)_{es}$ values for the formation of the enzyme–substrate complex, which seem to indicate that the positive charge in the enzyme molecule is partially neutralized by the negative charge of HCO_3^-. This may imply that the anion does indeed bind very closely to the zinc ion in the transition state of the bicarbonate binding step.[4]

$$(\Delta S^*)_{es} \approx -3.7 Z_{enz} Z_{HCO_3^-} \text{ eu} \qquad (5)$$

Clearly, the above effects should not be confused with the inhibitory effect of SO_4^{2-} at high salt concentrations where an intimate enzyme–sulfate complex is formed in a mode that inhibits both CO_2 hydrase and the *p*-nitrophenyl acetate hydrolase activities (Simonsson & Lindskog, 1982; Y. Pocker & C. T. O. Fong, unpublished results; Y. Pocker and C. H. Miao, unpublished results). These latter effects are presumably due to the capacity of the protonated enzyme to bind SO_4^{2-} ion. In fact, His-64 in CAII can create on protonation an additional cationic site capable of binding anionic inhibitors, while in its neutral form it can provide both acceptor and donor functions in the catalytic mechanism of hydrase and hydrolase reactions (Pocker & Dickerson, 1968; Pocker & Stone, 1968). At high salt concentrations, SO_4^{2-} may interact strongly with both $Zn–OH_2$ and the protonated imidazole group of His-64[5] and inhibit the enzymatic reaction (eq 6). Other divalent

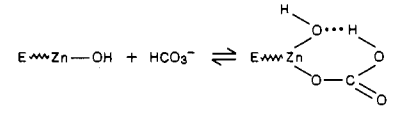


(6)

anions have also been found to modulate enzymatic processes in very specific ways. Phosphate ions, in particular, have been shown to exhibit larger k_{cat} and K_m values in CAII-catalyzed CO_2 hydration and HCO_3^- dehydration when used as buffers (Pocker & Bjorkquist, 1977). Powerful control of enzyme catalysis by phosphate is especially noticeable in the case of the plant isozyme (Pocker & Ng, 1973; Pocker & Miksch, 1978). The proposed binding of SO_4^{2-} to the enzyme wherein both $Zn–OH_2$ and His-64 are protonated may serve as a widely applicable model for the interaction of CAII with dianions.

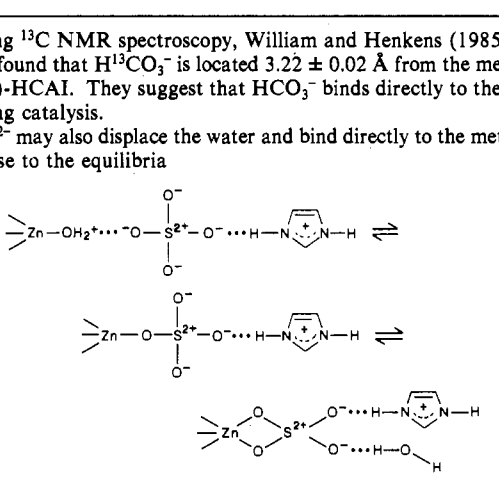
In conclusion, an exact prediction of electrostatic effects on ionic equilibria over a wide range of pH is possible at low salt

[3] HCO_3^- was found to inhibit the carbonic anhydrase catalyzed CO_2 hydration reaction. It was proposed that bicarbonate may bind to the basic form of the enzyme and form the adduct (Pocker et al., 1981)

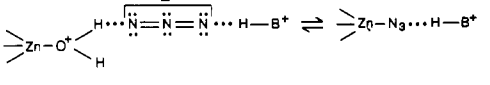


[4] Using ^{13}C NMR spectroscopy, William and Henkens (1985) have recently found that $H^{13}CO_3^-$ is located 3.22 ± 0.02 Å from the metal ion in Co(II)-HCAI. They suggest that HCO_3^- binds directly to the metal ion during catalysis.

[5] SO_4^{2-} may also displace the water and bind directly to the metal ion, giving rise to the equilibria



A similar case was considered by Pocker and Dickerson for N_3^- inhibition in CA-catalyzed hydration of aldehydes (1968):



concentrations. Our measurements show that control of ionic strength is a matter of considerable importance in the enzymatic reactions of carbonic anhydrase. The effects depend on the pH of the solution, suggesting that the results must be explained in terms of the ionizing groups concerned in the catalytic process and the manner in which they interact with added ions. At low ionic strengths, the transition-state theory predicts rate constant corrections in good agreement with our results. At high ionic strength, an inner-sphere enzyme–SO_4^{2-} interaction takes place. A "frozen" anion theory in which SO_4^{2-} is assumed to be unable to move on the time scale of the barrier top crossing successfully predicts the outcome of the enzyme-catalyzed trajectory of the HCO_3^- dehydration.

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