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Interaction of Bovine Carbonic Anhydrase with (Neutral) Aniline, Phenol, and Methanol†

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ABSTRACT: We have investigated the interaction of bovine carbonic anhydrase with neutral aniline, phenol, and methanol molecules. The measurements are of optical spectra and solvent water and methanol proton magnetic relaxation rates of solutions of Co^{2+}-substituted enzyme. We recently proposed a model [Koenig, S. H., Brown, R. D., & Jacob, G. S. (1980) *Proceedings of the Symposium on Biophysics and Physiology of Carbon Dioxide*, Springer-Verlag, West Berlin and Heidelberg], based on the interaction of enzyme with monovalent anions, that accounts for the pH dependences observed for a wide variety of phenomena, including the apparent pK_a for enzymatic activity. We now extend the model to include the observed effects of neutral molecules. Aniline and phenol, though isoelectronic, shift the observed pK_a values in opposite directions, and both appear to bind at the aromatic binding site to which sulfonamide inhibitors and aromatic esters are known to bind. The resulting binary complexes behave as altered enzymes, with different values of the pK_a for activity, but otherwise are similar to the native enzyme. In terms of our model, aniline and phenol alter the relative affinities of water and anions for the same coordination position of the metal ion at the active site. The effect is opposite in sign for the two molecules because of the differing proton affinities of the NH_2 and OH moieties of the phenol ring in each case. By extension, our results indicate that data from experiments using aromatic buffers such as imidazole and lutidine should be analyzed with some care; effects previously attributed to buffer in solution may well be due to binding of neutral buffer molecules to the aromatic binding site in the active region of the enzyme. The interaction of methanol with carbonic anhydrase is quite different, and very weak. Methanol does displace water at the metal, but to first order there is little, if any, preferential binding of methanol compared to water. Observations by others that alcohols inhibit esterase activity with inhibition constants on the order of 1 M are not attributable to binding of alcohol to enzyme but rather, in our view, result from the increased solubility of aromatic ester substrates in the alcohol-modified solvent.

The zinc-containing enzyme carbonic anhydrase (EC 4.2.1.1), which catalyzes the reversible reaction $CO_2 + H_2O \rightleftarrows H^+ + HCO_3^-$, has also been found to catalyze the hydrolysis of a variety of esters (Tashian et al., 1964; Malmström et al., 1964; Pocker & Stone, 1965, 1967) and the hydration of aliphatic aldehydes (Pocker & Meany, 1965a,b). A longstanding question has existed regarding the identity of the metal-activated ligand in the enzyme that is responsible for the nucleophilic attack on a bound CO_2 during the hydration reaction. A widely held model for enzymatic activity, originally proposed by Davis (1959), identifies this nucleophile as a zinc-bound hydroxide ion. The observed pH dependence of the catalysis (greater hydration and esterase activity at higher values of pH) is then attributed to ionization of a metal-coordinated water ligand to produce the zinc-bound hydroxide nucleophile. This model, as well as most others proposed for the activity-controlling ionizing group (cf. Pocker & Sarkanen, 1978), cannot explain the observed rate of magnetic relaxation of solvent water protons first reported by Fabry et al. (1970) (cf. Koenig & Brown, 1972). Moreover, Lindskog, a long-time proponent of the "hydroxide" mechanism, concludes a recent review by noting that "... as long as unequivocal evidence for the existence of Zn^{2+}-bound OH^- in the enzyme is lacking, this model must be continually questioned and tested against alternative models" (Lindskog, 1980).

An alternate explanation for the pH dependence of enzymatic activity was recently proposed by us (Koenig et al., 1980). In our model, there is no ionization on the enzyme that determines the observed pH-dependent activity; rather, the pH-dependent phenomenon can be explained by the existence of both active enzyme and anion-inhibited enzyme, with relative concentrations that depend on the type and concentration of monovalent anion present in solution, and on pH. The active enzyme was proposed to have a zinc-bound water ligand, capable of exchanging rapidly with solvent, that can be replaced by a monovalent anion and an associated proton from solution to produce inactive enzyme. Thus, active enzyme predominates at high pH, and anion-inhibited enzyme predominates at low pH, with the pK_a for activity being determined by the composition of the solvent.

The essence of the model is that a constant charge environment is maintained in the active site, both statically and

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dynamically. We proposed that a molecule can bind at the active site only if the charge is not altered. In those preparations in which monovalent anions are excluded, it appears that HSO_4^- anions, in equilibrium with the SO_4^{2-} generally present to maintain ionic strength, inhibit the enzyme at low pH. To test these ideas further, as well as to generalize their applicability to a wider range of phenomena, we have investigated in detail the interaction of carbonic anhydrase with small neutral molecules. We chose three compounds: aniline and phenol, aromatic and isoelectronic, which can interact with the aromatic binding site of the enzyme, and methanol, a weak inhibitor, at least of esterase activity, that presumably mimics H_2O in its mode of binding.

Very little is known about the mechanisms by which neutral molecules interact with carbonic anhydrase. Indeed, an extensive study of both hydration of CO_2 and hydrolysis of esters in the presence of neutral molecules such as methanol and phenol has not yet been reported. The inhibitory effect of neutral molecules on the esterase activity of the enzyme was first reported by Verpoorte et al. (1967) for the human enzymes, and investigated by Pocker & Stone (1968) for the bovine enzyme. More recently, Appleton & Sarkar (1975) observed that the inhibition constant for methanol decreased with increasing pH; that is, the apparent affinity of the enzyme for methanol increases with increasing pH. In addition, they reported a pK_a for inhibition by methanol of 6.4, the same as the pK_a for enzymatic activity. Both shifted to higher pH in the presence of increasing concentrations of monovalent anions. Methanol appeared to bind to enzyme in a one to one stoichiometry, competitively with monovalent anions. Aniline, too, was found to interact at a single binding site but, unlike methanol, has a greater affinity for enzyme at low pH, much like monovalent anions. However, the question of whether the neutral molecules interact by binding directly to the metal was left unanswered. Recently, Westerik et al. (1978) reported additional results for the interaction of methanol and aniline with carbonic anhydrase. Aniline was reported to bind directly to the metal of Co^{2+}-substituted enzyme, yet did not effect the water proton magnetic relaxation rate. They proposed that aniline either binds at a fifth coordination site or, alternatively, displaces one of the histidine ligands of the metal. On the basis of ^{13}C relaxation rate studies, it was claimed that methanol binds at a hydrophobic region of the enzyme ~6 Å from the metal.

In the experiments reported below, we used Co^{2+}-substituted bovine carbonic anhydrase in order to monitor changes in the active-site region. The experiments were designed to demonstrate, first, that the pH-dependent properties of the samples depend on anion concentration in the expected manner, and then to measure the alteration of these properties in the presence of aniline, phenol, or methanol. We conclude that aniline and phenol bind near, but not to, the active metal to produce a binary complex that is essentially an altered, but otherwise active (with regard to hydration and dehydration), enzyme. The functional group of the inhibitor has the effect of altering the relative affinities of water and monovalent anions (with their associated protons) for the metal. By contrast, we find that methanol competes directly with H_2O for a ligand of the metal. Our results will be compared with those of Westerik et al. (1978), and a number of apparent contradictions between the conclusions of the two reports will be resolved.

Experimental Procedures

Materials. Native bovine anhydrase, purchased from Sigma and purified by chromatography on DEAE-cellulose (Kandel et al., 1970) to yield the B enzyme (akin to the human C), was demetallized in the usual way (Lindskog & Malmström, 1962) and lyophilized after extensive dialysis against distilled water (Bertini et al., 1978). Co^{2+}-substituted enzyme in deionized water (Co^{2+}-BCA)[1] was prepared by dialysis of apoprotein against 1 mM $CoCl_2$ for 1 day and then against deionized water, with frequent changes, for 6 days at 5 °C. (The small amount of Cl^- anion introduced with the metal has negligible inhibiting effect, since the affinity of Cl^- for the active enzyme is relatively low.) The final pH, 5.4–5.7, is near the isoelectric point of the enzyme. Sample pH was measured with a Radiometer PHM65 meter and combination electrode from Microelectrodes, Inc.[2] Ultra-high purity sodium chloride and sodium sulfate were obtained from J. T. Baker and EM Laboratories, respectively. Deuterated methyl alcohol (CD_3OD) and methyl alcohol-*d* (CH_3OD) were from Sigma and Merck, respectively. All other chemicals were reagent grade.

Absorbance Measurements. Optical absorption spectra were measured at room temperature in cuvettes of 1-cm path length, using a Cary Model 14 recording spectrophotometer equipped with 0 to 0.1 and 0 to 1 optical density slide-wires. Protein concentration was computed using $A_{1cm}^{1\%} = 18$ at 280 nm. All glassware used in the titration experiments was immersed in 0.1 M EDTA solution for at least 1 day and rinsed with deionized water before use.

The pH dependence of the optical properties of the exhaustively dialyzed enzyme in the presence of chloride was measured by adding chloride as a concentrated salt solution to the dialyzed sample to give the desired anionic concentration. The pH was then adjusted with HCl, and a series of optical measurements taken as the pH was raised by the addition of NaOH.

The optical titration of Co^{2+}-BCA in the presence of aniline was done in a similar fashion. The neutral molecule was added to give a concentration of 20 mM, and a series of optical spectra taken as the pH was raised with NaOH; the same sample was subsequently used for titrations in the presence of chloride and aniline. Chloride was again added as a concentrated salt solution; the pH was lowered with HCl to a minimum pH starting point and a titration carried out by raising the pH with NaOH. Titrations were performed at 0.06 and 0.6 M chloride in the presence of 20 mM aniline.

Titrations of Co^{2+}-BCA in the presence of phenol or methanol were performed in a fashion similar to the aniline titration. Final concentrations of the individual components are given in the legends to the figures. For the experiments using CH_3OH/D_2O, pD values are the uncorrected meter readings.

The dilution of any sample at the end of a titration was always less than 1%. The fraction of active enzyme at each pH, here defined as the fraction of enzyme with the high-pH optical spectra, was calculated from the amplitude of the 640-nm peak relative to the initial plateau observed at high pH. This procedure is somewhat arbitrary, since the optical absorption does not remain perfectly pH independent at values of pH well beyond the upper (high pH) end of a particular titration. The procedure generally used was first to make a rough estimate of the pK_a of a particular titration curve, then to take the amplitude of the absorption either 1.5 or 2 pH units above the estimated pK_a as being at either 97 or 99% of the titration limit, respectively. The data were then fit to a single

[1] Abbreviation used: BCA, bovine carbonic anhydrase.

[2] The KCl flow rate from the reference electrode is guaranteed to be no greater than 0.01 μL per 24 h, thus obviating the possibility of contamination of the sample by chloride.

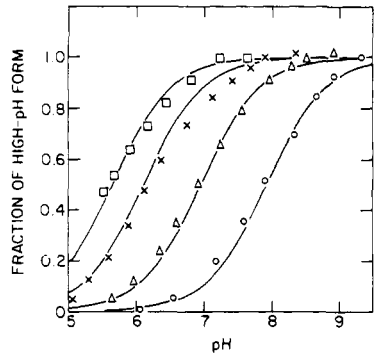

FIGURE 1: Dependence on pH of the fraction of the high-pH form of Co^{2+}-BCA, determined spectroscopically, as a function of added NaCl: (□) no added salt; (×) 0.006 M; (△) 0.06 M; (○) 0.6 M. The solid lines represent the results of a least-squares comparison of all the data points with the two-parameter model, eq 1 of the text. The respective values of pK_a are 5.6, 6.1, 6.9, and 7.9.

titration using a least-squares criteria. The uncertainty in the end point is typically in the range 5–10%, and the relative shifts in the derived values of pK_a obtained by varying the procedural details vary by no more than ±0.2 pH unit at most. Our procedure has the advantage of being well defined, and follows the protocol of our earlier experiments (Jacob et al., 1978; Koenig et al., 1980). In the phenol and methanol experiments there was no net absorbance at 640 nm in the limit of low pH. However, for the chloride and aniline titrations a correction was necessary for absorbance at 640 nm, attributable to the chloride–enzyme and (presumably) aniline–anion–enzyme complexes, respectively.

Relaxation Measurements. Longitudinal nuclear magnetic relaxation rates of water protons and CH_3OD protons, as a function of magnetic field,[3] were measured using apparatus and procedures that differed only slightly from those described previously (Hallenga & Koenig, 1976; Brown et al., 1977; Wells et al., 1979).

Results

Cl^-–Enzyme Interactions. Figure 1 shows the fraction of the total Co^{2+}-BCA that has an optical spectrum identical with that of the active enzyme,[4] as a function of pH, for extensively dialyzed samples of enzyme with no added anions, and with 0.006, 0.060, and 0.60 M NaCl. In the absence of added anions the optical transition has a pK_a of 5.6, significantly lower than other values in the literature (Lindskog, 1963, 1966; Wells et al., 1979), presumably due to the exclusion of sulfate and the inhibitory effects of HSO_4^- (Koenig et al., 1980). The solid lines through the data points result from a fit to the model in the Theory section that has two adjustable parameters, one that measures the affinity of Cl^- and a second that accounts for the residual pK_a in the absence of deliberately added anions. It should be noted that the agreement of data and theory is very good despite the significant variation in ionic strength from one sample to another; the theory does not consider possible influences of ionic strength.

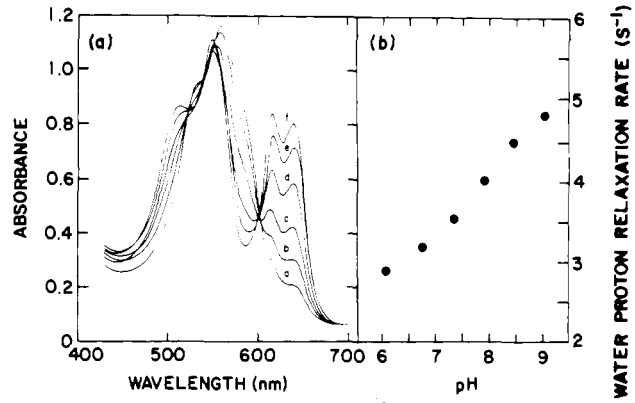

FIGURE 2: (a) Spectra of 3.9 mM Co^{2+}-BCA containing 0.03 M aniline, with no added anions, at the following pH values: (a) 6.10; (b) 6.77; (c) 7.36; (d) 7.91; (e) 8.45; (f) 9.05. (b) Plot of solvent water proton spin–lattice relaxation rate at 0.02 MHz as a function of pH for the samples used to obtain the spectral data. From other measurements, the diamagnetic contribution is 1.7 s^{-1}.

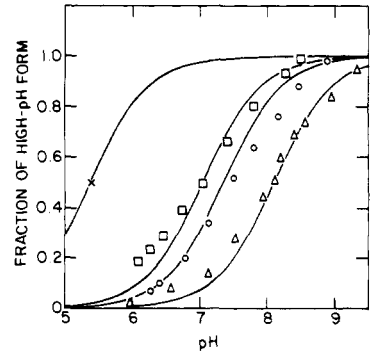

FIGURE 3: Dependence on pH of the fraction of the high-pH form of Co^{2+}-BCA, determined spectroscopically, as a function of added aniline and chloride: (×) no added aniline or chloride; (□) 0.02 M aniline; (○) 0.02 M aniline and 0.06 M Cl^-; (△) 0.02 M aniline and 0.6 M Cl^-. The solid lines represent the results of a least-squares comparison of all the data points with the model, eq 12 and 13 of the text. The respective values of pK_a for the three titrations are 7.0, 7.4, and 8.1.

Aniline–Enzyme Interactions. Figure 2a shows the effect of 30 mM aniline on the optical spectrum of a 3.9 mM Co^{2+}-BCA sample, as a function of pH. As reported by Bertini et al. (1978), the cobalt-substituted enzyme in the presence of aniline has a spectrum at low pH that is more intense than in the absence of aniline. As the pH is raised, the spectrum reverts to that of the active enzyme. Note the presence of at least one well-defined isosbestic point.

Figure 2b shows the spin–lattice relaxation rate of solvent water protons $1/T_1$ (at 0.02 MHz) in the same aniline-containing samples, as a function of pH. These rates are total observed rates, uncorrected for the diamagnetic contributions of the protein and buffer. As the pH is raised and a greater percentage of the active Co^{2+}-BCA is detected optically, the solvent proton relaxation rate also increases; qualitatively, it is clear that a strong correlation exists between the para-

[3] We measure the magnetic field intensity in units of the Larmor precession frequency of protons in that field, as well as in oersteds. For protons, the conversion is 4.26 kHz = 1 Oe = 1 G.

[4] Previous workers have used the terms "low-pH form" and "high-pH form" to identify the two spectroscopically distinguishable species of cobalt carbonic anhydrase that contribute to the optical spectra in the observable pH range. However, many authors go on to propose that the high-pH form catalyzes the hydration of CO_2, and that the low-pH form catalyzes the reverse reaction, the dehydration of bicarbonate. We find this view to be patently unacceptable on the basis of microscopic reversibility; a single active form must exist that catalyzes both reactions equally under any given set of conditions. A proton and a bicarbonate ion must be on an active enzyme molecule concurrently to be turned over to $CO_2 + H_2O$, precisely as CO_2 and H_2O must similarly find an active enzyme molecule to be converted to $H^+ + HCO_3^-$. Because the dehydration of HCO_3^- requires a proton as cosubstrate, lowering the pH produces an increase in dehydration velocity for fixed $[HCO_3^-]$. However, an active enzyme molecule with a proton cosubstrate attached should not be confused with the form of the enzyme that may exist at low pH which, in our view, is an inhibited enzyme, inactivated by monovalent anions. Throughout this paper we will identify the high-pH form with active enzyme, and the low-pH form with inactivated enzyme.

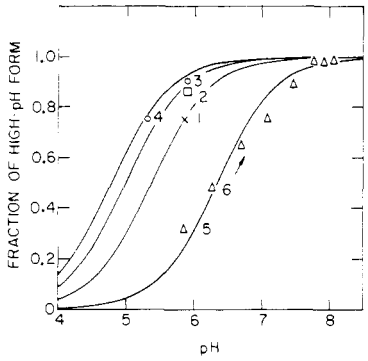

FIGURE 4: Dependence on pH of the fraction of the high-pH form of Co^{2+}-BCA, determined spectroscopically, as a function of added phenol and chloride: (×) no added phenol or chloride; (□) 0.02 M phenol; (○) 0.04 M phenol; (△) 0.04 M phenol and 0.06 M Cl^-. The numbers near the data points indicate the sequence of the experiments. The solid lines represent the results of a least-squares comparison of all the data points with the model, eq 12 and 13 of the text. The respective values of pK_a for the left- and right-most curves are 4.8 and 6.3.

magnetic contribution to $1/T_1$ and the amount of active Co^{2+}-BCA enzyme present (as determined optically). From previous measurements (Wells et al., 1979), the diamagnetic contribution is 1.7 s^{-1} and is essentially pH independent in the range considered. Thus, in the presence of aniline, there is a significant paramagnetic contribution, about 0.8 s^{-1}, to the relaxation rate of the inactivated enzyme (at low pH) that is not there in its absence.

Figure 3 illustrates the more quantitative aspects of the effects of additional aniline, in the absence and presence of Cl^-. Here the pH dependence of the fraction of Co^{2+}-BCA with an optical spectra the same as that of the active enzyme is shown for samples containing no aniline, 0.02 M aniline, 0.2 M aniline plus 0.06 M Cl^-, and 0.02 M aniline plus 0.6 M Cl^-. The sample with no additions, as prepared by dialysis, was measured at one value of pH, its isoelectric point. This single data point is shown by ×; the solid titration curve near this point is from the analogous sample of Figure 1. In the present case the dialysis resulted in a slightly lower pK_a than in the former. Addition of neutral aniline (essentially the high pH form of a buffer with a pK_a of 4.6) raised the pH to 6.1, the first point of the titration in the presence of aniline. In the absence of both aniline and Cl^-, the optical transition has a pK_a of 5.4. Addition of aniline to a final concentration of 0.02 M produces a shift of the pK_a to 7.0. Subsequent additions of 0.06 and 0.6 M Cl^- chloride produce further shifts of the pK_a to pH values of 7.4 and 8.1, respectively. It should be noted that the pK_as of the curves in the presence of aniline and chloride together are higher than the analogous curves in the absence of aniline (Figure 1) and, moreover, that for a given pK_a, the ionic strength of an aniline–chloride sample is much lower than for the corresponding chloride sample. The solid lines through the data points result from a simultaneous fit of all the data to the model for interactions of carbonic anhydrase with aromatic neutral molecules developed in the Theory section. Three parameters are involved: one, the dissociation constant of aniline from the active enzyme; the others, the dissociation constants of aniline from the anion-inhibited forms of the enzyme. A point to note here is that a consistent theoretical description of the behavior of binary and ternary complexes of enzyme with small anions and neutral molecules can be made without attention to variations of ionic strength. Ionic strength is difficult to control in a satisfactory fashion without introducing other anions whose interactions with the enzyme are uncertain. The theory ignores effects of ionic strength, which are regarded as possibly occurring only in second order.

Phenol–Enzyme Interactions. Unlike aniline, phenol produces no alteration in the optical spectrum of Co^{2+}-BCA at the extremes of the pH range investigated. Both the limiting low- and high-pH spectra are unaffected, but with increasing amounts of phenol, the pK_a shifts downward. This is seen in Figure 4, where a final concentration of 0.04 M phenol lowers the optically derived pK_a to 4.8. Such behavior differs from that of aniline and indeed from any small molecule or ion studied to date. (In contrast to addition of aniline, addition of phenol does not alter the pH, since the pH of 5.85 is so far from the pK_a = 10 of phenol). Subsequent addition of 0.06 M chloride to the 0.04 M phenol sample raises the optical pK_a to 6.3, a value lower than the corresponding value of 6.9 for a sample in the absence of added aromatic neutral molecules, Figure 1. The solid line near the data point for the initial sample, once again from Figure 1, is included as a reference curve. The other solid lines through the data points result again from a least-squares fit of all the data in the figure to the model in the Theory section for the phenol–carbonic anhydrase interaction. As in the case of aniline, three parameters are involved.

Methanol–Enzyme Interactions. Initial experiments with undeuterated samples showed that 1 and 2 M added methanol shifted the (optically derived) pK_a for activity less than 0.1 pH unit. Figure 5a shows the optical spectra of Co^{2+}-BCA as a function of pH in the presence of 9% (by volume) CH_3OD in D_2O solvent, corresponding to 2.2 M methanol. The sample, as freshly prepared, was at pD 6.73. To start the titration experiment, concentrated D_2SO_4 was added to lower the pD

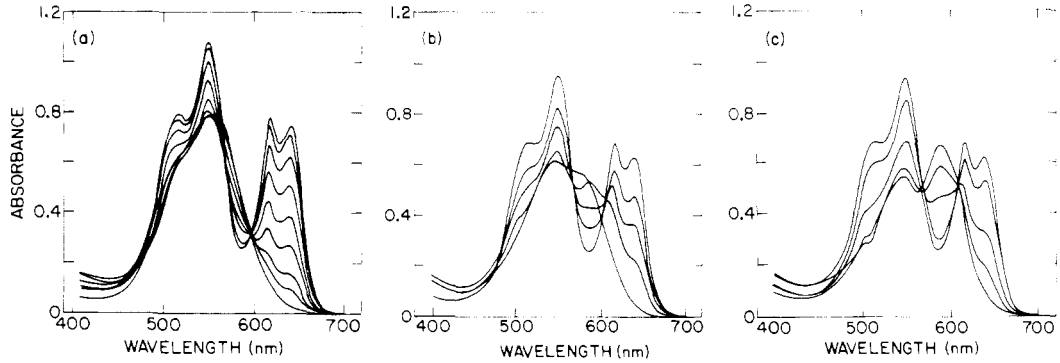

FIGURE 5: (a) Spectra of 2.8 mM Co^{2+}-BCA in 9% CH_3OD and 91% D_2O, with no added anions, at the following pD values (uncorrected meter readings), reading from the lowest to the topmost curve near 625 nm: 5.11, 5.51, 5.77, 6.06, 6.20, 6.42, 6.83, 7.26, and 7.84. (b) Spectra with 0.06 M NaCl added, at the following pD values: 5.29, 6.39, 6.87, 7.20, and 7.83. (c) Spectra with 0.6 M NaCl added, at the following pD values: 6.11, 6.96, 7.54, 8.23, and 8.69.

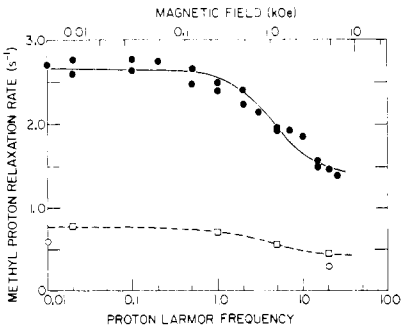

FIGURE 6: Relaxation dispersion data for solvent methyl protons for 2.8 mM Co^{2+}-BCA in 9% CH_3OD and 91% D_2O (2.2 M methanol): (●) at pD 6.73; (□) at pD 5.11. Two points (○) are shown for Zn^{2+}-BCA at pD 5.74. The solid line through the upper points results from a least-squares comparison of the data with relaxation theory (see text).

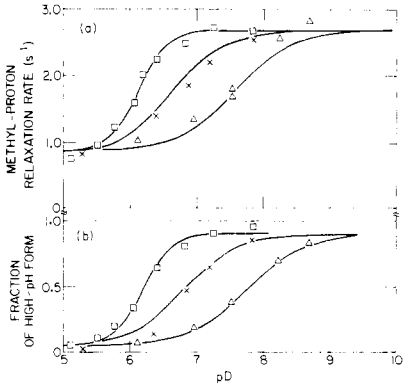

FIGURE 7: (a) Dependence on pH of the solvent CH_3OD proton spin–lattice relaxation rate at 0.02 MHz for 2.8 mM Co^{2+}-BCA in 9% CH_3OD and 91% D_2O (2.2 M methanol): (□) no added salt; (×) plus 0.06 M Cl^-; (△) plus 0.6 M Cl^-. (b) Dependence of the fraction of the high-pH form of 2.8 mM Co^{2+}-BCA on pH, determined spectroscopically, for the same samples as in a, derived from the data in Figures 5a–c. The solid lines are analogous to a, with pK_a values of 6.1, 6.8, and 7.8. The steeper rise of the left-most of the curves in both a and b relates to the titration of $[DSO_4^-]$, the only added monovalent anion in these samples.

to 5.11, at which point there was essentially no high-pH fraction detectable. Figures 5b and 5c show similar data for the same sample with 0.06 and 0.6 M added NaCl; the sample was brought back each time to low pD by the addition of D_2SO_4 in amounts too small to influence the pK_a of the samples under the particular conditions. The high concentration of methanol used was chosen because previous data indicate that the addition of 1 M alcohol is needed to halve the esterase activity at pH values above 7 (Pocker & Stone, 1968; Westerik et al., 1978). The deuterated solvent is used in anticipation of the measurements of magnetic relaxation of the methyl protons of methanol, reported below.

The spectra in Figure 5a are essentially the same as those observed in the absence of methanol, in agreement with the report by Westerik et al. (1978) that addition of methanol does not alter the optical spectra of the Co^{2+}-substituted enzyme at high pH. Both parts a and c of Figure 5, with no added Cl^- and with a high concentration of Cl^-, respectively, show clear isosbestic points (indicating the presence of only two optically distinct species in varying proportions) over the pH range considered. These are the high-pH form, and either the residual anion–enzyme complex, or the Cl^--enzyme complex, in our view. This is in contrast to Figure 5b, for an intermediate concentration of Cl^-, which has no such points, and thus represents a mixture of all three species in significant concentrations.

The samples used for obtaining the optical spectra were also used to measure the magnetic field dependence (dispersion) of the spin–lattice relaxation rate of the methyl protons of methanol. This is one of the few ways of investigating the possible binding of methanol, since the optical data show no distinctive methanol–enzyme spectrum. Though this is the first report of such dispersion measurements for other than water nuclei, the experiments are completely analogous to those for solvent water protons (Wells et al., 1979) and for water deuterons (Koenig et al., 1978) in solutions of Co^{2+}-substituted carbonic anhydrase. Figure 6 shows dispersion data, from which the paramagnetic contribution to the relaxation can be obtained. From the intensity of the proton signal before and after addition of methanol, it can be calculated that over 90% of the signal intensity is due to methanol methyl protons. This was substantiated from the methyl and hydroxyl proton signals observed on a 100-MHz high-resolution spectrometer. The sample for Figure 6 was at pD 6.73, which, from the titration data (Figures 7a,b below), is in the high-pH regime. Also shown in Figure 6 are several points for the same sample at pD 5.11, the low-pH regime, and two points for a comparable Zn-BCA sample at pD 5.74. From these dispersion data, and application of the usual (simplified) Solomon–Bloembergen–Morgan theory (cf. Koenig, 1978), a value of 8.6×10^{-11} s for the correlation time for the interaction is obtained (a shorter value of 1.2×10^{-11} s, based on the early data of Fabry et al. (1970) for Co^{2+}-BCA in the absence of methanol, had to be assumed by Westerik et al. (1978)), as well as values for the mean distance $\langle r \rangle$ (suitably defined) of the methyl protons from the Co^{2+} ion when methanol is bound to enzyme, and the residence time τ_M of the methanol. The solid line through the upper data points, Figure 6, illustrates the quality of the least-squares fit of the theory, incorporating the above assumptions, to the data. Absolute values for $\langle r \rangle$ and τ_M can only be obtained if the dissociation constant for the methanol–enzyme complex is known, whereas this information is not needed to obtain the correlation time, or the fit itself. This point is discussed extensively below.

Figure 7a shows the titration of the low-field values of the total methanol proton relaxation rates of the samples used for the spectra shown in Figures 5a–c as a function of pH, while Figure 7b shows the optical titration of these samples, in terms of the fraction of enzyme with high-pH optical spectra. The solid lines through the data result from a least-squares comparison of the data with the theory developed below, as will be discussed.

Ancillary Experiments. Measurements of the solvent water proton relaxation rates were made for solutions of Co^{2+}-substituted carbonic anhydrase with either added phenol or methanol, as well as aniline, motivated in part by the report of Westerik et al. (1978) with whose interpretations we disagree (see below). One observation is that solvent water relaxation is essentially unaffected by the presence of high concentrations of methanol in the high-pH region (as judged optically). However, it cannot be deduced from these results whether protein-bound water is or is not displaced by methanol, since the exchangeable methanol proton can be relaxed by the Co^{2+} ions, and this magnetization change could be conveyed to the solvent proton reservoir by proton exchange in a time short compared to the observed relaxation time, much as suggested below for aniline at low pH. Thus, little quantitative information can be obtained from such measurements without a more extensive study.

Data were also obtained for the influence of added CN^- on the relaxation of the methyl protons of methanol. We find

that addition of 2 mM KCN to a 1.9 mM solution of Co^{2+}-substituted carbonic anhydrase in 9% CH_3OD in D_2O solvent eliminates the paramagnetic contribution to the methyl proton relaxation, and eliminates the peak at 640 nm that characterizes the high-pH optical spectrum, for values of pH < 8. Both attributes return with increasing pH, with a $pK_a \simeq 11$, as best as can be estimated. Moreover, the optical spectra show the usual isosbestic points over this range. Thus, once again the relaxation of methyl protons correlates with the optical spectra; when the spectra is that of the active enzyme (high-pH limiting form), the paramagnetic contribution to the relaxation is maximum, and when the spectra is that of the low-pH limiting form, the relaxation is minimum.

Similar results are obtained for solvent proton relaxation in the presence of enzyme and either aniline or phenol. The correlation of relaxation rate with activity, as defined by the titration of the optical spectra, is again observed, and is independent of the pK_a, as was found earlier for enzyme inhibited by various concentrations of anions (Jacob et al., 1978; Koenig et al., 1980).

Finally, measurements were made of the relaxation of methanol methyl protons as a function of methanol concentration in the range 0.5–2.5 M methanol. The purpose was to measure the dissociation constant of methanol. The results were confounded by the (known) dependence of thermodynamic activity of methanol on concentration (cf. Moelwyn-Hughes, 1964). Attempts were made to simplify the problem by measurements of relaxation caused by Mn^{2+} ions in solutions of CH_3OD/D_2O in various proportions. No results helpful in the interpretation of the present work were obtained.

Theory

In a recent communication (Koenig et al., 1980), we presented a model for the activity of carbonic anhydrase and its inhibition by monovalent anions. The essence of the model is that, over the pH range of interest (about 6–10), there is a single active form of the enzyme that can be inhibited by monovalent anions, and that the net charge in the region of the active-site metal ion is maintained upon ligand exchange. Either there is a water molecule ligand on the metal ion, corresponding to the active enzyme, or else there is a monovalent anion ligand with an associated proton that resides on a nearby proton acceptor, producing an inactivated or inhibited enzyme. Because of the restrictions of charge neutrality, divalent anions, for example, do not bind. The bound proton–anion pair may be regarded as competing with the water molecule ligand of the metal ion in the active enzyme, or the anion may be regarded, in a formal sense, as competing with the OH^- moiety of the water molecule. In either view, the binding of anions will be greater at lower values of pH, and the pH dependence of the fraction, f, of active enzyme can be readily derived (cf. Koenig et al., 1980):

$$f = \frac{1}{1 + [H^+]/K_{Aeff} + [H^+]/K_0} \quad (1)$$

where

$$K_{Aeff} = K_A/[A^-] \quad (2)$$

The pH- and concentration-independent constant K_A is a measure of the relative competition between the particular proton–anion pair and water for the available coordination site of the metal ion. K_{Aeff} characterizes the pH dependence of the activity of the enzyme for a given concentration of anionic inhibitor A^-. For an exhaustively dialyzed Co^{2+}-BCA sample in the absence of deliberately added anion, the titration exhibits a pK_a value in the range of 5.4 to 5.7 that we account for by the empirical constant K_0, which may be due to residual anions or may be the true pK_a of the proton acceptor in the active site; it is sufficiently low so that it is of little concern to the present considerations.

The attraction of this new model is that it affords a totally consistent interpretation of all data published on bovine carbonic anhydrase of which we are aware; in particular, it is the only model for enzyme action that can explain the results of solvent nuclear magnetic relaxation (Fabry et al., 1970) in a natural way, e.g., without invoking an unusual ligand configuration for the metal ion at the active site (cf. Silverman et al., 1979).

We now extend the model to include interactions of carbonic anhydrase with neutral molecules, in both the presence and absence of monovalent anions. These neutral molecules are of two classes: the first are molecules that are competitive with water and anion–proton pairs for the metal–ion ligand. We have in mind molecules such as methanol and ethanol that are chemically very much like water; it remains to argue, however, whether these particular molecules should be included in this class. The second class consists of neutral molecules, which are small and aromatic, with highly polar moieties, and are assumed to bind at the aromatic binding site with which, for example, the sulfonamide inhibitors interact. These molecules are not to be so large that they displace ligands of the metal ion as do the sulfonamides; however, they might interact with these ligands and alter the values of K_{Aeff}, eq 1. A binary complex of carbonic anhydrase with this second class of neutral molecule may have an altered pK_a for hydration–dehydration activity. However, such molecules would be competitive inhibitors of, for example, esterase activity for aromatic ester substrates. We believe that aniline, imidazole, phenol, and similar (neutral) molecules belong to this second class of molecules, but again it remains to be argued from the data that this is so for these particular molecules. We first develop the implications of the model for the two classes of neutral molecules, which we will call "waterlike" and "aromatic", and then compare the implications of the model with the foregoing data.

Interactions with Waterlike Neutral Molecules. When considering these molecules it becomes necessary to refine the definition of f, eq 1. Replacement of a water ligand of the Co^{2+} of Co^{2+}-BCA by a waterlike neutral molecule will, according to the model, eliminate enzymatic activity and (in principle) the paramagnetic contribution to solvent water proton relaxation. However, it will not grossly alter the ionicity of the bond and therefore will not alter the optical spectra at high pH (Koenig et al., 1980). Thus, we define three quantities, f_{spec}, f_{enz}, and f_{rel} for the fraction of "active" enzyme as measured spectrometrically, by enzymatic activity, and by magnetic relaxation. f_{spec} will be determined by molecules with the same optical spectra as the active enzyme, which includes active enzyme and enzyme with this class of bound neutrals. Thus, according to the model, $f_{spec} > f_{enz} = f_{rel}$. Specifically, we define

$$[EH^+A^-]/[EH_2O] \equiv [H^+][A^-]/K_A \quad (3)$$

and

$$[EW]/[EH_2O] \equiv [W]/K_w \quad (4)$$

where $[EH^+A^-]$, $[EW]$, and $[EH_2O]$ are the concentrations of anion-inhibited, waterlike inhibited, and active enzyme, respectively, $[W]$ is the concentration of waterlike neutral molecules in solution, and K_A and K_W are dissociation constants. It is straightforward to derive the expressions

$$f_{enz} = f_{rel} = \frac{1}{\left[1 + \frac{[W]}{K_W} + \frac{[H^+][A^-]}{K_A} + \frac{[H^+]}{K_0}\right]} \quad (5)$$

and

$$f_{spec} = (1 + ([W]/K_W))f_{enz} \quad (6)$$

These expressions are valid in the limit of negligible substrate binding, and implicitly assume that the neutral molecule has a significantly greater affinity for the metal than does water, since changes in the thermodynamic activity of water upon addition of, for example, methanol are neglected. It follows from the form of the denominator in eq 5 that, for any method of observing enzymatic activity, there will be a decrease in the observed pK_a upon addition of W that is given by

$$\Delta pK_a = -\log (1 + [W]/K_W) \quad (7)$$

Essentially, ions will not bind as well in the presence of these waterlike neutral molecules because of the (assumed) greater affinity of the latter for the metal compared to that of water. Concomitant with the decrease in pK_a will be an apparent pH-dependent dissociation constant for anions in the presence of W given by

$$K_A^{(W)} = K_A(1 + [W]/K_W) \quad (8)$$

Additionally, if one measures the apparent value of K_W as a function of pH by activity measurements, for example, by measuring the value of [W] that decreases enzymatic activity by a fixed factor, then one will obtain an effective dissociation constant for the waterlike neutral molecules given by

$$K_{Weff} = K_W\left[1 + \frac{[H^+]}{K_{Aeff}} + \frac{[H^+]}{K_0}\right] \quad (9)$$

That is, the dissociation constant of the neutral molecule (a true inhibitor, in this case) will titrate with a pK_a precisely equal to that of the activity of the enzyme.

Interactions with Aromatic Molecules. The algebraic expressions that describe the titration behavior of the enzyme in the presence of neutral aromatic molecules are somewhat more complex than the above, but nonetheless straightforward to derive. One must consider not only the dissociation constant K_{Ar} of the neutral aromatic molecules from the active enzyme, but also from the anion-inactivated enzyme, $K_{Ar}^{(A)}$. Moreover, the value of $K_{Ar}^{(A)}$ can be (and is found to be, as argued below) anion specific. Because of this, it is convenient to define the factors q_A and q_0 such that

$$K_{Ar}^{(A)} = q_A K_{Ar} \quad (10)$$

$$K_{Ar}^{(0)} = q_0 K_{Ar} \quad (11)$$

Then q_0 determines the shift of the pK_a of the enzyme in the absence of deliberately added monovalent anion but in the presence of aromatic neutral molecules, and q_A describes the relative dissociation of the aromatic neutral molecules from the anion-inhibited enzyme and the active enzyme. (A value of q_0 much different from 1 would strongly suggest that indeed K_0 is determined by an external anion and not an intrinsic pK_a.) The results, analogous to eq 5 and 6, are

$$f_{rel} = 1/[1 + [H^+]/K_0\{1 + [Ar]/q_0K_{Ar}/1 + [Ar]/K_{Ar}\} + [H^+][A^-]/K_A\{1 + [Ar]/q_AK_{Ar}/1 + [Ar]/K_{Ar}\}] \quad (12)$$

if binding of Ar to the active enzyme does not alter the water off-rate too drastically, and

$$f_{spec} = f_{enz} = f_{rel} \quad (13)$$

if binding of Ar to the active enzyme neither alters the spectra nor the enzymatic activity (by competing with substrate, for example).

In all cases, the shift of pK_a upon adding aromatic molecules when $[A^-] = 0$ is given by

$$\Delta pK_a = \log\left\{\frac{1 + ([Ar]/q_0K_{Ar})}{1 + ([Ar]/K_{Ar})}\right\} \quad (14)$$

and may be readily generalized to the case when $[A^-] \neq 0$. The sign of the shift depends upon whether the neutrals bind better to the active or inactivated enzyme; if $q_0 < 1$, the dissociation is less from the inactivated enzyme and the sign of ΔpK_a is positive (i.e., the pK_a rises upon addition of neutrals), and if $q_0 > 1$, the reverse is true.

Comparison of Data and Theory

Binding of Cl^-. The solid lines in Figure 1 result from a least-squares comparison of the data with eq 1; the values obtained are $K_0 = 2.5 \times 10^{-6}$ M and K_{Cl^-} ($\equiv K_A$ for chloride) $= 7.2 \times 10^{-9}$ M. The latter is an averaged best fit to *all* the data, rather than a separate fit for each titration. The variation of ionic strength among samples is ignored, since we have not found any results that correlate (to first order) with ionic strength, which we therefore consider of secondary importance.

Binding of Aniline. Aniline is neutral throughout the pH range of Figure 3; it becomes positively charged at lower values of pH, with a pK_a of 4.60. The solid lines through the data points are from a least-squares comparison of the data with eq 12 and 13. The value of K_0 is taken from the fit relating to Figure 1. The results for the binding of aniline to the active enzyme and to the inactivated forms of the enzyme are described by, respectively, K_A ($\equiv K_{Ar}$ for aniline) = 0.02 M, $q_A = 0.57$, and $q_0 = 0.011$. Note that as indicated under Theory, $q_0 < 1$, consistent with the upward shift of the pK_a upon binding of aniline in the absence of added anions.

Binding of Phenol. Phenol is isoelectronic with aniline, and is also neutral in the pH range considered here. It ionizes and loses its proton, to become negatively charged, with a pK_a of 9.98. Once again the solid lines, Figure 4, result from a comparison of data and eq 12 and 13. The results are K_{Ph} ($\equiv K_{Ar}$ for phenol) = 0.014 M and $q_A = q_0 = 530$. Note that $q_0 > 1$ for phenol, corresponding to a downward shift of the pK_a for activity, in contrast to the results for aniline.

Binding of Methanol. We consider methanol to be waterlike and, therefore, to compete with water for the available metal–ligand. The solid lines in Figures 7a,b result from a comparison of the data with eq 5, which incorporates this view. The optical data and relaxation data are fit independently, but in each case all data of one type are used to derive values of K_{Cl^-} and K_0, or, more correctly, a value for these parameters divided by $D \equiv (1 + ([W]/K_W))$, since K_W is unknown. Only a value for the apparent inhibiton of esterase activity by methanol is known; however, as we show below, this is in reality a solvent effect unrelated to the binding of methanol. The values for K_{Cl^-}/D from the relaxation and optical data, respectively, are 1.5×10^{-8} and 0.99×10^{-8} M^2. K_0 we regard as resulting from the presence of DSO_4^-, in turn due to the D_2SO_4 added to lower the pH at the start of the experiments. The sharper titration of the leftmost curve in each instance results from this assumption, a point consistent with these data and with earlier results (Koenig et al., 1980). The values

derived for $K_{DSO_4^-}$ are 1.3×10^{-12} and 1.8×10^{-12} M^2, respectively.

The results for K_{Cl^-}/D compare reasonably with that obtained from the data of Figure 1 (7.2×10^{-9} M^2), and the value for $K_{DSO_4^-}/D$ compares well with the average 1.6×10^{-12} M^2 obtained earlier (Koenig et al., 1980). A quantitative comparison requires knowledge and incorporation of possible alterations of charge in the active-site region due to deuteration, something that we have not considered. Nonetheless, the agreement for both anions is sufficiently close to suggest that $[W]/K_W$ is small for the conditions used, even though [W] = 2.2 M. The derived values of pK_a for the several curves are 6.1, 6.6, and 7.6 for the relaxation data, and 6.2, 6.8, and 7.8 for the optical data. Within the uncertainty of the data themselves, and the method of data reduction, these sets of pK_a values can be considered the same.

It remains to derive values for the parameters of the theory of relaxation from the fit to the dispersion data for methanol methyl protons, Figure 6. This also requires knowledge of K_W for the methanol–enzyme complex, since the ratio of the concentrations of free to bound methanol enters the equations that describe the relaxation. (These equations appear throughout the literature on the subject, and will not be repeated here; cf. Koenig (1978).) We assume for the moment, and argue the point in the Discussion, that the affinities of methanol and water for the metal ion are about equal, i.e., that methanol is so "waterlike" that the metal ion does not distinguish significantly between methanol and water. This corresponds to $K_W \simeq 28$ M. The solid line, Figure 6, through the upper data points results from a least-squares comparison of the data and theory. (The diamagnetic correction was first subtracted from the data, a fit made to the net paramagnetic contribution, and the diamagnetic part then added back to give the curve shown.) The form of the fit does not require knowledge of K_W and gives the correlation time τ_c (field independent, in this case) of 7.5×10^{-11} s. Using $K_W = 28$ M gives $\langle r \rangle = 3.2$ Å and $\tau_M = 16$ μs.

Discussion

The nature of the binding of small molecules to the active region of carbonic anhydrase is of interest not only for the understanding of inhibition of enzymatic activity at the molecular level; in a larger sense, such understanding can help clarify the mechanisms of enzymatic action, a question that is currently the subject of much contention (cf. Lindskog, 1980). We have recently proposed (Koenig et al., 1980) a model for the active site of carbonic anhydrase that accounts for, in a straightforward and unambiguous manner, the pH dependence of a wide variety of observations: hydration and dehydration activity, whether measured by traditional stopped-flow methods, line widths of ^{13}C NMR resonances of labeled substrate (Koenig et al., 1974; Simonsson et al., 1979), or isotope loss and mixing (Silverman & Tu, 1976; Silverman et al., 1979); anion binding and the associated changes in the optical spectra of the Co^{2+}-substituted enzymes (Lindskog, 1966); complexiметric titration upon addition of anions (Coleman, 1967); and (for the first time) magnetic relaxation of solvent protons in solutions of the Co^{2+}-substituted enzyme. Though the model was based on data obtained for the bovine enzyme, it applies equally well to the very similar human C enzyme and, though not obviously without some rethinking, to the human B enzyme. Our intention in the current work was to obtain data for the interaction of (bovine) carbonic anhydrase with small neutral molecules, and to extend or elaborate the model in a natural way to include any new findings.

It is generally agreed that small anions, e.g., Cl^-, CN^-, etc., inhibit by directly binding to the essential metal ion of the enzyme. This has been shown to be the case for Cl^- (Ward & Fritz, 1970), CN^- (Feeney et al., 1973), F^- (Taylor et al., 1971), and acetate (Lanir & Navon, 1974; Bertini et al., 1976). Sulfonamides, potent inhibitors of carbonic anhydrase that are neutral in solution below about pH 10, are also known to bind to the metal ion of the active site (Liljas et al., 1972). However, these inhibitors have been shown by a number of physical methods to be in the ionized RSO_2NH^- form when bound (Chen & Kernohan, 1967; King & Burgen, 1970; Kumar et al., 1976). Indeed, there is as yet no established case, backed by crystallographic and biophysical evidence, of a molecule in the neutral state binding at or near the position of the exchangeable ligand site to the metal ion of bovine carbonic anhydrase, other than (we assert) water itself. (However, associated with the binding of anions is the uptake of a proton that, on our model, resides on a proton acceptor in the active site near the metal ion.)

A number of neutral molecules (other than the sulfonamides) have been shown to be weak inhibitors of esterase activity of both bovine carbonic anhydrase (Pocker & Stone, 1968) and human carbonic anhydrase C (Verpoorte et al., 1967). Of these inhibitors (inhibitors in a strictly formal or operational sense, namely, that their addition to a sample reduces the observed enzymatic activity), those that have an aromatic group, such as phenol and aniline, are significantly stronger inhibitors than the alcohols. Since phenol is a product of hydrolysis of phenyl acetate, it is reasonable to expect that it acts as an inhibitor by competing directly with free ester for the hydrophobic binding site. Phenol, aniline, and by extension imidazole and other aromatic neutrals then need not interact directly with the essential metal of BCA to inhibit esterase activity. The question of whether these neutral molecules inhibit or influence hydrase activity has not yet been answered, but if they belong to the proposed class of molecules that bind at the aromatic site, they would not. On the other hand, waterlike molecules such as ethanol and methanol, as a class, would not bind to the aromatic binding site of carbonic anhydrase and, indeed, though they inhibit esterase activity, the formal inhibition is 100-fold weaker than for the aromatic neutrals, corresponding to values of $K_I \simeq 2$ M. However, weak as this is, an inhibition constant of this magnitude corresponds to very successful (and unlikely) competition if the waterlike molecules are regarded as competing with 56 M solvent water for a ligand of the metal. These considerations lead us to the following proposals to account for the interaction of small neutral molecules with bovine carbonic anhydrase.

We extend our earlier views of carbonic anhydrase by including the binding of two classes of small neutral molecules. We assert that phenol and aniline belong to the earlier defined class of small aromatic neutral molecules that bind to the aromatic binding site of the enzyme and interact with, but do not displace, the ligand of the metal involved in activity and anion inhibition. By contrast, we consider that methanol, ethanol, and similar molecules compete with water and small monovalent anions for the same ligand of the metal ion at the active site. Arguments for this view, including predictions of the model, follow.

Aniline, Phenol, and Similar Molecules. Though aniline and phenol are often mentioned as inhibitors of carbonic anhydrase activity, they have only been studied with regard to enzymatic hydrolysis of aromatic esters. Additionally, judging from the binding of the sulfonamides to demetallized carbonic anhydrase by their aromatic moieties (King & Burgen, 1976),

one would also expect aromatic esters, as well as aniline and phenol, to bind similarly. Thus, it is not unexpected (certainly, with hindsight) that aniline and phenol are inhibitors of esterase activity. However, their influence on the hydration of CO_2 and dehydration of HCO_3^-, we predict, must be of another sort. Since aniline and phenol are isoelectronic, have singly-substituted phenyl groups, and very similar dissociation constants, one would expect a great deal of similarity in the way they bind, yet our study points up an interesting difference: they shift the optical pK_a for the Co^{2+}-substituted bovine enzyme in opposite directions. Since the value of the pK_a is determined by the relative binding of anion–proton pairs and water to the same ligand of the metal ion, it must be that aniline and phenol influence this relative binding in an opposite fashion. This is understandable as follows. The OH and NH_2 moieties of neutral phenol and aniline differ mainly in that the OH is a poor proton acceptor but readily forms hydrogen bonds, whereas the converse is true for the NH_2 group. The phenol–enzyme complex would then tend to favor water on the metal, by hydrogen-bond formation, rather than anions and thereby have a lower optical pK_a than the enzyme itself; by contrast, the hydrogen-accepting properties of the NH_2 would cause the aniline complex to favor anion binding to the metal and thereby raise the optical pK_a. We would predict the analogous influence on the hydrase activity of the enzyme, something that can and should be measured.

Furthermore, since the interaction of bound phenol is mainly with the liganded water (on this view), the shift of pK_a should be independent of the anion that is responsible for the pK_a. Thus, the shift of pK_a, in the absence of intentionally added anions, should be the same as the shift of the pK_a in the presence of, say, chloride. In the language of the model, this implies that $q_A = q_0$, as indeed was found in the fit of the data shown in Figure 4.

By contrast, since the interaction of the bound aniline is mainly with the anion–proton pair, the interaction would be expected to be anion specific. Again, this is as found in the comparison of the model theory with the data, Figure 3. The ratio $q_A/q_0 = 51$, indicating that the interaction of the aniline–enzyme complex with the (presumed) adventitious anion that produces pK_a is 51-fold stronger than with Cl^-. We suspect that this anion is HSO_4^-, or something of a similar size (conceivably HCO_3^-), in which case the anion–proton–aniline geometry would simulate that of a bound sulfonamide, for which the enzyme has a strong affinity.

The conclusion that aniline binds best to the anion-inhibited enzyme may afford an explanation for the excess relaxation contribution, observed as a relatively high value for the relaxation rates at low pH, Figure 2b. The NH_2 protons of bound aniline would be relaxed by the Co^{2+} ion, and the exchange of these aniline molecules with solution would convey this loss of magnetization to the solvent protons by proton exchange, all of which can occur rapidly compared to the magnitude of T_1.

The measured dissociation constants for aniline and phenol from the active bovine enzyme, 20 and 14 mM, respectively, though they are not very different from the values 28 and 30 mM obtained by Pocker & Stone (1968) from studies of the inhibition of esterase activity, cannot be directly compared with these since they are in fact measures of different quantities. However, they can be compared with the influence of (neutral) imidazole on the off-rate of water as obtained by Silverman et al. (1979) from measurements of carbonic anhydrase catalyzed isotope exchange between labeled CO_2 and HCO_3^-. Imidazole, as well as lutidine, apparently labilize the water ligand of the metal ion, increasing its off-rate by an order of magnitude; they find a value of about 2 mM (their Figure 5) for 50% effect. Though imidazole and lutidine are used as buffers, and these authors implicitly regard their influence as due to buffer effects, we suggest that the effect is due to binding of the neutral forms of the buffers to the aromatic binding sites of the (human C) enzyme. We would attribute the effect to an increase in the hydrophobic character of the active site upon binding of buffer, with a concomitant labilization of the water bound to the metal ion. We would expect a similar influence upon binding of other small aromatic molecules, and would urge that these measurements be done as a test of our ideas.

In summary, aniline and phenol can simply be viewed as reacting with enzyme at a hydrophobic site to produce binary complexes that have altered relative affinities for monovalent anions and water. The phenol interacts predominately with water via hydrogen-bond formation, whereas aniline interacts predominately with the anions in an ion-specific fashion. This view explains the opposite direction of the shift of pK_a upon complex formation, and is also consistent with the observation that binding of phenol does not alter the optical properties of the Co^{2+}-substituted enzyme, whereas aniline influences the ionicity of the metal–ion–anion bond, and thereby the optical spectra of the anion–cobalt complex. These results imply that aromatic buffers such as imidazole and lutidine, often used to avoid anionic effects, could well be interacting with enzyme to form complexes with altered enzymatic properties.

Methanol Binding. The view that methanol, very similar to water both in its size and its chemistry, competes with water for the same ligand of the metal ion is an appealing one, and one that is consistent with our data. However, there are some subtleties involved regarding the dissociation constant of methanol that must be considered. The titration of the relaxation rate of the methyl protons, Figure 7a, certainly indicates that methanol behaves very much like water: it is relaxed well at high pH when, in our model, it is a ligand of the metal and is not relaxed at all (by paramagnetic interactions) at low pH when it is displaced by Cl^-. It was also shown above that addition of CN^- eliminates the paramagnetic part of the relaxation. If the methanol were not in the first coordination sphere of the metal, and the relaxation were due to outer-sphere effects, then the relaxation rate of the methyl protons would not titrate unless some additional mechanisms were invoked; as examples, the magnetic properties of the cobalt could vary with pH, or the access of methanol to the outer-sphere region could depend on the liganding of the metal ion. We regard these possibilities as unlikely, and indeed unnecessary, assumptions.

The titration of the optical data, Figure 5, shows that the presence of methanol at a pH at which the methyl protons are relaxed does not alter the optical spectra. This would be expected on our model because methanol, when bound, should not alter the nature of the metal–oxygen bond. On the other hand, it could also be that only a small fraction of the metal ion sites contain methanol when 2 M methanol is in the solvent, so that the spectra remain dominated by that of the active enzyme. We favor this latter view, despite contradictory inferences that may be drawn from the results of the effect of methanol on the esterase activity (using *p*-nitrophenyl acetate) of carbonic anhydrase.

Both Pocker & Stone (1968) and Westerik et al. (1978) show that $\sim$1 M alcohol reduces the esterase reaction velocity at values of pH above 7 by a factor of two. However, since the substrate is poorly soluble in water (typically, acetone or

acetonitrile is added to solubilize the substrate), only the ratio (k_{cat}/K_M) can be measured for these preparations, and not the value of K_M itself. There is little question that addition of ~1 M alcohol halves this ratio. However, we submit that the influence is not on k_{cat} but on K_M; the substrate dissolves well in methanol and poorly in water (as is readily seen in standard tables of solubilities), so that K_M, essentially the equilibrium dissociation constant of the enzyme–ester complex in this case, increases upon addition of methanol to the solvent. It is straightforward to show that, in the range where the alteration of K_M is linear in [Me], the form of a Dixon plot (cf. Figure 1 of Westerik et al., 1978) will be identical in form with that which results from competitive inhibition. The apparent K_I is then identified with the concentration of alcohol that increases K_M for the ester twofold, and will be very sensitive to the composition of the solvent. (This effect is akin to that reported by Steiner & Lindskog (1972); they found that esterase activity is enhanced upon addition of salt, because of the decreased solubility of the ester.)

This view is further substantiated by the relaxation data. The dispersion data, Figure 6, from which an estimate of a Co^{2+}-methyl proton distance can be obtained, yields the following self-consistent set of results. We assumed, as a start, that methanol and water bind equally well to tetrahedral Co^{2+} in carbonic anhydrase. Then the theoretical curve, Figure 6, gave a Co^{2+}–methyl proton distance $\langle r \rangle$ of 3.2 Å and a residence lifetime τ_M of 16 μs for the methanol molecule. The extremal Co^{2+}–methyl proton separations are 3 and 3.8 Å for a bound methanol, and the residence lifetime of a water molecule under similar conditions of buffer is about 6 μs (S. H. Koenig and R. D. Brown, unpublished results). Thus, if the affinities of water and methanol for tetrahedral Co^{2+} in carbonic anhydrase are assumed to be the same, then the derived value of $\langle r \rangle$ is completely reasonable, and the methanol off-rate is close to the water off-rate. This implies that the on-rates are similar as well, completing the circular argument that assumed that water and methanol behave almost identically in the present instance. A more refined result, assuming that methanol binds precisely half as well as D_2O, gives $\langle r \rangle$ = 3.3 Å and τ_M = 7.9 μs.

The same results can be obtained from the relaxation of ^{13}C in labeled CH_3OH, as measured by Westerik et al. (1978). They derive a value of 6 Å for the separation of the ^{13}C from the Co^{2+} ion in the substituted bovine enzyme using, however, the value K_W = 1 M. If the value 28 M is used instead, the distance will be reduced by the factor $28^{-1/6}$ = 0.57 to give 3.4 Å, in agreement with our results from methyl proton relaxation. (The metal ion–^{13}C distance is essentially the same as the averaged proton distance.)

Luz & Meiboom (1964) measured the relative affinities of methanol and water for the (octahedrally coordinated) Co^{2+} aquo ion, and found that the preference is about 50-fold in favor of water at −60 °C. However, with an expected activation energy on the order of 10 kcal mol^{-1} for the relative affinities, the preference would vanish before room temperature was reached, a result consistent with our interpretations. Certainly, it appears unlikely that the affinities could be reversed, as one might (incorrectly) infer from the inhibition of esterase activity by methanol. The observed inhibition, as noted above, is a result of a change of interaction of substrate with solvent, and not with enzyme.

Finally, it is necessary to comment further, and in some detail, on the recent work by Westerik et al. (1978), who reported a study of the influence of methanol and aniline on the esterase activity of carbonic anhydrase. Though, as discussed below, they present no data that are inconsistent with our model for carbonic anhydrase activity, they draw many conclusions that are at variance with our ideas. For example, they report a series of measurements of the esterase activity of the bovine enzyme, and the human B and C forms as well, in the presence of methanol and aniline, and report "both inhibitions were noncompetitive with respect to substrate". By contrast, we would expect methanol to be noncompetitive with respect to the ester and aniline to be competitive, in ostensible disagreement with Westerik et al. (1978). However, the issue is readily resolved: these workers used substrate concentrations of at most 1 mM, and generally less, whereas Pocker & Stone (1967) report that K_M for the same substrate (and bovine enzyme) is greater than 30 mM. Westerik et al. (1978) would never observe competition since they never use sufficient substrate to compete with aniline. (That this is the case for all three enzymes is readily seen by the intercept on the vertical axes of their Dixon plots.)

Additionally, for reasons never stated, they did the aniline-binding experiments at values of pH well below the pK_a for esterase activity. Though this does not effect the above arguments, it makes much of the algebra somewhat complex, since the majority of the enzyme molecules are inactive, and aniline binds differentially to the active and inactive forms. Nonetheless, this does resolve another apparent inconsistency between our expectations and their interpretations: in a Dixon plot in which the influences of methanol and aniline on esterase activity are compared at fixed substrate concentration, the two neutral molecules appear competitive. We would expect them to bind noncompetitively to active enzyme molecules, since they compete for different sites. The explication is that methanol competes with water at the active site, and thus with the active enzyme, whereas aniline binds preferentially to the anion-inactivated enzyme, as deduced from the data of Figure 3 above. Though methanol and aniline are not in themselves competitive, they each bind to forms of the enzyme that are formed when anion–proton pairs and water compete for the same ligand. This situation is reflected as an *apparent* competition between methanol and aniline when they are both added to enzyme samples at a pH near the pK_a for activity. The quantitative aspects of this can readily be verified by combining eq 5 and 12.

The above holds if the Dixon plots of Westerik et al. (1978) are interpreted to mean that methanol binds to enzyme under the conditions used. But, as argued above, the major effect of methanol is to increase the solubility of ester substrate in the experiments reported, which in turn appears as an increase of K_M proportional to the concentration of methanol. As already remarked, the Dixon plot for this interpretation is identical with the interpretation of formal, noncompetitive inhibition by methanol. Analogously, the Dixon plot comparing methanol and aniline interactions for fixed substrate, at low pH, will again appear, formally, as competitive, since the methanol affects the binding of substrate to active enzyme, and aniline binds to the inactive enzyme. However, for both cases, the inhibition constant derived for methanol is in reality a measure of the increased solubility of substrate in the solvent (now in the range 5–10% methanol). Thus, all the kinetic data of Westerik et al. (1978) are consistent with our model.

In addition to the kinetic data, Westerik et al. (1978) present optical and relaxation data that must be reinterpreted. They show the optical absorption of Co^{2+}-BCA as a function of aniline concentration at low pH (i.e., below the pK_a for activity) both in the presence and absence of 30 mM KBr, and argue from these results for the "mutually exclusive nature

of the aniline and Br^- interactions". However, there are isosbestic points in both sets of data, indicating that in the absence of Br^- the samples contain mixtures of the low pH forms with and without bound aniline, whereas in the presence of Br^- the samples contain mixtures of the low pH, Br^--inhibited forms with and without bound aniline. The data are entirely compatible with our views; we see no evidence for mutual exclusivity. Moreover, Westerik et al. (1978) report that the addition of 30 mM aniline, a concentration ostensibly equal to the observed inhibition constant, does not alter the relaxation rate of solvent water protons. However, the inhibition studies are done at low pH where aniline binds relatively well, whereas the relaxation studies are done at high pH so that the paramagnetic relaxation contribution can be observed. Unfortunately, at their pH, aniline does not bind well at all (500-fold weaker, as reported above), and no effect was observed because no binding occurred. Apparently not realizing this, Westerik et al. (1978) tried to reconcile these optical and relaxation data by invoking a five-coordinate complex of Co^{2+} with water, aniline, and three residues of the enzyme. Clearly, there is no need for this.

We have already remarked on the apparent interaction of methanol with carbonic anhydrase and noted that, while in a formal sense addition of methanol lowers the esterase activity, the influence of methanol is via an increase in the solubility of the substrate because the aromatic phenol ring is more soluble in methanol than in water. Analogous arguments hold for the more complex simultaneous influences of methanol and aniline on esterase activity; the several relevant conclusions of Westerik et al. (1978) in this regard must all be reexamined. The data themselves, as we have noted, are consistent with the predictions of our views.

There is only one set of results of Westerik et al. (1978) that does not seem to fit into our scheme, and this is the influence of added CN^- on the relaxation rate of solvent water and methanol methyl protons. We have repeated these measurements (as noted above), with ±1% precision contrasted to their ±10% precision, and find that we disagree with their data. We find, as expected, that when CN^- binds, as confirmed by changes in the optical spectra, the relaxation rate of solvent protons is reduced proportionately. By contrast, Westerik et al. (1978) do not present optical spectra which would, independently, indicate the state of the samples. Moreover, for the cases in which their methyl proton data are presented, CN^- is added stoichiometrically rather that in excess. They see little effect on either water or methanol protons, suggesting that $[CN^-]$ is too low to give the desired result. For the one instance where they add excess CN^-, as do we, they only report water proton relaxation data; here the effect is almost a factor of two. It is not clear why the methanol proton relaxation data for this sample are not shown.

Summary

We have investigated the interaction of (neutral) aniline, phenol, and methanol with Co^{2+}-substituted bovine carbonic anhydrase, with the intent of generalizing our recently proposed model for the interaction of anions with the enzyme (Koenig et al., 1980) to include effects due to interactions with small neutral molecules. Our model for enzyme–anion interactions was proposed to explain the pH dependence of the activity in a manner consistent with the implications of the magnetic relaxation results for solvent water protons, which indicate that there is an exchanging water molecule (rather than OH^-) as a ligand of the metal ion when the enzyme is active. By contrast, the enzyme is inactivated by binding of monovalent anions to the metal ion; the anions bind preferentially at low pH because a proton binds simultaneously nearby to maintain charge neutrality in the region of the active site. In the absence of deliberately added monovalent anions, we demonstrated that HSO_4^-, in equilibrium with (bivalent) sulfate generally used as buffer anion, has been the determinant of the pK_a for activity.

We now find that aniline and phenol, isoelectronic aromatic neutral molecules that inhibit esterase activity, influence the pK_a for hydrase activity (as inferred here from optical and relaxation measurements) in opposite ways; aniline raises the pK_a and phenol lowers it. We argue that both aniline and phenol bind at the well-known hydrophobic site to which aromatic ester substrates and sulfonamide inhibitors are known to bind. The resulting binary complexes of enzyme plus neutrals then behave as altered enzymes, the alteration being expressed as a shift in the pK_a for activity, due to a change in the relative affinities of the metal ion for water and monovalent anions. Phenol, we suggest, increases the affinity for water by formation of a hydrogen bond to the metal-bound water, whereas aniline increases the proton-accepting capacity of the active-site region and thereby increases the affinity for anions. All other properties of these binary complexes follow directly from this view when combined with our earlier model. By extension, we expect (neutral) imidazole and lutidine to bind as do aniline and phenol, and alter the pK_a of the enzyme analogously. Thus, care must be exercised when interpreting data obtained when these aromatic molecules are used as cationic buffers; what have been called "buffer effects" (cf. Silverman et al., 1979) may be due to the formation of enzyme–buffer complexes.

By contrast to the aromatic neutrals, the influence of methanol on the properties of carbonic anhydrase is totally different. Methanol is very much like water and, indeed, competes with water about equally well for the fourth ligand of the metal ion. Thus, for 1 M methanol in solution, corresponding to the observed K_I for inhibition of esterase activity by methanol, only about 2% of the metal-bound water is displaced by methanol. Moreover, the relaxation of the methanol methyl protons behaves precisely as does that of water protons: the correlation of the pH dependence with the optical spectra is the same; inhibition by anions is analogous; the magnitude of the relaxation effects yields methanol methyl proton distances that are consistent with the methanol oxygen being in the same position as that of water oxygens, i.e., bonded to the metal ion at the active site. The reported inhibition of esterase activity is attributed to alteration of the properties of the solvent: methanol is a relatively good solvent for molecules with phenol rings, including phenol itself, aniline, and *p*-nitrophenyl acetate, whereas water is not. Thus, 1 M methanol, corresponding to almost 5% of the solution by volume, decreases the affinity of substrate for the enzyme by increasing its solubility.

As a final point, we note that now several of the observed properties of solutions of carbonic anhydrase depend on the composition and properties of the solvent rather than on the properties of the enzyme. Thus, we showed earlier that the pK_a for activity, long the basis for a search for a group on the enzyme that ionizes, is in actuality due to an ionization in the solution (Koenig et al., 1980); it may be regarded, formally, as a competition between added monovalent anions and OH^- (that results from the ionization of solvent water) or, equivalently, between anion–proton pairs and water molecules. Now we find that altering the solvent to increase the solubility of substrate produces effects that have also long been the basis for a search for methanol-binding sites on the enzyme. Thus,

when one observes altered behavior of solutions of enzymes upon addition of small molecules (substrates, inhibitors, etc.), one must be sensitive to the possibility that the interaction may well be with the solvent rather than, or as well as, with the enzyme.

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