monodentate and the chelated catecholate complexes is striking and has implications as to the nature of the enzyme–substrate interaction in these enzymes. Resonance Raman studies on the dioxygenases indicate that the substrate catechol is chelated to the active-site iron;[7,25] our results suggest that this form is not reactive. However, studies on substrate analogues indicate a preference of the iron center for the coordination of one catecholate hydroxyl over the other.[30] Perhaps oxygen binding induces protonation of the catecholate, rendering the complex reactive with oxygen. Further studies on both model and enzyme systems are in progress.

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Observation of a Novel Effect of pH on the Anionic Inhibition of Carbonic Anhydrase: Implications for Enzymatic Catalysis

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The inhibition by anions of the catalysis of the reversible hydration of CO_2 and dehydration of HCO_3^- by carbonic anhydrase (EC 4.2.1.1) was noted by the earliest investigators of this extremely powerful catalyst.[1] The catalytic properties of this enzyme have recently been reviewed.[2] Carbonic anhydrase activity is quite pH dependent; at low pH, catalysis of CO_2 hydration is negligible, while at high pH, CO_2 hydration is catalyzed with maximal efficiency. The reverse holds for HCO_3^- dehydration activity. An ionization with pK_a near neutrality appears to control this change in activity.[2]

The mechanism and pH dependence of the anionic inhibition of the HCO_3^- dehydration activity of bovine erythrocyte carbonic anhydrase (BCA) has recently been investigated in these laboratories.[3] We are engaged in a corresponding study of the anionic inhibition of the BCA catalysis of CO_2 hydration, covering for the first time a significant range of pH values. The results of this study are sufficiently surprising to warrant a preliminary communication. Using stopped-flow initial rate techniques previously described,[3,4] inhibition by the acetate anion has been investigated over the pH range 6.15–9.00. At each of 9 pH values, a full Lineweaver–Burk plot ($1/V$ vs. $1/[S]$) has been obtained by using inhibitor concentrations spanning the observed K_i values. Primary plots and secondary slope and intercept replots[5] exhibit excellent linearity (correlation coefficients >0.99).

At pH values below 7, the mechanism of inhibition observed is best described in the nomenclature of Cleland[6] as intersecting (slope and intercept linear) noncompetitive inhibition; both the slope and ordinate intercepts of the Lineweaver–Burk plots increase with increasing inhibitor concentration. The slope and intercepts are not necessarily equivalently influenced. Two K_i values can be isolated; one describes the effect of inhibitor on the slope of the Lineweaver–Burk plot and will be referred to as K_i^{slope}. The other term, K_i^{int}, describes the effect of inhibitor on the ordinate intercept of Lineweaver–Burk plots. In simple one-substrate enzymes under conditions of rapid equilibrium between enzyme species, K_i^{slope} measures the dissociation of enzyme–inhibitor complex in the absence of substrate, while K_i^{int} reflects the dissociation of the ternary enzyme–substrate–inhibitor complex.[5]

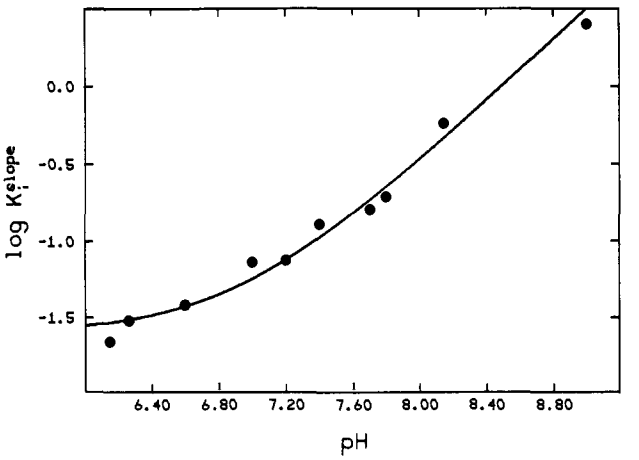

Figure 1. Plot of log K_i^{slope} vs. pH for the inhibition of BCA-catalyzed CO_2 hydration by $CH_3CO_2^-$. The line is a theoretical curve representing a change from a limit low pH value for S_i^{slope} of 0.025 M to negligible binding at high pH, governed by a single ionization of pK_a 6.9.

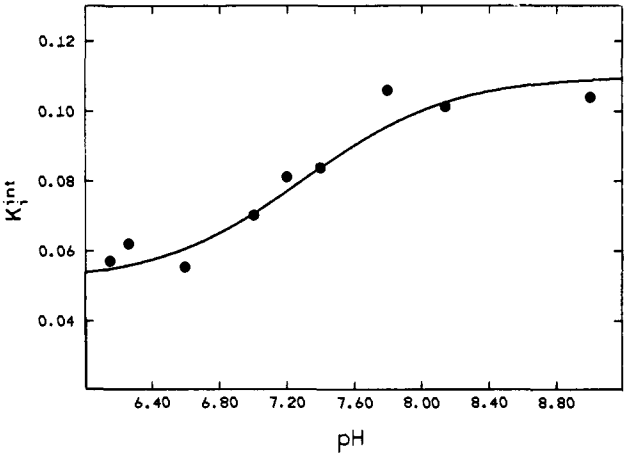

Figure 2. Plot of K_i^{int} vs. pH for the inhibition of BCA-catalyzed CO_2 hydration by $CH_3CO_2^-$. The line is a theoretical curve representing a change from a limit low pH value for K_i^{int} of 0.05 M to a limit high pH value of 0.11 M, governed by a single ionization of apparent pK_a 7.3 (see ref 8).

As the pH increases above 7.0, the mechanism of inhibition changes to a limiting pattern best described[5,6] as linear uncompetitive inhibition; increasing inhibitor concentration increases the ordinate intercept, but not the slope, of the Lineweaver–Burk plot. Such a mechanism of inhibition of carbonic anhydrase has not been previously reported under any experimental conditions. This important and unprecedented result is quite general. With K_i values ranging over four orders of magnitude, the anions Cl^-, N_3^-, ClO_4^-, formate, and hexanoate all exhibit the same uncompetitive pattern at high pH. Anionic inhibition is established extremely rapidly. No change in degree or mechanism of inhibition is observed whether enzyme and inhibitor are preincubated for several minutes or allowed to interact for only 10 ms before initial rate measurements.

For illustration of the smooth transition between the mechanisms observed at low and high pH, log K_i^{slope} values for acetate inhibition of BCA-catalyzed CO_2 hydration are plotted vs. pH in Figure 1. The line drawn is a theoretical curve describing the binding represented by K_i^{slope} as controlled by a single ionizable group of pK_a 6.9, with inhibitor binding only to the acidic form of enzyme. This is exactly the pattern observed in studies of the anionic inhibition of BCA-catalyzed HCO_3^- dehydration[3] and *p*-nitrophenyl acetate hydrolysis,[7] both of which display significant

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pH dependent K_i^{slope} values, apparently governed by the same ionization.

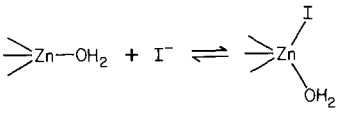
Figure 2 presents the behavior of the K_i^{int} term for acetate inhibition of BCA-catalyzed hydration of CO_2 as a function of pH. This pattern is unique in carbonic anhydrase activity and of fundamental importance as the reversible hydration of CO_2 represents the only known physiological catalysis by this enzyme. The theoretical curve drawn models this term as representing a change from a low pH value for K_i^{int} of 0.05 M to a high pH limit value of 0.11 M, governed by a single ionization of pK_a 7.3. A twofold diminution of inhibitor binding potency accompanies the increase in pH, but anion inhibition is still quite significant at the high pH plateau.

Although the observed pK_a values governing the changes in anion dissociation measured by K_i^{slope} and K_i^{int} differ by 0.4 pK_a units, the ionization governing K_i^{slope} can be shown to represent only an apparent pK_a value. The shift from the pK_a of the activity linked ionization of 6.9 is a consequence of the binding of inhibitor to the enzyme–substrate complex with differing affinities at low and high pH. A simple rapid equilibrium analysis demonstrates this necessary influence and predicts quantitatively the magnitude of the effect, given the difference in K_i^{slope} values measured at low and high pH.[8] Interestingly, this same formal analysis predicts that at pH values near the pK_a of the activity linked ionization, 6.9, inhibition by anions of BCA-catalyzed CO_2 hydration should exhibit a pure noncompetitive mechanism ($K_i^{slope} = K_i^{int}$), consistent with an earlier report from these laboratories on anionic inhibition at pH 7.0.[9] A more complete description of this formal analysis is forthcoming.

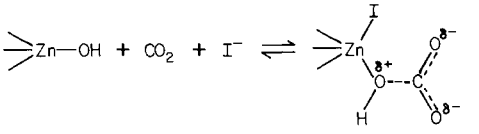
Numerous spectral and X-ray crystallographic studies place the site of inhibitory anion binding in the inner sphere of the catalytically essential Zn(II) ion, while CO_2 is bound near but not within the Zn(II) inner sphere.[2] No study to date has demonstrated significant interaction of anions with carbonic anhydrase at high pH. This observation must now be revised; the binding of CO_2 is sufficient to restore a considerable interaction between monoanions and BCA, via the Zn(II) ion, in the high pH region.

We wish to outline a working hypothesis that attempts to account for this new binding interaction, incorporating some features of earlier proposals[2] with the addition of a novel mechanism for organometallic catalysis. We propose that an expansion of the coordination number of the Zn(II) ion is the means by which anions bind to the enzyme. Further, we propose that the activity linked ionization controlling substrate activity and anion binding is the ionization of a zinc(II) aquo ligand to a zinc(II) hydroxide. A number of spectral and kinetic studies have suggested a variable coordination number for the metal ion in carbonic anhydrase.[10,11]

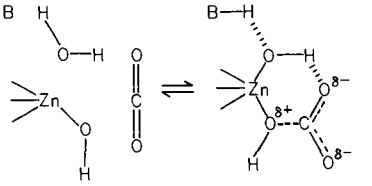
In the acidic form of the enzyme, the Zn(II) ion is liganded by three imidazoles of histidine residues and a water molecule.[2] We propose that a fifth ligand, a monoanion, can bind reversibly with little influence by bound substrate CO_2.[12]



This accounts for the noncompetitive inhibition of BCA-catalyzed CO_2 hydration by anions at low pH. Bicarbonate could occupy this fifth coordination site, accounting simply for the competitive inhibition of BCA-catalyzed HCO_3^- dehydration at all pH values.[3] This mode of binding is abolished at high pH as the formal charge on the Zn(II) is increased by deprotonation to zinc(II) hydroxide. The Zn(II) ion in the active site apparently cannot accommodate two negative charges in its inner sphere. Consistent with this model, dianions are negligibly inhibitory toward carbonic anhydrase.[2] CO_2 binding to the basic form of BCA must therefore modify the formal charge of the zinc(II) hydroxide to permit anion binding. We propose that this occurs by an interaction between the zinc(II) hydroxide and the CO_2 substrate molecule bound nearby. A fifth ligand could then enter the inner sphere while the excess formal charge is distributed over an incipient bicarbonato ligand, with the possibility of other stabilizing interactions with neighboring amino acid residues.



This complex must be inactive, since release of a bicarbonate anion from this complex would turn over the CO_2 substrate.[13] An additional step must precede product release, which likely consists of one or more proton-transfer steps involving the group that would normally occupy this anion binding site during effective catalysis. Our hypothesis is that this incoming group is an incipient hydroxide, generated by an adjacent basic moiety acting as a general base catalyst. This base, designated as B below, could be the



conserved active site (nonligand) histidine,[2] possibly operating through one or more bridging water molecules, consistent with the observed solvent deuterium isotope effects on enzyme activity.[3,14-16] Subsequent proton transfers release the bicarbonate

(8) The full velocity expression for the anionic inhibition of BCA-catalyzed CO_2 hydration, according to this model, is given by eq 1.

$$\frac{1}{V} = \frac{1}{[CO_2]}\frac{K_M}{V_{max}}\left[1 + \frac{[H^+]}{K_a}\left(1 + \frac{[I]}{K_i^{slope}}\right)\right] + \frac{1}{V_{max}}\left[1 + \frac{[I]}{\beta K_i} + \frac{[H^+]}{K_a}\left(1 + \frac{[I]}{\alpha K_i}\right)\right] \quad (1)$$

where αK_i and βK_i are, respectively, the limit values at low and high pH for K_i^{int}. The intercept Y_i of a plot of $1/V$ vs. $1/[CO_2]$ is a linear function of inhibitor concentration, which, from eq 1, is given by eq 2.

$$Y_i = \frac{1}{V_{max}}\left[1 + \frac{[I]}{\beta K_i} + \frac{[H^+]}{K_a}\left(1 + \frac{[I]}{\alpha K_i}\right)\right] \quad (2)$$

The value of K_i^{int} is then the abscissa intercept of a plot of the Y_i (eq 2) vs. [I] which can be simply derived from the above linear equation, yielding eq 3.

$$K_i^{int} = \left(1 + \frac{[H^+]}{K_a}\right)\left[\frac{1}{\alpha K_i}\left(\frac{\alpha}{\beta} + \frac{[H^+]}{K_a}\right)\right]^{-1} \quad (3)$$

Rearranging, and setting $K_a' = (\alpha/\beta)K_a$, we obtain eq 4.

$$K_i^{int} = \frac{\alpha K_i}{1 + (K_a'/[H^+])} + \frac{\beta K_2}{1 + ([H^+]/K_a')} \quad (4)$$

The change with pH from αK_i to βK_i is governed by an apparent K_a equal to $(\alpha/\beta)K_a$. From our data, $\alpha/\beta = 0.4$ and $K_a = 1.26 \times 10^{-7}$ ($pK_a = 6.9$). Therefore, $K_a' = 5.0 \times 10^{-8}$ ($pK_a' = 7.3$).

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(12) For simplicity, certain charges are not explicitly represented in the accompanying diagrams.

(13) Such a mechanism would be inconsistent with the observed pH dependence of BCA-catalyzed CO_2 hydration and HCO_3^- dehydration. Moreover, by microscopic reversibility, HCO_3^- would necessarily bind to Zn(II) by its neutral hydroxyl ligand, contrary to the evident anion affinity of BCA.

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anion and reionize the enzyme for another catalytic cycle.

This mechanistic sketch offers a dynamic explanation for the catalytic steps of CO_2 hydration. A 4-coordinate $Zn^{II}OH$ is expected on the basis of model studies to be less basic than a similar 5-coordinate complex.[10] The approaching fifth ligand increases the basicity and nucleophilicity of the $Zn^{II}OH$, facilitating attack on the previously bound CO_2. Carbonic anhydrase can thereby retain in the resting state a hydroxide ligand of pK_b near 7 while utilizing catalytically a 5-coordinate hydroxide of increased nucleophilic power. The dilemma of assigning a weakly basic pK_b of 7 to the Zn-bound OH while simultaneously invoking its participation as a nucleophile is resolved by the proposed mechanism. Moreover, a fundamental connection is established for the first time between the anionic inhibition of carbonic anhydrase and its catalytic mechanism.

We believe that the observed high pH uncompetitive inhibition of the CO_2 hydration activity of carbonic anhydrase is not readily accomodated by any previously proposed mechanism of action of the enzyme. We therefore offer this new proposal for carbonic anhydrase catalysis. In a forthcoming work we shall demonstrate more fully the congruence of this mechanism with previous results.

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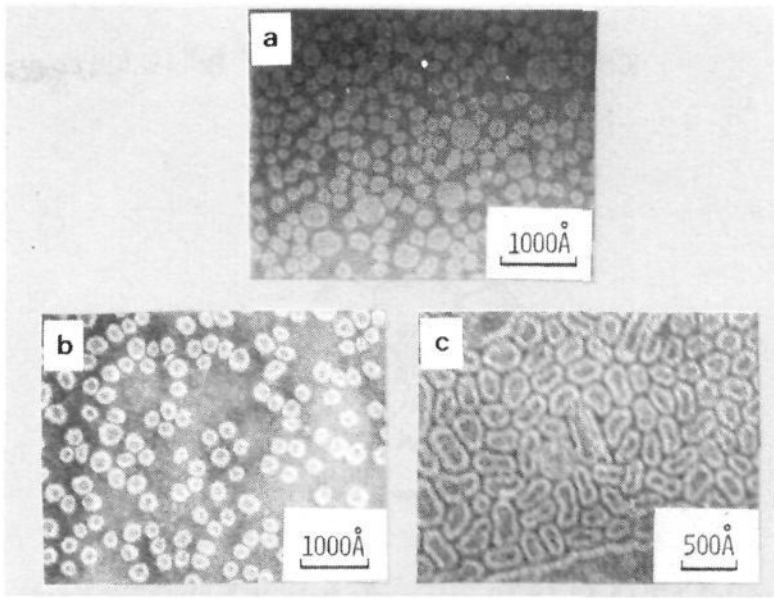

Figure 1. Electron micrographs of single-compartment vesicles negatively stained with uranyl acetate: (a) 5.0×10^{-3} M aqueous solution of **1** sonicated for 60 s with a probe-type sonicator at 30-W power (W-220F, Heat Systems-Ultrasonics) and allowed to stand for 10 min at 5 °C (magnification, ×75 000); (b) 5.0×10^{-3} M aqueous solution of **1** containing **2** (8.3×10^{-5} M) sonicated as above (magnification, ×64 000); (c) 5.0×10^{-3} M aqueous solution of **1** containing **2** (1.7×10^{-4} M) sonicated as above (magnification, ×107 000).

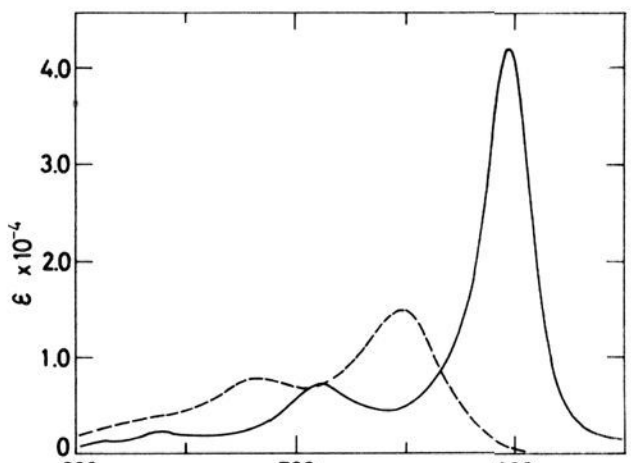

Figure 2. Electronic absorption spectra of **2a** (---) and **2b** (—) incorporated into single-compartment vesicles of **1** (1.0×10^{-3} M) in water containing KCN (5.0×10^{-4} M) at 20 °C.

Novel Substrate-Binding Property of Synthetic Membrane Vesicles Involving an Amino Acid Residue as a Molecular Component

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Currently, there is increasing interest in the behavior and characterization of naturally occurring[1] and synthetic bilayer membranes.[2] Although various probe techniques have been widely adopted to obtain information on the physical properties (microviscosity, micropolarity, fluidity, and so on) of these assemblies and particularly on the effects of their phase transitions thereupon,[3] there are only very scattered data as to the nature of hydrophobic substrate-binding characteristic of these bilayer assemblies. Recently, we have shown that amphiphiles involving an amino acid residue (Ala or His) interposed between a polar head group and an aliphatic double chain form *stable single-compartment vesicles* in aqueous media.[4,5] In the present work, we have studied the binding interaction of bilayer assembly, formed with cationic amphiphile **1**, with a hydrophobic Co(III) complex. The present bilayer aggregate provides two binding sites greatly different from each other in microenvironmental property. The substrate incorporated into these two sites is subjected to a novel distribution law, and its translocation between the two sites is practically prohibited due to the presence of a significant barrier provided at the so-called hydrogen-bonded region.

Amphiphile **1** (*N*,*N*-didodecyl-N^{α}-[6-(trimethylammonio)hexanoyl]-L-alaninamide bromide),[6] like its zwitterionic analogue,[4] forms single-compartment vesicles with relatively uniform size (130–400 Å) in aqueous media upon sonication (Figure 1a). A Co(III) complex used here as a guest molecule is dicyano(8,12-

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(6) Prepared from reaction of $(CH_3)_3N$ with *N*,*N*-didodecyl-N^{α}-(6-bromohexanoyl)-L-alaninamide:[4] liquid crystal with final mp 195 °C; $[\alpha]^{20}_D$ −18.5° (*c* 0.91, EtOH); ^1H NMR ($CDCl_3$, Me_4Si) δ 0.88 (6 H, br t, $(CH_2)_{11}CH_3$), 1.25 (40 H, s, $CH_2(CH_2)_{10}CH_3$), 1.35 (3 H, s (sh), $CH(CH_3)$), ~2.00 (6 H, m, $NCH_2(CH_2)_3CH_2$), 2.26 (2 H, br t, $N(CH_2)_4CH_2CO$), 3.42 (9 H, s, $N^+(CH_3)_3$), 3.04–3.79 (6 H, m, $CH_2N^+(CH_3)_3$ and NCH_2-$(CH_2)_{10}CH_3$), 4.78 (1 H, br q, $CH(CH_3)$), and 7.04 (1 H, br d, NH). Anal. Calcd for $C_{36}H_{74}N_3O_2Br$: C, 65.42; H, 11.28; N, 6.36. Found: C, 65.07; H, 11.27; N, 6.09. Critical micelle concentration by surface tension method of the Wilhelmy principle, 1.5×10^{-5} M.

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