Effects of pH on the Anionic Inhibition of Carbonic Anhydrase Activities

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Abstract: We present the first investigation of the inhibition of bovine carbonic anhydrase (BCA) by simple monoanions as a function of both substrate employed and variation in pH. At low pH (pH <7) the CO_2 hydration, HCO_3^- dehydration, and *p*-nitrophenyl acetate (PNA) esterase activities of BCA are all strongly inhibited by anions. The formal mechanism of inhibition for CO_2 is noncompetitive, for HCO_3^- competitive, and for PNA probably noncompetitive. We employ a linear free-energy approach to anion inhibition to determine that despite these variations in apparent mechanism of inhibition, anions interact with BCA in all cases by binding at the same site, and we show that K_i values determined with these substrates can be accurately considered as representing true ligand dissociation constants for the catalytically essential Zn(II) ion of BCA. At high pH (pH $\simeq$9) anionic inhibition of BCA-catalyzed HCO_3^- dehydration and PNA hydrolysis is abolished, while anionic inhibition of BCA-catalyzed CO_2 hydration persists, only slightly diminished in potency. The mechanism of anionic inhibition of BCA-catalyzed CO_2 hydration at this pH is linear uncompetitive. This is the first report of this mechanism of inhibition with carbonic anhydrase and the first report of anion interaction with CA at high pH. By comparison with K_i values determined at low pH, we show that this inhibition occurs at the same binding site as observed in kinetic and spectral studies at low pH. A formal mechanism is proposed, employing rapid equilibrium assumptions, to account for the above data, based on a competition between HCO_3^-, OH^-, and other anions for the Zn(II) binding site. This formal mechanism permits quantitative predictions for the pH dependence of anionic inhibition of BCA; these predictions are confirmed in a study of the inhibition of BCA-catalyzed CO_2 hydration by $CH_3CO_2^-$ from pH 6.15 to 9.00. To account for this unusual uncompetitive inhibition as well as other data on CA catalysis, a mechanism of associative competition between anions for an optional fifth coordination site on Zn(II) is proposed, incorporating an interaction between CO_2 and Zn(II)-bound OH^- as the means by which anions uncompetitively inhibit CO_2 hydration at high pH. This interaction forms the basis for a sketch of the molecular mechanism of CA catalysis in which OH^-, formed by general base catalysis, enters the fifth coordination site of the basic form of the enzyme, enhancing the nucleophilicity of $Zn^{II}OH$ and catalyzing attack on bound CO_2. The implications of this mechanism for BCA catalysis are discussed.

Erythrocyte carbonic anhydrase (EC 4.2.1.1) catalyzes the reversible hydration of CO_2 and dehydration of HCO_3^-, a reaction of fundamental physiological importance. Carbonic anhydrase (CA) is a Zn metalloenzyme of exceptional catalytic efficiency, with a maximal turnover rate near 6×10^5 s^{-1}, whose mechanism of action has been the object of continuing scrutiny by an arsenal of physical and chemical techniques.[1-3]

The long-recognized inhibition of CA by a variety of simple anions[4] has been repeatedly exploited as a probe of its catalytic activity. The localization of inhibitory anion binding in the inner sphere of the essential Zn(II) ion by X-ray,[5,6] NMR relaxation,[7-9] and other spectral techniques[10-12] serves to confirm the intimate association between anion binding and CA catalysis. The reversible inhibition of CA by anions has been thoroughly studied for the esterase[13-15] and HCO_3^- dehydrase[16-18] activities of the native enzyme and for the CO_2 hydrase activity of the Co(II)-substituted enzyme.[11] On the basis of these and other studies, anions have been considered to bind only to carbonic anhydrase at low pH values, with diminution of anion binding exhibiting the same pH dependence as CA catalytic activity.[2]

No study to date has, however, explored in detail the relationships between the anionic inhibition of the various activities of CA, nor has the CO_2 hydration activity of the native Zn(II) enzyme been examined over a pH range comparable to that employed in other kinetic inhibition studies. Using a computer-interfaced stopped-flow system with which large quantities of highly accurate kinetic data can be collected, we have investigated the anionic inhibition of bovine erythrocyte carbonic anhydrase (BCA) catalyzed ester hydrolysis, HCO_3^- dehydration, and CO_2 hydration for a series of anions of diverse structure over a significant pH range (pH 6.15-9.00).

By applying a linear free-energy approach we have utilized the entire series of anions tested as an active site probe to compare the mechanism of anionic inhibition under different experimental conditions. The results of this study demonstrate the validity of important simplifying assumptions regarding the mechanism of CA.

In the course of this investigation, we have detected a heretofore unnoted mode of inhibition by anions, unique to the catalysis of CO_2 hydration by CA at high pH, conditions under which catalytic efficiency is maximal. Such a mode of inhibition is neither predicted by nor easily accommodated under any previously proposed scheme of anionic inhibition or mechanism of CA ca-

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talysis. We therefore offer a new mechanistic proposal for CA catalysis that integrates the phenomenon of anionic inhibition and the catalytic mechanism of CA and appears to possess exceptional explanatory capacity to reconcile the extant literature of this enzyme.

Experimental Section

Materials. Reagent grade dimethylimidazole and *N*-methylimidazole were distilled under N_2 and stored in dark well-sealed bottles under N_2 at 4 °C. Reagent grade *p*-nitrophenol was purified by sublimation. Sodium salts of C_3–C_7 carboxylic acids were prepared by precipitation with NaOH from solutions of dried and distilled reagent grade carboxylic acids. CO_2 solutions were prepared by bubbling Anco Coleman Grade CO_2 through distilled deionized H_2O thermostated at 30 °C, yielding a CO_2 concentration of 0.30 M.[16] Bovine carbonic anhydrase (Worthington Biochemicals) was used without further purification. Active enzyme concentration was determined by inhibition studies with the potent inhibitor acetazolamide, plotting the resulting data by the method of Henderson.[19] Other materials employed were of reagent grade and were used without further purification.

Methods. Stopped-Flow System. Initial rate determinations for studies of BCA-catalyzed CO_2 hydration and HCO_3^- dehydration were performed on a Durrum-Gibson stopped-flow apparatus, modified in these laboratories. Modifications include interfacing to a PDP 8L computer for data acquisition, permitting real time acquisition of 125 transmittance data points in periods as short as 11 ms. Data from up to 25 kinetic runs can be stored before data reduction commences, considerably facilitating data acquisition. Initial rates are determined by unweighted linear least-squares analysis of the transmittance data, converted to absorbance readings, and plotted against time. A pneumatic electronically controlled drive system has also been incorporated. Drive pressure and duration are under operator control, eliminating artifacts due to release of the drive piston during data collection and enhancing reproducibility of drive motion.

Initial rate determinations were performed by employing the changing pH indicator method,[20] as previously described.[16,21] Two buffer–indicator pairs, dimethylimidazole (pK_a 8.22) with metacresol purple (pK_a 8.43) and *N*-methylimidazole (pK_a 7.19) with *p*-nitrophenol (pK_a 7.07), were employed. Buffer concentration in the reaction cell was maintained at 0.02 M in all experiments. Ionic strength was maintained at 0.2 with Na_2SO_4. In those inhibition experiments where the concentration of added inhibitory anion would significantly (greater than 5%) perturb the total ionic strength, the concentration of added Na_2SO_4 was adjusted to compensate. Indicator concentrations were chosen at each pH to give an initial absorbance of 0.3–0.4 for optimal photomultiplier response yielding indicator concentrations near 10^{-5} M, varying somewhat with pH.

Buffer factors[20] were determined experimentally by titration at one pH value for each buffer–indicator pair and found to agree well with buffer factors (Q) calculated by the formula[22]

$$Q_{\text{calcd}} = \frac{[\text{Buff}]}{[\text{In}]L\Delta\epsilon}\frac{K_a^{\text{Buff}}}{K_a^{\text{In}}}\left(\frac{K_a^{\text{In}} + [H^+]}{K_a^{\text{Buff}} + [H^+]}\right)^2$$

[Buff] and [In] represent total buffer and indicator concentrations, respectively, and K_a^{Buff} and K_a^{In} represent buffer and indicator ionization constants. $\Delta\epsilon$ is the difference in molar extinction coefficient between the acidic and basic forms of the indicator, and L is the reaction cell optical path length in centimeters. For metacresol purple, $\Delta\epsilon = 3.2 \times 10^4$ at 578 nm; for *p*-nitrophenol, $\Delta\epsilon = 1.9 \times 10^4$ at 400 nm.

CO_2 and HCO_3^- solutions were prepared as previously described.[16,22] Substrate concentrations employed ranged from 0.015 to 0.0025 M for CO_2 and from 0.06 to 0.006 M for HCO_3^-. Enzyme concentration was adjusted to give velocities at least tenfold larger than the uncatalyzed rates of reaction, typically $1–3 \times 10^{-7}$ M. All kinetic data was collected at 25 ± 0.01 °C. In all kinetic runs, a 3–5 ms delay following solution mixing was programmed to eliminate mixing artifacts. Initial rate data were taken for 15–20 ms following the mixing delay. For a typical K_i determination, 4 to 5 inhibitor concentrations were employed at each of 4 or 5 substrate concentrations, giving a matrix of 16 to 25 data points. For each substrate–inhibitor concentration pair, 15–20 replicate kinetic runs were performed. The standard deviation of such replicate sets averaged 3% of the mean value. The velocities so determined were corrected for the contribution from the uncatalyzed background rate to yield net enzymatic velocities.

Enzyme velocities so determined were plotted as reciprocal velocity vs. reciprocal substrate concentration, the Lineweaver–Burk plot. For each inhibitor concentration, slopes, intercepts, and correlation coefficients of the plots were determined by unweighted linear least-squares analysis, yielding lines with correlation coefficients exceeding 0.995. Replots of the slopes and ordinate intercepts of each line vs. inhibitor concentration were also analyzed by unweighted linear least-squares techniques, with the correlation coefficients of the replots exceeding 0.99. K_i values determined from slope replots will be referred to below as K_i^{slope} values; those from ordinate intercept replots will be referred to as K_i^{int}.

For the purpose of graphical display of inhibition data, the kinetic parameters determined from the above plots and replots were substituted into the velocity equation describing the observed inhibition pattern. The lines thereby generated for each inhibitor concentration were then plotted along with the data points used to generate the parameters.

Lineweaver–Burk plots have been criticized on statistical grounds as giving too much weight to less accurate data taken at low substrate concentrations.[23,24] With the data of the present study, alternate linearized forms of the Michaelis–Menten velocity equation, recommended as statistically superior,[24,25] yielded essentially identical kinetic parameters.

Three techniques were used to assess the effect of rapid mixing of enzyme and inhibitor as a probe of the rate of inhibitor equilibration. In the preincubation technique, enzyme, inhibitor, and buffer–indicator solutions were mixed and placed in one syringe of the stopped-flow apparatus, and the substrate solution was placed in the other mixing syringe.

In the rapid-mixing technique, enzyme and buffer–indicator solutions filled one syringe, while substrate and anion solutions were placed in the second syringe. Under these conditions, upon reaction initiation by mixing, enzyme and inhibitor may interact only for the 5 ms mixing period before initial rate data are taken for 15–20 ms.

In the pH-jump technique, anion and buffer occupied one syringe. In the other syringe, enzyme and substrate solution were premixed. This technique was employed with CO_2 as substrate only. CO_2 substrate solutions have an equilibrium pH near 4, the lower limit of BCA stability. For ensurance of enzyme stability, small calculated aliquots of NaOH solution were added to adjust the pH of these solutions to 5.0. Substrate concentration varies only slightly with this manipulation; the change in concentration was calculated and corrected substrate concentrations employed in kinetic studies. In these pH-adjusted solutions, BCA was stable over the time period required for kinetic studies. Upon mixing of the enzyme–substrate solution with the buffer–inhibitor solution, the enzyme undergoes a shift in pH of up to 4 pH units in the 5 ms prior to initial rate determination, depending on the pH under study. Despite this drastic change, in control experiments without added inhibitor the K_m and V_{max} values obtained were identical within experimental error with those determined by the two other techniques described above.

Spectrophotometric Studies of PNA Hydrolysis. The anionic inhibition of BCA-catalyzed *p*-nitrophenyl acetate hydrolysis was studied at two pH values: pH 6.6, using 0.02 M phosphate buffer, and pH 8.81, using 0.02 M borate buffer, both at ionic strength 0.2 with added Na_2SO_4. Rates were initiated by injection of 0.3 mL of a solution of PNA in acetone into 2.7 mL of buffer solution, giving a 10% concentration of acetone as cosolvent. Reactions were initiated directly in a 1-cm spectrophotometer cuvette, and initial rates were followed at 400 nm, monitoring the appearance of the hydrolysis product, *p*-nitrophenoxide ($\epsilon_{400} = 5.9 \times 10^3$ at pH 6.6 and 1.81×10^4 at pH 8.8). Data was collected on a Cary 210 recording spectrophotometer. The linear initial portion of the reaction progress curve was used to determine the initial velocity. BCA concentrations were typically near 2×10^{-5} M. Observed velocities were corrected for nonenzymatic background hydrolysis. Kinetic runs were performed in triplicate. The resulting velocity data were analyzed as described above for stopped-flow data.

Results

A. Anionic Inhibition of BCA Catalysis, pH 6.6. For an accurate comparison of the anionic inhibition of BCA-catalyzed HCO_3^- dehydration, CO_2 hydration, and *p*-nitrophenyl acetate (PNA) hydrolysis, kinetic studies were undertaken at pH 6.6. At this pH, BCA catalyzes both CO_2 hydration and HCO_3^- dehydration with nearly equal efficiency and the catalysis of PNA hydrolysis can also be conveniently monitored.

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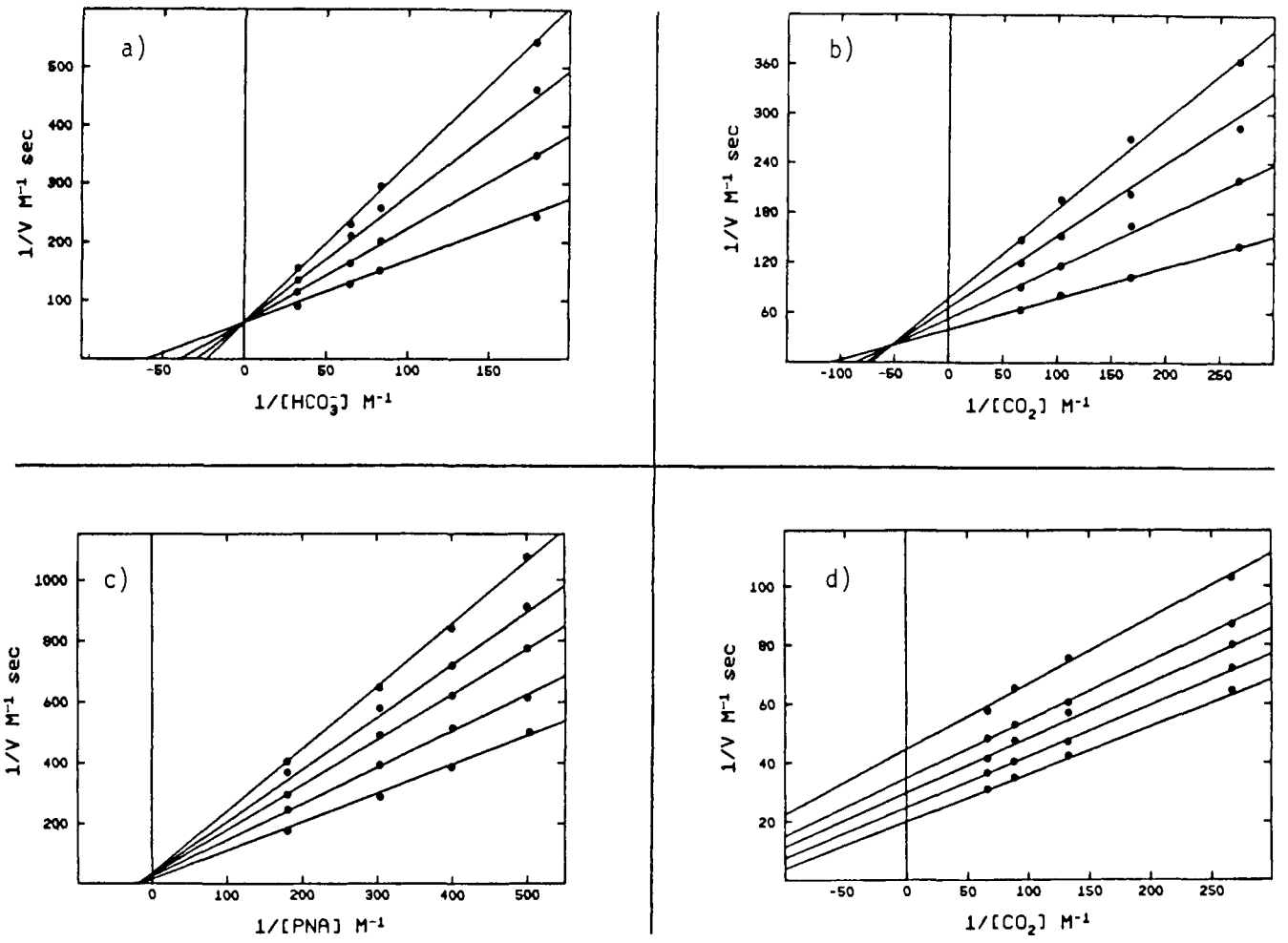


Figure 1. Inhibition by SCN^- of BCA catalysis of the following: (a) HCO_3^- dehydration, pH 6.60, $K_i^{slope} = 1.8 \times 10^{-3}$ M; (b) CO_2 hydration, pH 6.60, $K_i^{slope} = 1.6 \times 10^{-3}$ M, $K_i^{int} = 3.2 \times 10^{-3}$ M; (c) PNA hydrolysis, pH 6.60, $K_i^{slope} = 1.6 \times 10^{-3}$ M; and (d) CO_2 hydration, pH 9.00, $K_i^{int} = 5.5 \times 10^{-3}$ M.

1. Anionic Inhibition of BCA-Catalyzed HCO_3^- Dehydration, pH 6.6. Figure 1a presents the Lineweaver–Burk plot for the inhibition of BCA-catalyzed HCO_3^- dehydration by SCN^-, consistent with a formal linear competitive mechanism. Of the 12 anions tested, only N_3^- exhibited a slight reproducible deviation in the ordinate intercept, i.e., a measurable K_i^{int} value (see Methods), consistent with a previous report from the laboratories on this anion.[16] No change in mechanism or degree of inhibition was detected whether or not enzyme and inhibitor were preincubated.

2. Anionic Inhibition of BCA-Catalyzed CO_2 Hydration, pH 6.6. Under experimental conditions identical with those employed for HCO_3^-, anions inhibit BCA-catalyzed CO_2 hydration by a linear mixed noncompetitive mechanism. The Lineweaver–Burk plot for SCN^- is presented in Figure 1b. For all anions tested, a significant value for both K_i^{slope} and K_i^{int} could be determined. The nonequality of K_i^{slope} and K_i^{int} for each anion tested is equivalent to a variation in apparent K_m with inhibitor concentration. Similar variations in apparent K_m for the anionic inhibition of BCA-catalyzed CO_2 hydration have been reported at pH 7.0, using stopped-flow techniques,[17] and at pH 7.2–7.6, using the less precise Wilbur–Anderson assay.[18] Rapid mixing of enzyme and inhibitor had no effect on anion inhibition.

3. Anionic Inhibition of BCA-Catalyzed PNA Hydrolysis, pH 6.6. The limited solubility of PNA, even in the presence of 10% organic cosolvent employed in the present study, restricts kinetic studies to concentrations well below substrate saturation, making accurate K_m determinations difficult. All inhibitors tested exhibited apparently noncompetitive inhibition patterns, although the proximity of the extrapolated abscissa intercepts to the vertical axis of the Lineweaver–Burk plot means that a precise characterization of the formal mechanism of inhibition is difficult. Figure 1c presents such a plot for inhibition by SCN^-. K_i^{slope} values were readily measured, but K_i^{int} values could not be accurately determined under these experimental conditions. Stopped-flow studies to determine the effect of rapid mixing of enzyme and inhibitor were not performed on this substrate.

B. Anionic Inhibition of BCA Catalysis, pH 9.0. The catalytic activity of BCA is controlled by an essential ionization of pK_a near 7.[2] At pH 9.0, this ionizable group is entirely in its basic form, and the enzyme is maximally active toward CO_2 hydration and PNA hydrolysis and negligibly active toward HCO_3^- dehydration. The anionic inhibition of BCA-catalyzed HCO_3^- dehydration cannot therefore be studied by current techniques at this pH.

Consistent with previous reports,[13,14] anions do not measurably inhibit BCA-catalyzed PNA hydrolysis at pH 8.8. The anions Cl^-, I^-, HCO_2^-, and N_3^- were tested for inhibition at a concentration of 0.1 M. In no case was a significant reduction in velocity detected. The measurement of N_3^- inhibition required compensation for the nucleophilic catalysis of PNA hydrolysis by this anion.

In contrast, BCA-catalyzed CO_2 hydration is effectively inhibited by all anions tested at pH 9.0. Figure 1d presents the Lineweaver–Burk plot of the inhibition by SCN^- at this pH. The change in mechanism between this plot and those for the inhibition of other catalytic activities of BCA is evident. The formal mechanism of inhibition in this case is linear uncompetitive,[24] the first instance of this formal mechanism of inhibition in the literature of CA catalysis. All anions tested share this mechanism, with inhibition confined largely to the K_i^{int} terms. In most cases, a slight variation in the slope of the Lineweaver–Burk plots with increasing inhibitor concentrations could be detected, equivalent

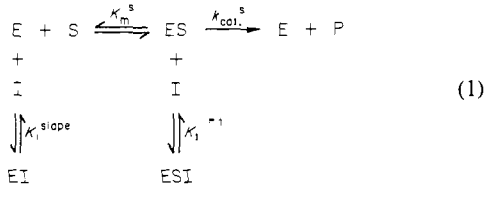
to a K_i^{slope} term approximately tenfold larger than the dominant K_i^{int} term. This effect will be discussed below.

For 10 of the 12 anions tested, preincubation of anion and BCA compared to rapid mixing in the stopped-flow apparatus showed no differences in mechanism or degree of inhibition. For Br^- and I^- (but not Cl^-), significant concave downward curvature of the Lineweaver–Burk plots occurred at CO_2 concentrations above approximately 0.01 M. When enzyme and either Br^- or I^- were rapidly mixed in the stopped-flow apparatus, the curvature was abolished and linear uncompetitive inhibition was restored. K_i^{int} values for Br^- and I^- were determined under these conditions of rapid mixing or inhibitor and BCA. Interestingly, when Br^- and I^- were tested by the pH-jump stopped-flow technique (see Methods), another means by which inhibitor and enzyme can be rapidly mixed, curvature was again observed, suggesting that factors other than the time of interaction of enzyme and inhibitor are contributing to the curvature. Despite the observed curvature in two sets of experiments, I_{50} values for Br^- and I^- inhibition could be reliably estimated from the curved plots and correspond reasonably well to the K_i^{int} values determined from the linear Lineweaver–Burk plots.

C. Realtionships among Mechanisms of Anionic Inhibition. It is essential to ascertain whether inhibition by anions of BCA-catalyzed CO_2 hydration at high pH shares any characteristics with the inhibitory patterns observed at low pH with CO_2, HCO_3^-, and PNA as substrates. For a quantitative probe of the effect of anions on the catalytic activity of BCA as pH and substrate are varied, a linear free-energy approach will be employed. With this technique, the entire series of anions tested under each set of conditions can function as an active site probe sensitive to alterations in the anion binding site.

We hypothesize that anion interactions with carbonic anhydrase occur on a sufficiently rapid time scale with respect to enzyme turnover for the establishment of equilibrium between enzyme and enzyme–inhibitor complex prior to initial rate determination. If this is the case, then the K_i values determined in these kinetic studies can be identified on a mechanistic as well as a formal kinetic level with the relevant equilibria of the Michaelis–Menten scheme.[24,26] The fact that rapid mixing within 5–10 ms in our apparatus provided sufficient time for establishment of a full degree of interaction between anions and BCA supports this hypothesis, as do a number of other kinetic and spectral studies indicating rapid enzyme–inhibitor association.[7,8,27]

Consider a version of the Michaelis–Menten formalism incorporating inhibition by a rapidly equilibrating inhibitor, eq 1.



(1)

It can be easily shown that at this level of analysis the velocity equation associated with the above scheme equates K_i^{slope} and K_i^{int} with the particular equilibria indicated above.[24] Note that under this analysis the K_i^{slope} term is an equilibrium dissociation constant which is independent of the substrate employed for its measurement. Since the substrates CO_2, HCO_3^-, and PNA all obey the Michaelis–Menten formalism for catalysis, the implication is that K_i^{slope} values determined under the same experimental conditions employing any of these three substrates should be numerically identical.

This prediction can be verified by a linear free-energy approach. Expressing the inhibitor dissociation constants determined under two different sets of experimental conditions, K_i and K_i', in terms of free energies ΔG and $\Delta G'$, where $\Delta G = -RT \ln K_i$, if the sites of inhibitor binding are similar under the two sets of conditions, a linear free-energy relationship of the form

$$\Delta G = D\Delta G' + C \qquad (2)$$

should hold. The hypothesis that identical interactions govern binding under the sets of conditions used to determine ΔG and $\Delta G'$ will be verified if D equals 1 and C is 0. This approach has been previously employed to compare ligand dissociation constants between different metal ions to probe similarities of interaction,[28] but has to our knowledge not been applied to enzyme inhibition.

Figure 2a presents such a plot comparing the K_i^{slope} value for inhibition of BCA-catalyzed CO_2 hydration at pH 6.6 with the K_i^{slope} value determined for HCO_3^- dehydration at the same pH. An excellent correlation is obtained, with a slope of 1 and a 0 intercept within experimental error consistent with the identity of anion binding sites measured.

The K_i^{slope} value for PNA hydrolysis can be similarly tested. In this instance, the rate constants for substrate binding, dissociation, and turnover differ by orders of magnitude for the catalysis of PNA hydrolysis from those involved in the natural activity of the enzyme. Under the hypothesis of rapid-equilibrium binding of inhibitors, such changes should have no effect on the kinetically determined K_i^{slope} values; for more complex mechanisms requiring derivation from the more restrictive steady state condition, substrate-related rate constants can significantly affect kinetically determined K_i values. Figure 2c presents a plot comparing K_i^{slope} values for CO_2 hydration and PNA hydrolysis inhibited by anions, demonstrating clearly the slope of 1 and a 0 intercept consistent with a rapid equilibrium mechanism and consistent with an identity of the anion binding milieu for inhibition of both enzymatic activities.

Thus far it has been demonstrated that anions behave toward BCA in a fashion that can be modelled accurately as a simple rapid ligand dissociation equilibrium with the free enzyme. The same technique can be used to probe the relationship between the K_i^{slope} and K_i^{int} values observed in the anionic inhibition of BCA-catalyzed CO_2 hydration. Figure 2b plots log K_i^{slope} vs. log K_i^{int} determined for BCA-catalyzed CO_2 hydration at pH 6.6. In this case, the same high degree of correlation and slope of unity, diagonostic of similar binding interactions between the two measured quantities, are observed, but a significant deviation in the ordinate intercept occurs.

From eq 2, this positive intercept value represents the value of C, which for this set of experimental conditions will be termed $C_{6.6}$. $C_{6.6}$ is a quantity which expresses a property of the enzyme active site and not a property of the individual anions comprising the correlation; it represents the destablization of the anion binding site induced by binding of substrate. This result permits a quantitative analysis of the modes of anion binding to two different forms of BCA and confirms that, apart from a general destabilizing effect, the steric and electronic environment for anion binding is largely unchanged upon substrate CO_2 binding at pH 6.6.

We are now in a position to test the hypothesis that the uncompetitive inhibition term observed at pH 9.0 is indeed the same binding interaction as that causing anion inhibition at lower pH values where most studies of carbonic anhydrase have been performed. Figure 2d presents the free-energy plot with the K_i^{slope} values determined at pH 6.6 as the abscissa and the K_i^{int} values for BCA-catalyzed CO_2 hydration at pH 9.0 as the ordinate. The close correlation seen in other plots is preserved, with slope unity and a positive intercept, $C_{9.0}$, equal to 0.72. The unusual mechanism of inhibition observed in this study at high pH evidently occurs by anion interactions substantially identical with those observed at lower pH values. Table I collects the K_i data employed above.

On the basis of the above analysis and the observation that the rapid-mixing experiments performed have no effect (with one exception to be discussed below) on the degree or mechanism of inhibition, we believe it is appropriate to identify the K_i values derived in a formal analysis of anion inhibition with actual ligand

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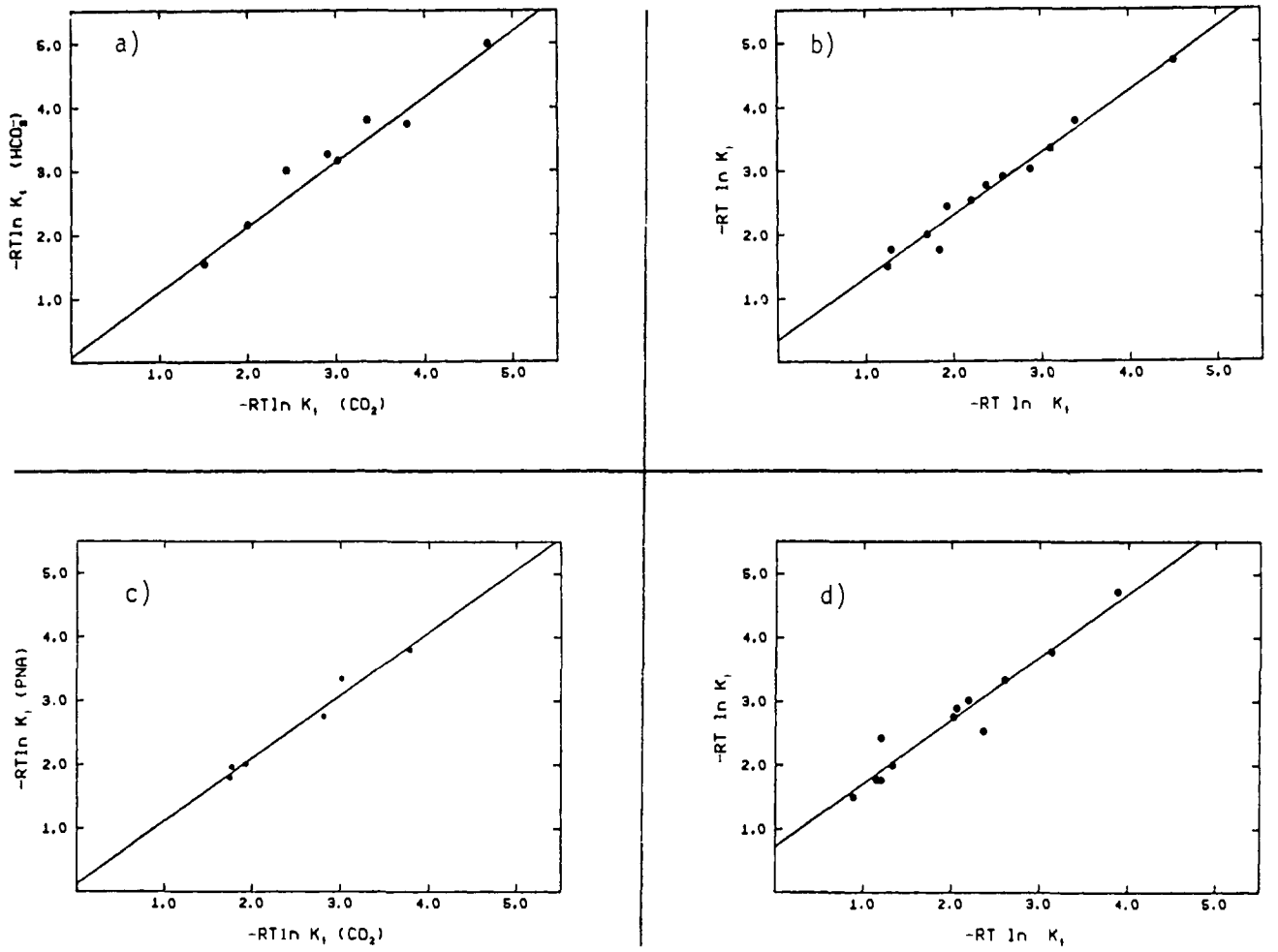

Figure 2. Comparisons of free energies of anion binding measured for the anionic inhibition of BCA catalysis under varying conditions of substrate and pH. K_i^{slope} values determined for the inhibition of BCA-catalyzed CO_2 hydration at pH 6.6 are employed as the reference state (x axis) for each comparison. (a) y axis: K_i^{slope} values for inhibition of BCA-catalyzed HCO_3^- dehydration, pH 6.60. Least-squares line: slope = 1.02 ± 0.08, y intercept = 0.08 ± 0.2, r = 0.98. Anions tested are (left to right) Cl^-, Br^-, $CH_3CO_2^-$, I^-, HCO_2^-, ClO_4^-, and N_3^-. (b) y axis: K_i^{int} values for inhibition of BCA-catalyzed CO_2 hydration, pH 6.60. Least-squares line: slope = 0.98 ± 0.05, y intercept = 0.35 ± 0.2, r = 0.98. Anions tested are (left to right) Cl^-, $CH_3CH_2CO_2^-$, $CH_3(CH_2)_2CO_2^-$, $CH_3CO_2^-$, Br^-, $CH_3(CH_2)_3CO_2^-$, $CH_3(CH_2)_4CO_2^-$, HCO_2^-, ClO_4^-, SCN^-, N_3^-. (c) y axis: K_i^{slope} values for inhibition of BCA-catalyzed PNA hydrolysis, pH 6.60. Least-squares line: slope = 0.99 ± 0.08, y intercept = 0.14 ± 0.21, r = 0.98. Anions tested are (left to right) $CH_3CH_2CO_2^-$, $CH_3(CH_2)_2CO_2^-$, $CH_3CO_2^-$, $CH_3(CH_2)_4CO_2^-$, HCO_2^-, SCN^-. (d) y axis: K_i^{int} values for BCA-catalyzed CO_2 hydration, pH 9.00. Least-squares line: slope = 0.99 ± 0.09, y intercept = 0.72 ± 0.2, r = 0.96. Anions tested are the same as those of Figure 2b.

Table I. Kinetic K_i Values for the Anionic Inhibition of BCA Catalysis

substrate	CO_2	CO_2	CO_2	HCO_3^-	PNA
reaction	hydration	hydration	hydration	dehydration	hydrolysis
pH	6.6	6.6	9.0[a]	6.6	6.6
K_i	$K_i^{slope} \times 10^2$ M	$K_i^{int} \times 10^2$ M	$K_i^{int} \times 10^2$ M	$K_i^{slope} \times 10^2$ M	$K_i^{slope} \times 10^2$ M
anion					
Cl^-	7.8[b]	12.0[b]	22.0[b]	7.3	
Br^-	1.6[b]	3.8[b]	13.0[c]	3.0	
I^-	0.73[b]	1.3[b]	3.0[c]	0.40	
SCN^-	0.16[b]	0.32[b]	0.50[b]	0.18[b]	0.19
N_3^-	0.034[b]	0.048[b]	0.14[b]	0.021[b,d]	
ClO_4^-	0.35	0.51	1.2[b]	0.16	
HCO_2^-	0.61	0.76	2.40	0.48	0.34
$CH_3CO_2^-$	3.4[b]	5.5[b]	10.4[b]	2.6	3.3
$CH_3CH_2CO_2^-$	5.1	11.0	14.0		4.7
$CH_3(CH_2)_2CO_2^-$	5.0	4.4	12.8		3.6
$CH_3(CH_2)_3CO_2^-$	1.4	2.4	5.6		
$CH_3(CH_2)_4CO_2^-$	0.95	1.8	3.2		0.86

[a] No significant K_i^{slope} term was observed at pH 9.0 (see Results). [b] Experiment repeated with and without preincubation (see Methods). No significant change in degree or pattern of inhibition detected. [c] Significant changes detected in pattern of inhibition with and without preincubation (see Results). [d] Significant K_i^{int} value detected for this anion only; $K_i^{int} = 2.6 \times 10^{-3}$ M. This result is consistent with previous reports on this anion.[16]

dissociation constants for the various enzyme species present during kinetic experiments. Thus, the competitive inhibition of BCA-catalyzed HCO_3^- dehydration is interpreted as an actual competition for the HCO_3^- binding site by other anions, and the uncompetitive inhibition at pH 9.0 represents obligate sequential binding of enzyme and substrate CO_2 prior to anion binding.

With one further simplifying assumption, a full velocity expression for BCA catalysis and anion inhibition can be derived.

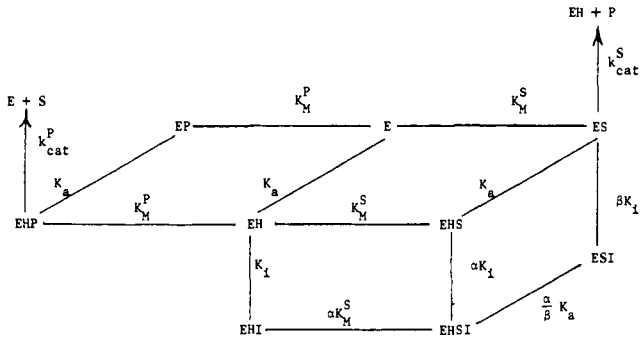

Figure 3. Formal kinetic scheme for analysis of the anionic inhibition of BCA-catalyzed CO_2 hydration and HCO_3^- dehydration. EH = form of BCA present at limiting low pH; E = form of BCA present at limiting high pH; S = CO_2, P = HCO_3^-, I = anionic inhibitor. Lines between enzyme species represent equilibria and those with arrows represent formal turnover steps. Kinetic constants are as defined for rapid-equilibrium kinetics.[24]

As at least a first-order approximation, we assume that, as is the case with simple anions, both HCO_3^- and CO_2 bind and dissociate from BCA rapidly compared to the rate of enzyme turnover. With the treatment of BCA catalysis in simple rapid-equilibrium fashion, K_m values for HCO_3^- dehydration and CO_2 hydration represent equilibrium dissociation constants for these compounds, and the pH independence of both K_m values reflects a true independence of substrate binding from the state of ionization of the enzyme.

With this assumption, a formal kinetic scheme for catalysis and anionic inhibition can be derived and is shown in Figure 3. Note that binding of inhibitor to the basic form of BCA is not indicated. There may be a slight binding term for anions detected as a slight variation in slope with inhibitor concentration at limiting high pH (see above). For purposes of simplicity, this variation will be neglected in the subsequent analysis, and a mechanistic rationale for such an effect will be proposed below.

D. Anionic Inhibition of BCA Catalysis at Intermediate pH Values. The anionic inhibition of BCA-catalyzed PNA hydrolysis[13] and HCO_3^- dehydration[16] have been previously investigated in detail in these laboratories over a significant pH range spanning the pK_a of the activity linked ionization. In both cases, anion inhibitory potency declined sigmoidally with no change in formal mechanism of inhibition, until at high pH values anion inhibition was abolished.

To complement these studies and to examine in detail the unusual transition in mechanism observed in the anionic inhibition of BCA-catalyzed CO_2 hydration, the inhibition by CH_3COO^- of BCA CO_2 hydration activity was investigated at 9 pH values spanning the pH range 6.15–9.00. Representative Lineweaver–Burk plots at four of the pH values tested are presented in Figure 4a–d; the smooth transition in mechanism with pH is evident. At no pH tested did rapid mixing of enzyme and inhibitor alter the pattern of inhibition observed compared to preincubation experiments.

Figure 5 presents plots of log K_i^{slope} and K_i^{int} vs. pH for the inhibition of BCA-catalyzed CO_2 hydration by $CH_3CO_2^-$. The sigmoidal increase in the value of K_i^{slope} with increasing pH can be clearly seen, as can the smooth transition between low-pH and high-pH K_i^{int} values. The apparent pK_a values governing these two transitions are significantly different, however, with the transition in K_i^{int} occurring at a higher pH value.

This difference in pK_a is only apparent, and is in fact a

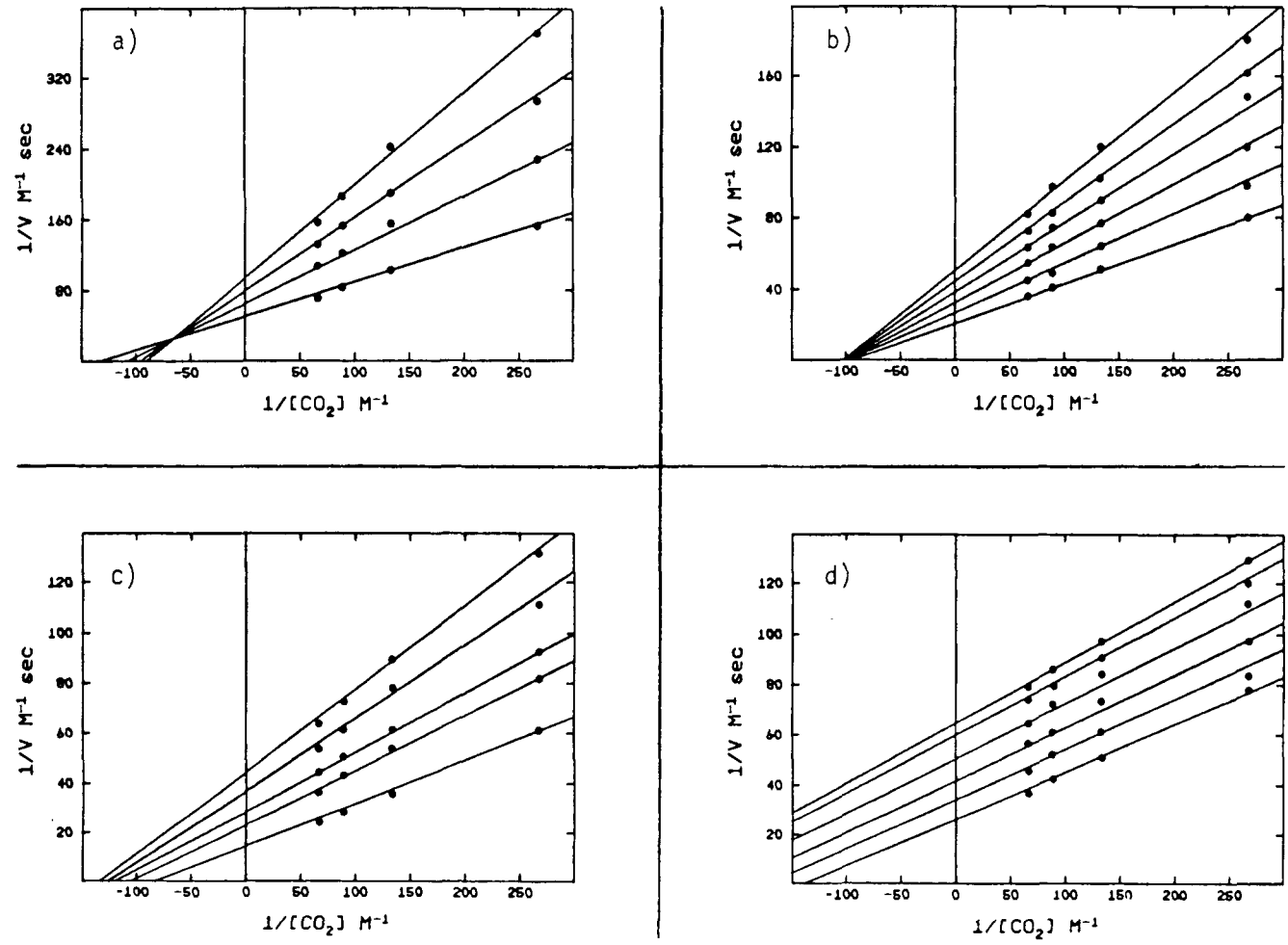

Figure 4. Inhibition of BCA-catalyzed CO_2 hydration of $CH_3CO_2^-$ as pH is varied. (a) pH 6.25; K_i^{slope} = 3.0 × 10^{-2} M, K_i^{int} = 6.2 × 10^{-2} M. (b) pH 7.00; K_i^{slope} = 7.2 × 10^{-2} M, K_i^{int} = 7.0 × 10^{-2} M. (c) pH 7.40; K_i^{slope} = 1.3 × 10^{-1} M, K_i^{int} = 8.4 × 10^{-2} M. (d) pH 8.14; K_i^{slope} = 5.8 × 10^{-1} M, K_i^{int} = 1.0 × 10^{-1} M.

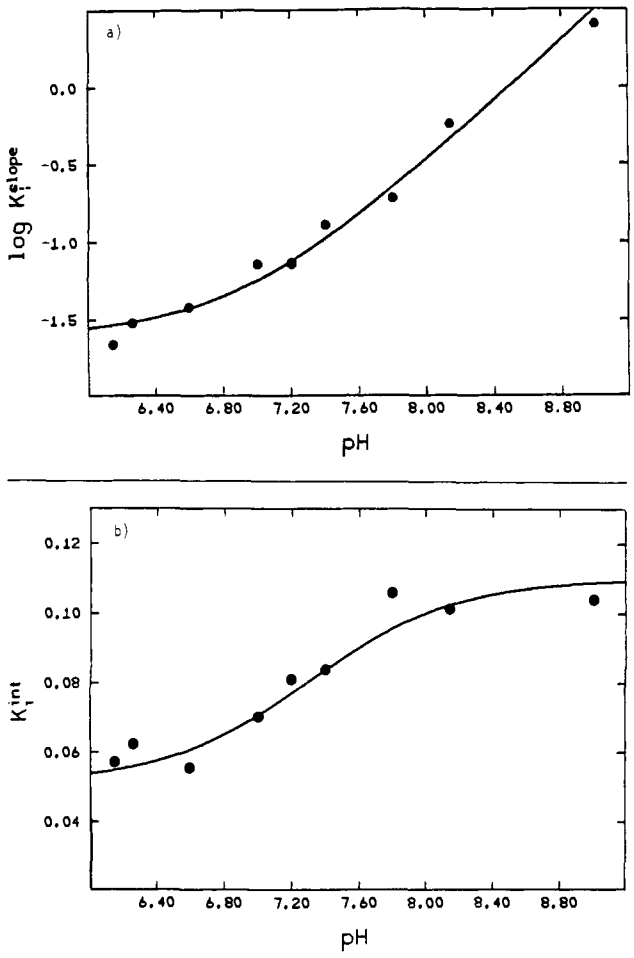

Figure 5. Plot of log K_i^{slope} vs. pH for the inhibition of BCA-catalyzed CO_2 hydration by $CH_3CO_2^-$. The line is a theoretical curve representing a change from a limit low-pH value for K_i^{slope} of 0.025 M to negligible binding at high pH, governed by a single ionization of pK_a = 6.9. (b) plot of K_i^{int} vs. pH for the inhibition of BCA-catalyzed CO_2 hydration by $CH_3CO_2^-$. The line is a theoretical curve representing a change from a limit low-pH value for K_i^{int} of 0.05 M to a limit high-pH value of 0.11 M, governed by a single ionization of apparent pK_a = 7.3.

quantitatively predictable consequence of the kinetic inhibition scheme presented above (Figure 3). From Figure 3, applying rapid equilibrium assumptions, a full velocity expression for BCA catalysis and anionic inhibition can be simply derived[24] and is, in double reciprocal form, suitable for a Lineweaver–Burk plot,

$$\frac{1}{V} = \frac{1}{[S]}\frac{K_m^S}{V_{max}^S}\left(1 + \frac{[H^+]}{K_a}\left(1 + \frac{[I]}{K_i}\right)\right) + \frac{1}{V_{max}^S}\left(1 + \frac{[I]}{\beta K_i} + \frac{[H^+]}{K_a}\left(1 + \frac{[I]}{\alpha K_i}\right)\right) \quad (3)$$

The pH dependence of both K_i^{slope} and K_i^{int} can be derived by straightforward algebraic manipulation of eq 3. For K_i^{slope} the result is

$$K_i^{slope} = K_i\left(1 + \frac{K_a}{[H^+]}\right) \quad (4)$$

A plot of log K_i^{slope} vs. pH will therefore have a simple sigmoidal dependence with an inflection point at pK_a of the ionization controlling anion binding. Figure 5a demonstrates the close adherence to a theoretical curve generated from eq 5, using a pK_a value of 6.9 and a K_i value of 0.025. It should be noted that in the remainder of this discussion the term K_i, unadorned by superscripts, will refer to the particular ligand dissociation constant which characterizes anion binding to the acidic form of CA in the absence of substrate. Note also that the apparent pK_a of the ionization controlling acetate binding in the absence of substrate closely corresponds to the activity linked ionization measured in studies of the catalytic activity of BCA[16,29] and the high activity form of human carbonic anhydrase[20] for both substrates CO_2 and HCO_3^-, a result predictable from the above kinetic scheme (Figure 3).

The pH dependence of the K_i^{int} term (Figure 5b) can now be analyzed quantitatively. From inspection of Figure 3, it is clear that a consequence of the differing values for αK_i and βK_i is an effect on the apparent pK_a for the transition from enzyme form EHS to ES, imposed as a thermodynamic requirement of the equilibrium relationships between the enzymatic species considered.

This perturbation in apparent pK_a value will manifest itself in the pH dependence of K_i^{int}. The pH dependence of K_i^{int} is, from eq 3,

$$K_i^{int} = \left(1 + \frac{[H^+]}{K_a}\right)\left(\frac{1}{\alpha K_i}\left(\frac{\alpha}{\beta} + \frac{[H^+]}{K_a}\right)\right)^{-1} \quad (5)$$

which, upon rearrangement, yields

$$K_i^{int} = \frac{\beta K_i}{1 + \frac{\beta}{\alpha}\frac{[H^+]}{K_a}} + \frac{\alpha K_i}{1 + \frac{\alpha}{\beta}\frac{K_a}{[H^+]}} \quad (6)$$

setting $(\alpha/\beta)K_a = K_a'$, we have

$$K_i^{int} = \frac{\beta K_i}{1 + \frac{[H^+]}{K_a'}} + \frac{\alpha K_i}{1 + \frac{K_a'}{[H^+]}} \quad (7)$$

clearly demonstrating the transition for K_i^{int} from a limit low-pH value of αK_i to a high-pH value of βK_i, governed by an apparent K_a equal to $(\alpha/\beta)K_a$.

The magnitude of the term α/β can be determined from knowledge of the values from anionic inhibition of BCA catalysis of CO_2 hydration at limiting low- and high-pH values. The high-pH result has been obtained in the present study. However, CA is negligibly catalytic toward CO_2 hydration when entirely in its acidic form, and therefore the value of α/β can only be determined kinetically by extrapolation. Fortunately, a robust estimate of the ratio α/β can be derived from the data of the present study. From eq 2, we have, for the data in Figure 2c taken at pH 6.6,

$$\Delta G = D\Delta G' + C_{6.6} \quad (8)$$

or,

$$-RT \ln K_i^{int} = -DRT \ln K_i^{slope} + C_{6.6} \quad (9)$$

Since from Figure 2c D = 1, we can rearrange eq 9 to give

$$e^{C_{6.6}/RT} = K_i^{int,pH\,6.6}/K_i^{slope,pH\,6.6} \quad (10)$$

Similarly, from the data of Figure 2d

$$e^{C_{9.0}/RT} = K_i^{int,pH\,9.0}/K_i^{slope,pH\,6.6} \quad (11)$$

Next, we take the values of K_i^{int} at pH 6.6 as a zero-order approximation for αK_i and K_i^{int} as determined at pH 9.0 as a zero-order approximation for βK_i,

$$K_i^{int,pH\,6.6} = \alpha_0 K_i \quad (12)$$

$$K_i^{int,pH\,9.0} = \beta_0 K_i \quad (13)$$

Substituting eq 12 and 13 into eq 10 and 11, respectively, and taking the ratio of the equations so generated leads to

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$$\frac{e^{C_{6.6}/RT}}{e^{C_{9.0}/RT}} = \frac{\alpha_0 K_i}{K_i^{\text{slope,pH 6.6}}} \Big/ \frac{\beta_0 K_i}{K_i^{\text{slope,pH 6.6}}}$$

$$e^{(C_{6.6}-C_{9.0})/RT} = \alpha_0/\beta_0 \tag{14}$$

From the data of Figures 2c and 2d, $C_{6.6} = 0.35$ and $C_{9.0} = 0.72$. Therefore, $\alpha_0/\beta_0 = 0.47$. A more accurate estimate of the ratio α/β can be achieved utilizing this value and a more complete kinetic expression of the K_i^{int} values. From the full velocity expression for K_i^{int}, eq 6, the value of K_i^{int} at pH 6.6 can be written by substituting the values for K_a of 1.26×10^{-7} (pK_a 6.9) and $[H^+] = 2.56 \times 10^{-7}$ to give

$$K_i^{\text{int,pH 6.6}} = \frac{\beta K_i}{1 + \frac{\beta}{\alpha}\frac{2.56 \times 10^{-7}}{1.26 \times 10^{-7}}} + \frac{\alpha K_i}{1 + \frac{\alpha}{\beta}\frac{1.26 \times 10^{-7}}{2.56 \times 10^{-7}}} = \frac{\beta K_i}{1 + (2.0\beta/\alpha)} + \frac{\alpha K_i}{1 + (0.50\alpha/\beta)} \tag{15}$$

At pH 9.0, K_i^{int} is simply, from eq 6, equal to βK_i.

$$K_i^{\text{int,pH 9.0}} = \beta K_i \tag{16}$$

Since, as eq 14 shows, the ratio of K_i^{int} at these two pH values represents α_0/β_0, we take the ratio of eq 15 and 16, yielding

$$\frac{K_i^{\text{int,pH 6.6}}}{K_i^{\text{int,pH 9.0}}} = \frac{\alpha_0}{\beta_0} = \left(\frac{\beta K_i}{1 + (2.0\beta/\alpha)} + \frac{\alpha K_i}{1 + (0.50\alpha/\beta)}\right)\frac{1}{\beta K_i} \tag{17}$$

Equation 17 simplifies to

$$\frac{\alpha_0}{\beta_0} = \frac{\alpha}{\beta}\left(\frac{1}{(\alpha/\beta) + 2.0} + \frac{1}{1 + (0.50\alpha/\beta)}\right) \tag{18}$$

which, upon rearrangement, yields

$$\frac{\alpha}{\beta} = \frac{\alpha_0}{\beta_0}\left(\frac{1}{(\alpha/\beta) + 2.0} + \frac{1}{1 + (0.50\alpha/\beta)}\right)^{-1} \tag{19}$$

The right-hand side of eq 19 now represents the first-order approximation for α/β, which can be given an accurate numerical estimate by substituting α_0/β_0 for α/β in the denominator of the right-hand side. The result is

$$\frac{\alpha}{\beta} = \frac{\alpha_0}{\beta_0}\left(\frac{1}{(\alpha_0/\beta_0) + 2.0} + \frac{1}{1 + (0.50\alpha_0/\beta_0)}\right)^{-1} \tag{20}$$

Substituting the value of 0.47 derived above into eq 20 gives the numeric result $\alpha/\beta = 0.39$.

Since, as discussed above, $pK_a = 6.9$ for the activity linked ionization, the value for α/β of 0.39 derived above implies a shift in apparent pK_a of 0.4 pH units, to a value of 7.3. The curve drawn in Figure 5b is a theoretical curve based on eq 7 with the apparent pK_a controlling anion binding of 7.3 as predicted. The agreement with the experimental data is excellent.

Discussion

Monoanions of diverse structure inhibit BCA under a variety of conditions. The formal mechanisms by which these anions exert their effect varies considerably depending on the choice of pH and substrate (Figure 1). The most striking observation to emerge from this study is the hitherto unnoted uncompetitive inhibition of BCA-catalyzed CO_2 hydration at high pH. This effect represents the first inhibition of CA by simple anions at high pH; the uncompetitive mechanism observed is rare among single-substrate enzymes.

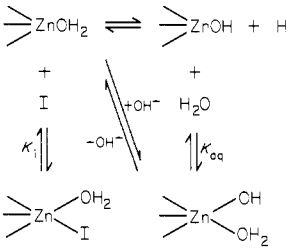
The linear free-energy approach employed above demonstrates clearly that this unusual uncompetitive inhibition occurs at an enzyme locus substantially identical in steric and electronic environment with that at which anions bind and inhibit at low pH (Figure 2). The ability to treat kinetic inhibition data quantitatively with simple rapid-equilibrium models as demonstrated for the CH_3COO^- inhibition of BCA-catalyzed CO_2 hydration reinforces the validity of this model (Figures 4 and 5). These results permit us to exploit the extensive literature[2] of physical studies on anion interactions with CA in the absence of substrate to seek an explanation for this uncompetitive inhibition term.

X-ray crystallographic,[5] NMR relaxivity,[7] and spectral studies of native Zn(II)[12] and highly active, colored, Co(II)-substituted[8,10,11] carbonic anhydrases are consistent with an inner-sphere ligation of simple anions to the catalytically essential Zn(II) ion in the active site of the enzyme. In the absence of added anions, X-ray crystallographic studies show that the Zn(II) ion is approximately tetrahedrally ligated by a water molecule and the imidazole moieties of three histidine residues.[5] This inner-sphere water molecule has been frequently invoked as the activity linked ionization of pK_a 6.9 controlling CA catalysis.[2] The conversion from a Zn(II)-aquo to a Zn(II)-hydroxo ligand as the pH is increased creates a potentially powerfully nucleophilic hydroxide, elegantly accounting for the increase in hydrolytic efficiency of the enzyme with increasing pH.

The proton relaxivity of CA is negligible at low pH, implying minimal inner-sphere ligand exchange, and increases sigmoidally with a pK_a near neutrality, to a maximal value at high pH.[30] In contrast, anion binding, detected by anion NMR relaxivity[7] or spectral changes with the Co(II) enzyme,[10,11] diminishes sigmoidally with increasing pH, consistent with the diminishing inhibition of CA-catalyzed PNA hydrolysis[13,14] and HCO_3^- dehydration.[16] These and other experiments have been interpreted as demonstrating a competition between inhibitory anions and hydroxide for coordination to the Zn(II) ion, accounting for the complementary interactions of hydroxide and other anions with changing pH.

The apparent competition of hydroxide and anions for the Zn(II) ion could, in principle, take place via either a dissociative mechanism, in which Zn(II) retains 4-coordinate structure, or through an association of anions with a fifth coordination site,[31,32] available only when the fourth ligand is in the acidic form, $Zn^{II}OH_2$. Recent spectral studies have suggested that CA has the capacity to participate in mobile equilibria[2,8,33] between 4- and 5-coordinate forms, consistent with an associative mechanism. Comparison with model systems has also been adduced as evidence for associative anion interactions.[32]

The ionization of $Zn^{II}OH_2$ to $Zn^{II}OH$ would, under an associative mechanism, increase the charge density of Zn(II) to such an extent that anions could no longer be accommodated in the inner sphere. The maximal proton relaxivity at high pH can also be explained in terms of control of ligand binding by charge density. Although the presence of $Zn^{II}OH$ is sufficient to block anion binding, the association of an additional neutral ligand, in this case H_2O, would not increase the net formal charge density of Zn(II), but could permit rapid proton relaxation by ligand exchange at high pH.[35] Thus, in the absence of substrate, the ligand interactions of CA can be sketched as eq 21.[36a] The



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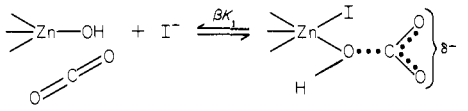
(35) Jacobs, G. S.; Brown, R. D., III; Koenig, S. H. *Biochemistry* **1980**, *19*, 3754.

competitive inhibition of CA-catalyzed HCO_3^- dehydration by anions is thereby explained as a competition of HCO_3^- and inhibitory anions for the fifth coordination site. Bound CO_2 has only a slight effect, measured by the parameter α defined above, on anion inhibition at low pH.

At high pH, the situation is dramatically different. As discussed above, the uncompetitive inhibition of CO_2 by anions represents obligate substrate CO_2 binding prior to inhibitor binding. This kinetic result demonstrates for the first time a direct, substantial influence of CO_2 binding on the inner sphere of carbonic anhydrase. The excellent linear free-energy correlations of anion binding at all pH values and the close adherence of anion inhibition to rapid-equilibrium analysis ensures that the uncompetitive anion binding represents the same inner-sphere process as that observed at low pH. CO_2 binding must therefore in some way alter the properties of the Zn(II) inner sphere so as to permit anion association.

If, as is argued above, the charge density created in the inner sphere by the formation of $Zn^{II}OH$ is the factor preventing anion binding to the basic form of CA, then CO_2, when bound, must relieve this constraint. This line of reasoning leads directly to a mode of anion binding to the enzyme–CO_2 complex with significant implications for the catalytic mechanism of CA. Acting as a Lewis acid, CO_2 could readily reduce the charge density of the hydroxide ligand by direct addition, a crucial step in the catalysis of CO_2 hydration. This interaction is outlined below:



(22)

C–O bond formation may or may not be complete in the ternary complex, but a substantial interaction is likely required to maintain the appropriate formal charge on Zn(II) when the anion binds. This scheme places bound CO_2 in a position just outside the Zn(II) inner sphere, close enough to permit a nucleophilic interaction with $Zn^{II}OH$ but not so close as to interfere significantly with the expansion of the coordination sphere associated with inhibitor binding.

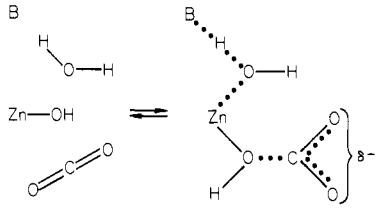
Not only does this binding interaction explain the anion inhibitions observed, but it provides an answer to paradox associated with the identification of $Zn^{II}OH$ as a nucleophilic component of CA catalysis, and thereby leads to a new mechanism in metalloenzyme catalysis for the exceptional catalytic activity of carbonic anhydrase.

A principal criticism of the identification of $Zn^{II}OH$ as a nucleophile is the pK_a near 7 which must be assigned to $Zn^{II}OH_2$ under this hypothesis. Although such a pK_a value is not unreasonable, based on model studies when appropriate corrections for tetrahedral coordination in the enzyme are made,[2,32] such an assertion creates a paradox. In achieving such a low pK_a, the basicity and nucleophilicity of the conjugate base, $Zn^{II}OH$, is correspondingly reduced. In order to create a $Zn^{II}OH$ in the physiological pH range, CA would appear to render the nucleophile less fit for its catalytic role.

This apparent paradox is readily resolved when the mobile coordination number porposed above is taken into account. Expansion of the coordination sphere provides a means by which carbonic anhydrase posesses a $Zn^{II}OH$ of $pK_b = 7$ with the nucleophilic power of a much more basic ligand. By adding a fifth ligand while CO_2 is bound, a process directly analogous to the mechanism proposed for uncompetitive inhibition at high pH, CA generates a more powerfully nucleophilic 5-coordinate $Zn^{II}OH$. The incoming fifth anionic ligand increases the charge density of Zn(II), which causes release of charge density to the bound hydroxide, increasing its nucleophilicity as it approaches the electrophilic carbon of CO_2.

Anions inhibit the enzyme at high pH, despite their apparent ability to facilitate the nucleophilic attack of $Zn^{II}OH$ on CO_2 (eq 22). Evidently other molecular rearrangements must be involved in effective catalysis. Proton-transfer steps are obvious candidates. As shown by arguments based on microscopic reversibility and diffusion limitations,[16,32,34] HCO_3^- must be released as the product of CO_2 hydration, not H_2CO_3. Therefore, the enzyme must effectively accept and release a proton in the net catalytic cycle to maintain the correct stoichiometry.

By analogy with the addition of an anion to the fifth coordination site of the enzyme–CO_2 complex, hydroxide should be the attacking ligand during effective catalysis. For the kinetic requirements of both the forward and reverse reactions to be satisfied, the incoming hydroxide could be provided not as a free hydroxide from solution but as an effective hydroxide, generated by a base acting on an incoming H_2O molecule. A role in catalysis for such a general base has long been postulated.[2] Two likely candidates for such a base exist: the conserved nonligand imidazole group of histidine or a bound buffer molecule. These two candidates are not necessarily mutually exclusive. Either base could function through one or more bridging H_2O molecules, a likely prospect in the case of the imidazole, located 6 Å from Zn(II).[5] As a shorthand notation encompassing all of these possibilities, the symbol B will be used in subsequent descriptions of the catalytic mechanism. The productive attack of $Zn^{II}OH$-bound CO_2 can therefore be represented by scheme 23

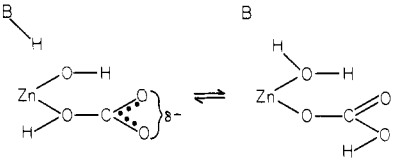


(23)

Proton transfer, C–O bond formation, and the formation of the fifth coordinate bond to $Zn^{II}OH$ need not be concerted. Indeed, coordination to $Zn^{II}OH$ may occur first, facilitating proton transfer by polarizing the incoming H_2O molecule.

As discussed above, the newly formed HCO_3^- shown above cannot leave the active site when bound as shown on the right side of eq 23. Another essential proton transfer must take place prior to product release—a proton transfer to the carboxylate moiety of the newly formed HCO_3^-.

Deprotonation of the oxygen currently protonated in the HCO_3^- ligand would, however, yield a species with excessive formal charge on Zn(II), analogous to a form of carbonic anhydrase binding two anions simultaneously. The anionic inhibition of BCA in the absence of substrate shows such a species to be highly unfavorable (see below). For the requirement for constant ligand electron density on Zn(II) at all times to be satisfied, a reprotonation by BH of the recently added hydroxide simultaneous with proton transfer to the carboxylic moiety of HCO_3^- could effectively switch the identity of the two ligands; the anionic hydroxyl becomes a neutral H_2O and the HCO_3^- becomes the permitted anionic ligand. The enzyme is now in the acidic form, with HCO_3^- bound as an anionic ligand.

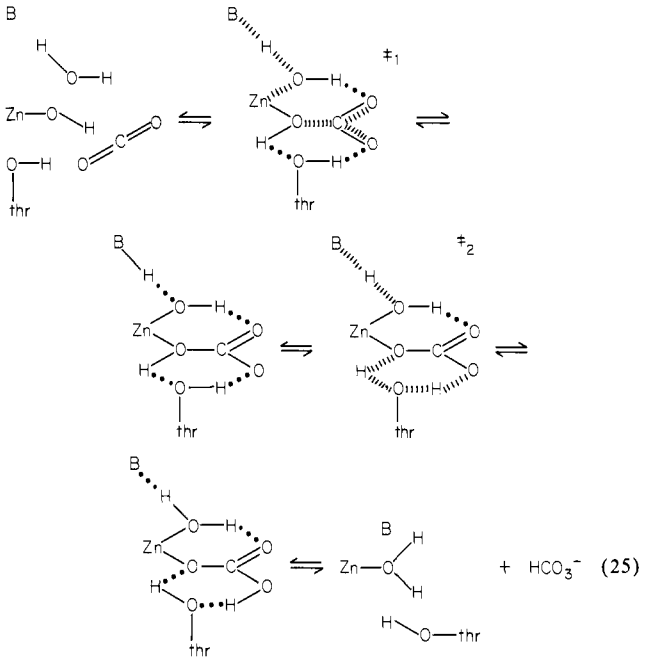


(24)

Equation 25 summarizes the above discussion by proposing a full molecular mechanism for catalysis of CO_2 hydration and HCO_3^- dehydration, with one added feature not yet discussed. The proton transfer between the two oxygens of the incipient HCO_3^- is presented as being facilitated by the conserved active

(36) (a) For simplicity certain charges are not explicitly represented in the accompanying diagrams. (b) Alternatively, the molecular organization involved in the catalysis of PNA hydrolysis may not take advantage of the full enzymatic machinery available for the catalysis of CO_2 hydration. Indeed, the steric requirements to form from PNA an sp^3-hybridized tetrahedral intermediate in the active site may be such as to preclude the special anionic interactions seen during CO_2 hydration at pH values above 7.0.

site threonine acting as the proton-transfer intermediate, as a hydrogen-bond donor stabilizing the bound CO_2, and as a hydrogen-bond acceptor stabilizing the $Zn^{II}OH$ ligand.



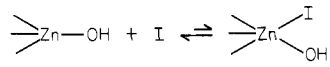
A water molecule would function equally well in this position, but the proximity of this threonine to the active site ligand (it is in fact found to be hydrogen bonded to the Zn(II) inner-sphere oxygen in X-ray crystallographic studies[6]) and its conservation across species makes assignment to it of a catalytic role appealing; the entropic cost of orienting a water molecule in this position may thereby be avoided in the catalytic cycle, although the degree of catalytic efficiency gained thereby is difficult to assess.

Inspection of the dynamic picture of catalysis presented in eq 25 shows that at all times the net formal charge on the ligands bound to Zn(II) never exceeds that contributed by one anion and one neutral ligand. The criterion that a constant charge environment be maintained in the active site of carbonic anhydrase has been recently employed to devise a model for the binding of anions and neutral inhibitors to carbonic anhydrase that explicitly rejects the hypothesis of an activity linked ionization at physiological pH.[35] The above analysis demonstrates that resort to such a model is not a necessary requirement for the retention of the important criterion that the static and dynamic states of carbonic anhydrase maintain constancy of electron density on Zn(II).

The uncompetitive inhibition by anions of BCA at high pH is confined to the CO_2 hydrase activity of the enzyme (see Results). The catalysis of PNA hydrolysis, also maximal at high pH, is unaffected by added anions at concentrations in considerable excess of those required to entirely inhibit BCA-catalyzed CO_2 hydration at the same pH. This distinction between the physiologically important hydrase activity of CA and its esterase activity provides some insight into the relationship between these two enzyme functions.

As discussed above, the enzymatic inhibition of BCA-catalyzed CO_2 hydration occurs by anion binding to an enzyme–CO_2 complex (eq 22). An economical interpretation of the lack of uncompetitive inhibition of BCA-catalyzed ester hydrolysis is that the enzyme–substrate complex in the case of ester hydrolysis is significantly different from that of CO_2. If the lifetime of the enzyme–PNA complex is so short as to preclude significant anion interaction during catalysis, no uncompetitive inhibition could be detected for this substrate. The fact that K_m values for PNA hydrolysis have been difficult to obtain reproducibly between different laboratories is consistent with this explanation, for without a significant fraction of the enzyme in the form of an enzyme–substrate complex during kinetic experiments, a meaningful K_m value cannot be determined. These kinetic observations may also imply a more general difference in the elementary steps defining kinetically determined apparent binding constants for ester substrates and CO_2.[36b]

Two observations made above (see Results) require brief comment. A minor K_i^{slope} term was detected in some instances at limiting high pH in the anionic inhibition of BCA-catalyzed CO_2 hydration. This term has been neglected for simplicity in the above analysis, as its value is generally an order of magnitude larger than the dominant K_i^{int} dissociation constant and is thereby difficult to identify or quantify. Such a term is, however, not an unreasonable feature of the above model. A limiting high-pH K_i^{slope} term would represent a highly disfavored equilibrium binding of the inhibitory anion to the basic form of the enzyme at high pH.



(26)

With high concentrations of potent anionic inhibitors at high pH, such a binding mode could be detected and indeed, under sufficiently forcing conditions, could become the principal enzyme species in solution in, for example, a spectral study of colored, Co(II)-substituted carbonic anhydrase. The difference between ligand dissociation constants measured under such conditions and the kinetic βK_i values determined in this investigation would provide a measure of the stabilizing effect of bound CO_2 on the high-pH 5-coordinate form of BCA.

The second observation, the curvature of Lineweaver–Burk plots detected in two of the 12 anions tested, Br^- and I^-, at high pH for inhibition of BCA-catalyzed CO_2 hydration, is also of considerable interest in terms of the proposed mechanism. One reasonable source of this curvature could be the breakdown of the rapid-equilibrium assumption in the case of Br^- and I^-, as suggested by the variation in observed curvature with differing methods of enzyme and anion preincubation.

Such effects have been previously noted in other enzyme kinetics studies. In a study of the inhibition of rabbit muscle phosphofructokinase, concave upward curvature of Lineweaver–Burk plots of inhibition data was attributed to slow rates of breakdown of enzyme–inhibitor complexes, based on computer simulation of inhibition kinetic schemes.[37] An observation made with the use of NMR relaxation techniques to examine the binding of halide ions to $Zn^{II}CA$[7] supports such a proposal. Br^- and I^- were found to dissociate from the inner sphere of the metal ion 10- to 100-fold more slowly than Cl^-. Qualitatively similar results have been obtained for the interaction of I^- with Cd(II) substituted BCA, monitored by ^{113}Cd NMR.[38] These observations do not appear to simply reflect the greater inhibitory potency of Br^- and I^-, and may be a significant factor contributing to the breakdown of the rapid-equilibrium assumption for these two anions.

A detailed study of the anionic inhibition of BCA by these two anions will be required to test the adequacy of this suggestion and to rationalize the differences observed between the results obtained with the various preincubation techniques employed. Such studies could provide a fruitful source of kinetic data on the details of anionic inhibition on the boundary between rapid equilibrium and steady-state inhibition systems.

The model for the catalytic mechanism of CA presented in this study brings together a larger body of kinetic, spectral, and structural properties of CA than other models proposed to date. For the first time, a catalytic mechanism provides a natural relationship between the catalytic and inhibitory processes of the enzyme on a molecular level. The implications of this model go well beyond those discussed above. We defer discussion of a number of features, including the details of the proton-transfer steps described, the interaction of the alkyl carbonates, $ROCO_2^-$, with carbonic anhydrase, and the nature of HCO_3^- binding to the enzyme for which further evidence will be presented.[39]

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Most of the above proposals follow directly from the assumption of a mobile equilibrium between 4 and 5 coordination, the requirement for steric and dynamic constancy of formal charge on Zn(II), and the special enzyme–substrate–anion complex characterizing the high-pH inhibition of BCA-catalyzed CO_2 hydration by anions. Throughout this study, the strongest simplifying assumption of rapid equilibrium between enzyme species has been employed, with excellent success. It is likely that expansion of this simplified scheme will make it possible to explain in detail certain other kinetic results, such as inhibition at high pH by Br^- and I^-, the diminution of CA catalytic activity at low buffer concentrations,[29,40-42] and other observations.[43] Consequently, we feel that the proposed mechanism is an appealing working hypothesis with intriguing implications for future investigations.

Acknowledgment. We are indebted to the National Science Foundation, the National Institutes of Health, and the Muscular Dystrophy Association for partial support of this research and to the Chevron Corporation for a Fellowship to T.L.D. We are grateful to Victor K. Hamasaki for his skillful performance of a number of PNA inhibition studies, to Donald B. Moore for his diligent assistance in all aspects of computer interfacing, programming, and repairing, and to Drs. Conrad T. O. Fong, Kenneth W. Raymond, and Simo Sarkanen for helpful discussions.

Registry No. Cl^-, 16887-00-6; Br^-, 24959-67-9; I^-, 20461-54-5; SCN^-, 302-04-5; N_3^-, 14343-69-2; ClO_4^-, 14797-73-0; HCO_2^-, 71-47-6; $CH_3CO_2^-$, 71-50-1; $CH_3CH_2CO_2^-$, 72-03-7; $CH_3(CH_2)_2CO_2^-$, 461-55-2; $CH_3(CH_2)_6O_2^-$, 10023-74-2; $CH_3(CH_2)_4CO_2^-$, 151-33-7; carbonic anhydrase, 9001-03-0.

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Cryptand Exchange Kinetics

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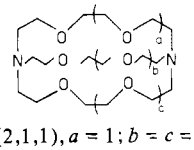
Abstract: The rate constant for some reactions between a metal cryptate $MCry_1^{n+}$ and a free cryptand Cry_2 has been determined. For the case when $M^{n+} = Tl^+, Ca^{2+}$, Cry_1 = (2,2,2), (2_B,2,2) and Cry_2 = (2,2,1) in water or water–methanol mixtures, the observed rate constant corresponds to that of the dissociation of $MCry_1^{n+}$. However, the exchange reactions $Pb(2,1,1)^{2+}$ + (2,2,1) and $Pb(2,1,1)^{2+}$ + (2,2,2) in MeOH present rates that are much larger than the dissociation rate of $Pb(2,1,1)^{2+}$. A mechanism involving a bimolecular reaction between cryptate and free cryptand is proposed.

Previous studies on the kinetics of macrobicyclic diamines (cryptands) and their metal complexes (cryptates) have been mainly concerned with the dissociation and formation rates of the complexes.[1] Proton-transfer reactions between hydroxide ions and some monoprotonated cryptands have also been investigated,[2] as well as the acid-catalyzed dissociation of metal cryptates.[3] As a result of NMR studies[4-6] some metal ion–metal cryptate exchange rates in various solvents and free ligand–complexed ligand exchange rates in water have been measured.

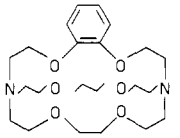
In this paper we wish to report a new aspect of cryptand kinetics. The reactions we have studied are of the type shown in eq 1, where M^{n+} is the metal ion and Cry_1 and Cry_2 are two different cryptands which form the metal cryptates $MCry_1^{n+}$ and $MCry_2^{n+}$, respectively.

$$MCry_1^{n+} + Cry_2 \xrightarrow{k_e} MCry_2^{n+} + Cry_1 \qquad (1)$$

The cations Ca^{2+}, Tl^+, and Pb^{2+} and cryptands (2,1,1), (2,2,1), (2,2,2), and (2_B,2,2) were used in the present study.



(2,1,1), $a = 1; b = c = 0$
(2,2,1), $a = b = 1; c = 0$
(2,2,2), $a = b = c = 1$



(2_B,2,2)

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Ligand-exchange reactions, where the ligands are macrocycles, may be of interest in relation to the transport of ions through membranes by macrocyclic carriers.[7] Simon et al. have shown, using ^{14}C-labeled valinomycin, that exchange of ligands occurs during transport of cations through membranes.[8] Studies on ion-permeability phenomena, such as Na^+ channels, have led others to propose kinetic models where bimolecular reaction with participation of a ligand is involved.[9]

Experimental Section

A. Materials. Cryptands (2,1,1), (2,2,1), (2,2,2), and (2_B,2,2) were purchased from Merck and used without further purification. Doubly-distilled water and dried methanol (Merck, GR, max. 0.01% H_2O) were

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