pH Dependence of Solvent Proton Relaxation in Carbonic Anhydrase Solutions: Paramagnetic and Diamagnetic Effects†

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ABSTRACT: The spin–lattice relaxation rate (T_1^{-1}) of solvent water protons has been investigated in solutions of zinc(II) and cobalt(II) bovine carbonic anhydrase B and two alkylated derivatives of the cobalt(II) enzyme. Both modified enzymes contain an acetazolamide residue bridging the metal ion and N^{τ} of histidine-63. At magnetic field strengths corresponding to proton Larmor frequencies of 0.02 and 20 MHz and in the presence of 25 mM Na_2SO_4, T_1^{-1} is enhanced equally by zinc(II) carbonic anhydrase and the alkylated cobalt(II) enzymes over the pH range from 5 to 12. The enhancement is pH independent below neutrality and drifts upward in a near-linear fashion above pH 8. With cobalt(II) carbonic anhydrase, the enhancement tends toward that of the zinc(II) and alkylated enzymes in the limit of low pH, increases sigmoidally between pH 5 and 8, and drifts upward at higher pH. The data are interpreted to indicate that zinc(II) carbonic anhydrase defines the diamagnetic component of the enhancement caused by the cobalt(II) enzyme and that covalently bound acetazolamide precludes either the presence or the rapid exchange of water from within the coordination sphere of the paramagnetic ion. Subtraction of the diamagnetic contribution from the enhancement obtained with uninhibited cobalt(II) carbonic anhydrase yields a sigmoid-like pH profile. The pattern can be described assuming that a maximal increase in T_1^{-1} requires the basic form of two ionizations exhibiting pK_as of 6.11 (standard error = 0.03) and 8.5 (standard error = 0.4) at the particular buffer concentration used. Approximately 10% of the total paramagnetic enhancement is controlled by the higher pK_a. Excess acetazolamide or Ethoxzolamide reverses only partially the increase in T_1^{-1} caused by the substitution of cobalt(II) for zinc(II). The residual paramagnetic enhancement is constant between pH 7.3 and 8.7, and equivalent to approximately 13% of that caused by the free cobalt(II) enzyme in the limit of high pH.

Elucidation of the catalytic machanism in carbonic anhydrase relates in part to the position of molecular water or hydroxide ion in the reaction pathway. This traditionally has been modeled in terms of a single water molecule or hydroxide ion bound at the fourth ligand position of a tetrahedrally coordinated metal ion (see, for example, Lindskog et al., 1971). As early as 1966, however, Dennard & Williams (1966) suggested that the metal ion could adopt a pentacoordinated geometry. Evidence for distortions from tetrahedral symmetry has been presented more recently for cobalt(II) carbonic anhydrase (Kaden et al., 1972; Cockle, 1974; Holmquist et al., 1975; Haffner & Coleman, 1975; Aasa et al., 1976; Bertini et al., 1976, 1977c, 1978b; Bauer et al., 1977) as well as for the copper(II) (Haffner & Coleman, 1975; Morpurgo et al., 1975; Bertini et al., 1977a), manganese(II) (Bertini et al., 1978a), and zinc(II) enzymes (Kannan et al., 1977a,b). The association of two solvent molecules with the metal ion has been implied by Mildvan (1974) and assigned a catalytic role in the mechanistic proposal of Wyeth & Prince (1977).

Fabry et al. (1970) have reported that the spin–lattice relaxation rate of water protons is enhanced in solutions of bovine and human cobalt(II) carbonic anhydrase B. More recently, Lanir et al. (1973, 1975) have obtained similar results using the manganese(II) bovine enzyme, although Bertini et al. (1978a) and Jacob (private communication) have questioned their interpretation in view of the tendency of manganese to dissociate from the enzyme. That portion of the cobalt(II)-induced enhancement inhibitable by aromatic sulfonamides arises from a magnetic dipolar interaction between solvent protons and the metal ion, and requires the alkaline form of an ionization occurring around neutrality. Whereas the increase in T_1^{-1} at high pH has been attributed to a single, rapidly exchanging water molecule at a coordination position on the metal ion (Koenig & Brown, 1972; Jacob et al., 1978), the absence of a paramagnetic effect at low pH remains unresolved. It appears, however, that the solvent molecule is either absent or in slow exchange relative to the time scale of the experiment. In the report of Fabry et al. (1970), saturating concentrations of Ethoxzolamide did not reverse fully the increase in T_1^{-1} accompanying the substitution of cobalt(II) for zinc(II) as the metal ion cofactor essential for enzymic activity; residual relaxativity exhibited by the inhibited enzyme was not considered in their interpretation of the data.

The present report describes the use of two alkylated derivatives of cobalt(II) bovine carbonic anhydrase to investigate further the enzyme-induced enhancement of the longitudinal relaxation rate of solvent protons. Greater experimental precision due to recent improvements in the relaxation apparatus has allowed us to reevaluate the residual enhancement reported by Fabry et al. (1970) for the sulfonamide inhibited enzyme, and to consider its possible relationship to water in rapid exchange at the active site.

Mono- and dialkylated cobalt(II) bovine carbonic anhydrase contain one and two acetazolamide residues, respectively, bound covalently at the active site (Wells et al., 1975, 1977b). In both derivatives, an acetazolamide residue is linked via the acetyl group to N^{τ} of histidine-63[1] and bound via the sulfonamide group at the fourth coordination position of the active site metal ion. Visible absorption spectra indicate that the relative positions of the sulfonamide group and the metal ion in monoalkylated cobalt(II) carbonic anhydrase are similar to those in the reversible, enzyme–acetazolamide complex (Wells et al., 1975). In the dialkylated enzyme, a second inhibitor residue is linked covalently to the 3-nitrogen atom in the thiadiazole ring of the first to yield an acetazolamide dimer at the active site. Dialkylated cobalt(II) bovine carbonic

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[1] The amino acid sequence in bovine carbonic anhydrase B is numbered according to Sciaky et al. (1974).

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anhydrase is spectrally and kinetically identical with the monoalkylated enzyme (Wells et al., 1977b). These derivatives have been used together with the native zinc(II) enzyme to define the diamagnetic component of the total increase in T_1^{-1}. A preliminary report of this work has appeared elsewhere (Wells et al., 1977a).

Experimental Procedure

Materials and Reagents. Acetazolamide was the gift of Lederle Laboratories and Ethoxzolamide was donated by Dr. R. W. King of the National Institute for Medical Research, London. Both were used without further purification. Bromoacetazolamide (bromoacetylamino-1,3,4-thiadiazole-5-sulfonamide) was prepared as reported previously (Kandel et al., 1968). L-[U-^{14}C]Leucine (0.324 Ci/mmol) was purchased from Amersham as an aqueous solution (50 μCi/mL) containing 2% ethanol.

Erythrocyte bovine carbonic anhydrase B was isolated from a commercial product (Worthington Biochemical Corp.) according to the method of Kandel et al. (1970). Apo and cobalt(II) carbonic anhydrase were prepared from the native enzyme as described by Lindskog & Malmström (1962). Bromoacetazolamide was used to prepare mono- and dialkylated cobalt(II) carbonic anhydrase as described by Cybulsky et al. (1973) and by Wells et al. (1977b), respectively. An affinity column (Kandel et al., 1974) was used to remove trace amounts of unmodified cobalt(II) enzyme from the alkylated derivatives obtained from initial purification on DEAE-cellulose. All enzyme preparations were stored at −20 °C as a powder lyophilized from deionized water.

Enzyme Assays. Enzyme used in the present investigation was characterized with respect to zinc and cobalt content, esterase activity toward *p*-nitrophenyl acetate, amino acid composition, electrophoretic mobility on starch and acrylamide gel, and visible absorption spectrum according to reported procedures (cf. Wells et al., 1975, 1977b). Preparations of zinc(II) and cobalt(II) bovine carbonic anhydrase B as well as the alkylated cobalt(II) derivatives were identical with those reported previously (Wells et al., 1975, 1977b). The cobalt(II) enzymes contained 0.96 g-ion of cobalt per mol of enzyme.

Protein concentration was determined from the absorbance at 280 nm of an aliquot (20 μL) of the sample solution diluted with 3.0 mL of Tris/sulfate buffer (0.1 M, pH 7.6). Molar absorptivities were taken as follows: zinc(II) and cobalt(II) bovine carbonic anhydrase, 56 000 M^{-1} cm^{-1} (Lindskog, 1960); monoalkylated cobalt(II) enzyme, 58 000 ± 1100 M^{-1} cm^{-1}; dialkylated cobalt(II) enzyme, 65 400 ± 1300 M^{-1} cm^{-1}. To determine the molar absorptivity of alkylated carbonic anhydrase, solutions of the zinc enzyme were assayed for both ultraviolet absorbance at 280 nm and zinc content. The latter value was obtained by atomic absorption spectrometry and provides an estimate of enzyme concentration when the molar ratio of metal ion to enzyme is known. In measurements on native carbonic anhydrase, however, atomic absorption indicated a metal ion content equivalent to 0.955 ± .017 g-ion of zinc per mol of enzyme when protein concentration was estimated spectrophotometrically using the value of 56 000 M^{-1} cm^{-1} reported by Lindskog (1960) for the molar absorptivitiy. For purposes of comparison, the zinc content of mono- and dialkylated carbonic anhydrase thus was taken as 0.955 when using atomic absorption data in the calculation of molar absorptivity.

Sample Preparation for Relaxation Measurements. Unless otherwise stated, enzyme solutions were prepared by dissolving the lyophilized protein in deionized water containing 25 mM Na_2SO_4 to yield a final enzyme concentration of 2.3–2.8 mM. pH was adjusted by the addition of either H_2SO_4 or NaOH using a Hamilton microsyringe as the solution was swirled on a vortex mixer. Values of pH around neutrality generally were obtained by mixing two solutions at equal enzyme concentration and initially adjusted to pH 6 and 9. Values above or below this range were obtained by the direct addition of NaOH or H_2SO_4 as required.

pH was measured using an electrode supplied by Activion Glass Limited, Kinglassie, Scotland, in which saturated K_2SO_4 was substituted for KCl as the salt bridge in the reference chamber. This precaution was prompted by the report of Campbell et al. (1974) that sufficient chloride ion can diffuse from the reference chamber into the sample solution to perturb appreciably the pK_a of the activity-linked ionization. Measurements were taken by inserting the electrode directly into the sample tube. As this procedure tended to alter slightly the protein concentration, absorbance measurements were made following each adjustment of the pH and as the final step prior to insertion of the sample tube into the relaxation apparatus.

To study the effect of reversibly bound sulfonamide inhibitors, stock solutions of acetazolamide and Ethoxzolamide (0.08 M) were prepared in either 25 mM Na_2SO_4 (pH >9) or Tris/sulfate buffer (0.1 M, pH 8.7). Aliquots were added to solutions of cobalt(II) bovine carbonic anhydrase using a Hamilton microsyringe and the pH was adjusted as required. During the various manipulations, care was taken to monitor all changes in the concentration of both enzyme and inhibitor; efforts particularly were made to avoid errors arising from the appreciable absorptivity of aromatic sulfonamides at 280 nm. When the experiment was performed in 25 mM Na_2SO_4 with frequent measurement of pH, dilution factors were determined using [U-^{14}C]leucine (<1 μM) introduced into the enzyme solution prior to the addition of inhibitor. All concentrations then could be related to the radioactive tracer by means of 5-μL aliquots removed at each manipulation and counted in a liquid scintillation spectrometer. Trial experiments indicated that introduction of the radiolabeled material had no effect on the relaxation rate. Changes in sample volume were monitored when required by weighing the sample tube and its contents. For inhibitor titrations of the enzyme carried out at constant pH in Tris/sulfate buffer (0.1 M, pH 8.7), initial protein concentration was determined spectrophotometrically and subsequent effects of dilution were calculated from a knowledge of the volume of inhibitor solution added at each stage.

Relaxation Measurements. Relaxation rates of solvent protons were measured at 25 °C in equilibrium with atmospheric CO_2 using apparatus and procedures described previously (Hallenga & Koenig, 1976; Koenig & Schillinger, 1969). Data are expressed as total relaxivity (R_t) according to eq 1, where T_1^{-1} is the observed relaxation rate of solvent water protons in the protein solution, V is the mole fraction occupied by the protein and taken as equal to zero, N is the molar concentration of protein, and T_{1w}^{-1} is the relaxation rate of the buffer.

$$R_t = (1 - V)(T_1^{-1} - T_{1w}^{-1})/N \quad (1)$$

At each pH, relaxivity was measured as a function of magnetic field, corresponding to a range of proton Larmor frequency from 0.01 to 20 MHz. Standard errors in the determination of T_1^{-1} typically were less than 0.6% and similar errors were found for the mean of several determinations.

Data Analysis. Theoretical expressions were fitted to experimental data using an iterative technique reported by Batchelor (1977) and based on the nonlinear, least-squares

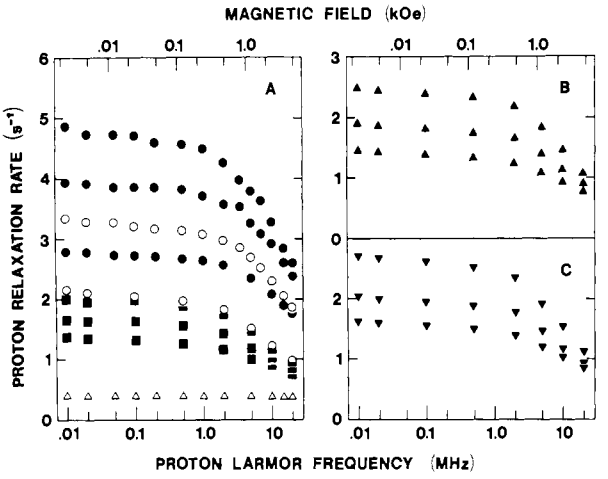

FIGURE 1: Dispersion of solvent proton relaxation rates for solutions of bovine carbonic anhydrase. Profiles are listed as shown from top to bottom. (A) (●) Cobalt(II) enzyme: 2.44 mM, pH 11.69; 2.49 mM, pH 8.72; 2.36 mM, pH 6.37. (○) Cobalt(II) enzyme plus Ethoxzolamide: 2.62 mM enzyme, 0.89 mM Ethoxzolamide, pH 8.64; 2.81 mM enzyme, 3.82 mM Ethoxzolamide, pH 8.73. (■) Zinc(II) enzyme: 2.26 mM, pH 11.41; 2.38 mM, pH 9.58; 2.40 mM, pH 5.73. (△) Protein-free solvent: 25 mM Na_2SO_4. (B) (▲) Monoalkylated cobalt(II) enzyme: 2.62 mM, pH 11.65; 2.75 mM, pH 9.29; 2.64 mM, pH 5.66. (C) (▼) Dialkylated cobalt(II) enzyme: 2.66 mM, pH 11.89; 2.72 mM, pH 9.62; 2.70 mM, pH 7.03.

algorithm of Marquardt (1963). Individual data points were weighted according to the inverse of the standard error. In all cases the statistical variation of experimentally determined mean values or parameters is indicated by the standard error.

Results

Dependence of T_1^{-1} on Magnetic Field and Protein Concentration. Representative examples in Figure 1 illustrate the frequency dispersion of T_1^{-1} for solvent protons in solutions of native and modified bovine carbonic anhydrase B. Data obtained with the native cobalt(II) and zinc(II) enzymes are in good accord with results reported previously (Fabry et al., 1970; Koenig et al., 1978). Substitution of the paramagnetic cation at the active site gives rise to a pH-dependent enhancement in the observed relaxation rate which is reduced in the presence of sulfonamide inhibitors bound either reversibly (Figure 1A) or covalently (Figures 1B and 1C) to the enzyme. While in all cases the enhancement is greater at higher pH, the shape of the dispersion profiles generally is preserved over the complete range of pH studied. In the absence of protein, the value of T_1^{-1} was found to be virtually independent of buffer and pH, and to decrease only slightly from 0.394 to 0.372 s^{-1} as the proton Larmor frequency increases from 0.01 to 20 MHz (Figure 1A).

The calculation of relaxivity according to eq 1 requires that the diamagnetic component of T_1^{-1} vary linearly with protein concentration. In view of nonlinear relationships reported for proteins of higher molecular weight such as liganded hemoglobin (Lindstrom et al., 1976) and apotransferrin (Koenig & Schillinger, 1969), this was checked using zinc(II) bovine carbonic anhydrase at pH 7.3 in 0.1 M Tris/sulfate buffer. At a magnetic field of 4.69 Oe, corresponding to a proton Larmor frequency of 0.02 MHz, T_1^{-1} was found to be a linear function of enzyme concentration over the range from 1.5 to 4.3 mM. Regression analysis yielded a slope of 0.346 ± 0.11 mM^{-1} s^{-1} and a y intercept of 0.390 ± 0.036 s^{-1}. The latter value is in good agreement with that of 0.394 s^{-1} obtained by direct measurement of the buffer solution in the absence of enzyme.

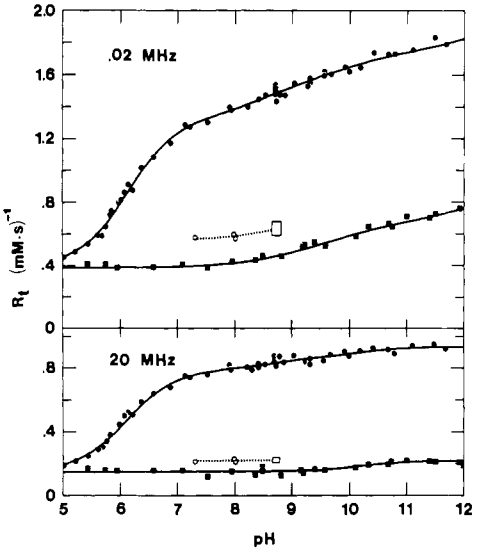

FIGURE 2: pH dependence of the total relaxivity for bovine carbonic anhydrase at two proton Larmor frequences. (●) Cobalt(II) enzyme; (■) zinc(II) enzyme; (○) cobalt(II) enzyme plus excess Ethoxzolamide; (□) cobalt(II) enzyme plus excess Ethoxzolamide (four samples) or acetazolamide (0.02 MHz, four samples; 20 MHz, one sample). At each frequency, the lower solid line represents the best fit of eq 3 to data obtained with the zinc(II) enzyme. The upper solid line represents a summation of the diamagnetic (R_d, eq 3) and paramagnetic (R_p, eq 4) contributions. R_d was taken as equal to R_t obtained with the zinc(II) enzyme; R_p was calculated assuming two ionizations and using the parametric values listed in Table I. The dotted line represents the summation of R_d, as determined with the zinc(II) enzyme, and the appropriate value of R_r from Table I. Molar ratios of inhibitor to enzyme varied between 1.08 and 1.85 in samples designated by the open symbols.

pH Dependence of the Total Relaxivity. The relaxivity–pH profile for bovine carbonic anhydrase is illustrated at two proton Larmor frequencies in Figure 2. With the cobalt(II) enzyme, the pattern is characterized by a sigmoid-like increase between pH 5 and 7 followed by a further upward drift under alkaline conditions. In contrast, the zinc(II) enzyme does not exhibit the transition at lower pH but retains the more gradual increase occurring above pH 8. With both enzymes, data were collected by decreasing as well as increasing the pH of the samples. The absence of hysteresis indicates that the changes in relaxivity are fully reversible over the entire pH range investigated. The different magnetic properties of cobalt(II) and zinc(II) carbonic anhydrase allow the total relaxivity (R_t) to be resolved according to eq 2 into a paramagnetic component (R_p) on one hand and a diamagnetic component (R_d) on the other.

$$R_t = R_p + R_d \tag{2}$$

Diamagnetic Component of the Relaxivity. Data obtained with zinc(II) carbonic anhydrase were smoothed for subsequent manipulations using eq 3 in which R_d is taken as equal to R_t. In this expression R_d is composed of two components,

$$R_d = R_d' + \frac{R_d'' K_d}{[H]Q + K_d} \tag{3}$$

designated R_d' and R_d'', which are pH independent and pH dependent, respectively. The pH-dependent component is controlled by a large number of titratable groups, the pKs of which are described by a bimodal distribution function defined according to Bruni et al. (1976). K_d is the weighted mean acid dissociation constant of the higher pH population and the quantity Q is a complex function of pH, K_d, the separation and relative size of the two populations, and the width of their

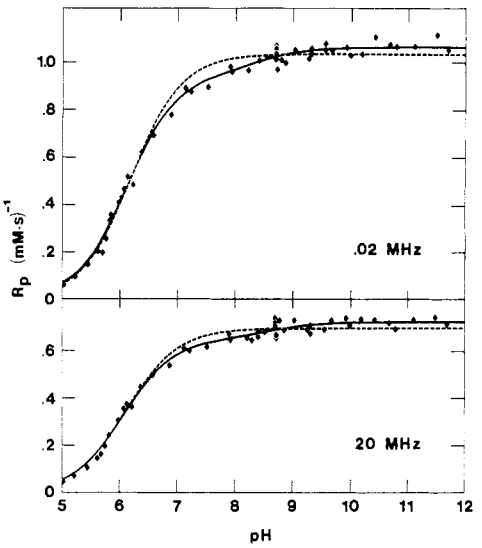

FIGURE 3: pH dependence of the paramagnetic relaxivity for cobalt(II) bovine carbonic anhydrase at two proton Larmor frequencies. Values plotted on the ordinate indicate the difference between total relaxivity and the diamagnetic component (R_d) determined with the zinc(II) enzyme and smoothed using eq 3. The curves represent the best fit of eq 4 to the data and are calculated using the parametric values listed in Table I. (---) One ionization ($R_p'' = 0$); (—) two ionizations.

distributions. Equation 3 was fitted to data obtained with the zinc(II) enzyme to yield the lower solid line shown at each frequency in Figure 2. In the limit of low pH, the function value for R_p decreases by approximately 62% from 0.389 ± 0.008 mM^{-1} s^{-1} at 0.02 MHz to 0.147 ± 0.005 mM^{-1} s^{-1} at 20 MHz. A similar decrease of 70% is obtained at pH 11.5 where the corresponding values of the function are 0.715 ± 0.013 mM^{-1} s^{-1} and 0.212 ± 0.009 mM^{-1} s^{-1}, respectively.

Paramagnetic Component of the Relaxivity. Subtraction of the smoothed, diamagnetic contribution from the data obtained with cobalt(II) bovine carbonic anhydrase yields the paramagnetic component (R_p) of the relaxivity shown in Figure 3. The dashed lines illustrate the best fit of eq 4 to the data points when R_p'' is set equal to zero. With this constraint

$$R_p = \frac{R_p'K_p'}{[H] + K_p'} + \frac{R_p''K_p''}{[H] + K_p''} \quad (4)$$

on R_p'', control of the relaxivity is assigned to a single ionization and the corresponding parametric values are listed in Table I. When described in this manner, however, the data deviate consistently from the computed curve and a better fit can be obtained when both terms of eq 4 contribute to the relaxivity. Use of a model encompassing two ionizations yields a best fit shown by the solid line at each frequency in Figure 3 and characterized by the parametric values listed in Table I. Summation of the results obtained with eq 4 for two ionizations and with eq 3 yields total relaxivity, illustrated by the upper solid line at each frequency in Figure 2.

Reversible Inhibition of R_p by Sulfonamides. The stepwise addition of 1 equiv of acetazolamide to cobalt(II) bovine carbonic anhydrase at pH 8.7 reduced the paramagnetic component of the relaxivity at 0.02 MHz from 1.06 mM^{-1} s^{-1} to 0.15 mM^{-1} s^{-1}, or approximately 14% of the native value (Figure 4). In a similar experiment, 1 equiv of Ethoxzolamide reduced R_p from 0.953 mM^{-1} s^{-1} to 0.144 mM^{-1} s^{-1}, or 15% of the native value. As illustrated by the open symbols in Figure 2, the relaxivity of cobalt(II) bovine carbonic anhydrase in the presence of excess Ethoxzolamide remains constant relative to that of the zinc(II) enzyme between pH 7.3 and 8.7.

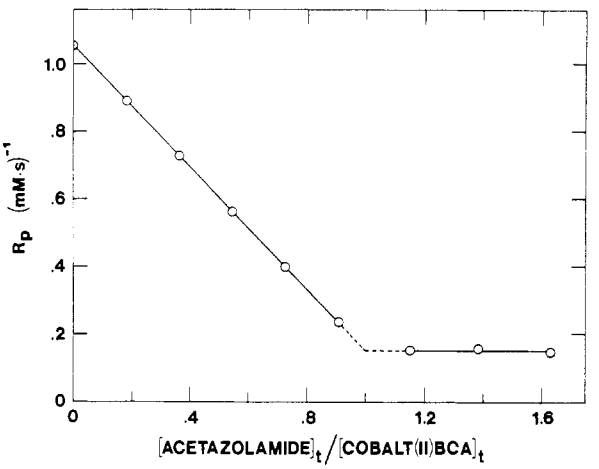

FIGURE 4: Titration by acetazolamide of the paramagnetic relaxivity of cobalt(II) bovine carbonic anhydrase B. Successive aliquots of acetazolamide (0.08 M) in Tris/sulfate buffer (0.1 M, pH 8.70) were added to a solution of the enzyme (2.63 mM) in the same buffer. Relaxivity was measured at a field strength of 4.69 Oe, corresponding to a proton Larmor frequency of 0.02 MHz. Concentrations of enzyme and inhibitor were corrected for the increase in sample volume occurring over the course of the titration. pH was not measured following initial preparation of the sample in order to avoid inserting the electrode into the probe. At the end of the titration, a value of 2.50 mM was calculated for the enzyme concentration and the pH was found to have remained constant at 8.70. Values plotted on the ordinate represent the difference between total relaxivity and the smoothed, diamagnetic component obtained with the zinc(II) enzyme.

Following the convention of Fabry et al. (1970), the paramagnetic relaxivity of the cobalt(II) enzyme in the presence of saturating concentrations of either acetazolamide or Ethoxzolamide is defined as R_r or "residual" relaxivity according to eq 5. Values of R_r thus are obtained by sub-

$$R_t = R_r + R_d \quad (5)$$

traction of the diamagnetic component, determined with the zinc(II) enzyme, from the total relaxivity of the complex. This quantity was found to be independent of pH within the range studied and to be identical for both Ethoxzolamide and acetazolamide. Average values obtained for R_r at 0.02 and 20 MHz are listed in Table I. The summation with R_d is illustrated by the dotted lines in Figure 2. Also included in Table I is the paramagnetic relaxivity (fR_p'') at pH 7.3 and 8.7 arising from that component (R_p'') controlled by a group or groups ionizing at around pH 8.5. These values have been calculated using the parameters obtained when eq 4 (two ionizations) is fitted to the data illustrated in Figure 3 and undergo an approximately tenfold increase within the limits of pH shown. It is noted that, while R_p'' and R_r are of comparable magnitude, the latter is pH independent under these conditions.

Relaxivity of Alkylated Cobalt(II) Bovine Carbonic Anhydrase. The relaxivity of mono- and dialkylated cobalt(II) bovine carbonic anhydrase is compared in Figure 5 with that of the native zinc(II) enzyme. While data obtained with the alkylated derivatives tend to lie slightly above the line approximating the behavior of the zinc(II) enzyme, the relaxivity of all three proteins is highly similar and significantly less than that of the reversibly inhibited enzyme. Differences in the effects of covalent and reversible inhibition are particularly marked at lower field.

Discussion

Inhibition by aromatic sulfonamides generally has been taken as evidence that events associated with carbonic an-

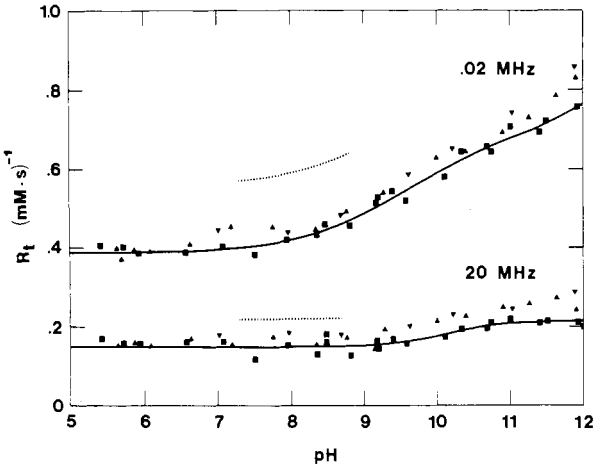

FIGURE 5: pH dependence of the diamagnetic relaxivity for bovine carbonic anhydrase at two proton Larmor frequencies. (■) Zinc(II) enzyme; (▲) monoalkylated cobalt(II) enzyme; (▼) dialkylated cobalt(II) enzyme. The solid lines represent the best fit of eq 3 to data obtained with the zinc(II) enzyme at either 0.02 or 20 MHz. The dotted lines represent the summation of eq 3, as defined by the zinc(II) enzyme, and the appropriate value of R_r from Table I.

hydrase occur at the active site. Accordingly, of the total increase in relaxivity accompanying the substitution of cobalt(II) for zinc(II), only that portion inhibitable by *p*-toluenesulfonamide or Ethoxzolamide has been considered by Fabry et al. (1970) and by subsequent investigators (Bertini et al., 1977b; Fabry, 1978) as relevant to the question of mechanism and catalysis. In contrast, two observations in the present report argue that the entire cobalt(II)-induced increase in relaxivity reflects water in association with the active site. Firstly, in the limit of low pH the relaxivity of the enzyme tends toward that of the diamagnetic, zinc(II)-substituted analogue. Secondly, two alkylated derivatives of cobalt(II) carbonic anhydrase exhibit relaxivity–pH profiles highly similar to that of the zinc(II) enzyme; in both cases the modification is known to occur within the region of the active site (Kandel et al., 1968, 1970; Wells et al., 1977b). Residual relaxivity retained by the reversible complex thus appears to reflect a magnetic interaction with the cobalt(II) ion, and reversibly bound sulfonamides thus do not eliminate the rapid exchange of water in and out of the active site cavity.[2] The alternative possibility that reversible inhibitors somehow increase the diamagnetic contribution of the protein can be ruled out by the observation of Fabry et al. (1970) that the relaxivity of zinc(II) bovine carbonic anhydrase is unaffected by the addition of Ethoxzolamide. Moreover, Bertini et al. (1978b) recently have reported that saturating concentrations of the bivalent anion oxalate reduce T_1^{-1} at 20 MHz to a greater extent than do monovalent anions or *p*-toluenesulfonamide. If specific for the active site, the difference is in accord with assignment of the residual relaxation to exchangeable protons not far from the paramagnetic center.

The virtually identical relaxivities of zinc(II) bovine carbonic anhydrase and the alkylated cobalt(II) derivatives confirm the conclusion of Wells et al. (1975, 1977b), based on the visible absorption spectrum, that the covalent incorporation of acetazolamide precludes the exchange of solvent protons at the active site. The relaxation data thus support the argument that esterase activity developing above pH 9 in both native and alkylated enzymes is unrelated to events occurring in the region of the active site (Wells et al., 1975, 1977b).

While several authors have measured the pH-dependent effects of a paramagnetic metal ion cofactor on the ability of bovine carbonic anhydrase to relax solvent protons (Fabry et al., 1970; Lanir et al., 1973, 1975; Wells et al., 1977a; Bertini et al., 1977b, 1978a,b; Fabry, 1978; Jacob et al., 1978), only Wells et al. (1977a) have reported that the paramagnetic component of the relaxivity may be controlled by more than one ionization. Although a similar possibility has been noted by Fabry (1978) for the human B cobalt(II) enzyme, the data were considered inadequate for analysis by more complex models and thus were interpreted in terms of a single ionization. Several factors in the present study permit refinement of the data to a degree not previously feasible: a considerably improved relaxation apparatus, the option of measuring T_1^{-1} at magnetic fields corresponding to proton Larmor frequencies as low as 0.01 MHz where relaxivity is maximal, the careful measurement of diamagnetic relaxivity over a broad range of pH using zinc(II) carbonic anhydrase. Characterization of the zinc(II) enzyme is particularly important in view of pH-dependent behavior unrelated to events occurring at the active site.

Resolution of the paramagnetic relaxivity of cobalt(II) bovine carbonic anhydrase into two, pH-dependent components implies that two ionizations control either one or two solvent exchange phenomena. As the minor component constitutes only about 10% of the total paramagnetic effect in the limit of high pH, a decision between these alternatives is not possible on the basis of the pH profile alone. X-ray analysis indicates, however, that sulfonamide inhibitors displace either a water molecule or a hydroxide ion on binding to a position within the inner coordination radius of the metal ion (Lindskog et al., 1971; Bergstén et al., 1972). Moreover, the inhibitor is expected to be in slow exchange relative to the time scale of the relaxation measurements (King & Burgen, 1976) and the residual relaxivity thus must reflect water protons exchanging at a site or sites distinct from the inhibitor binding site. If the phenomenon underlying residual relaxivity also exists in the free enzyme, there then must be at least two independent, additive contributions to the paramagnetic relaxivity of cobalt(II) bovine carbonic anhydrase. It perhaps is noteworthy that the residual paramagnetic relaxivity of the reversibly inhibited cobalt(II) enzyme is comparable in magnitude to the minor paramagnetic component of the free enzyme. The observed similarity may be fortuitous; alternatively, aromatic sulfonamides may inhibit only the major component controlled at pH 6.1 and affect the pH dependence but not the magnitude of the minor component. Caution is required, however, in comparing relaxivity in the native and inhibited cobalt(II) enzymes. Aromatic sulfonamides are believed to interact with carbonic anhydrase in a two-step mechanism culminating in a complex between the anionic form of the inhibitor and the acidic form of the enzyme (King & Burgen, 1976, and references therein). In its effect on solvent proton relaxation, however, the inhibited enzyme resembles neither the acidic nor the basic form of the free enzyme, at least in the pH range from 7.3 to 8.7. The presence of the inhibitor thus appears to evoke an exchange phenomenon that, while influenced by the paramagnetic center, is either nonexistent or not observable in the uninhibited enzyme at low pH.

The present data offer no insight into the identity of the ionization responsible for the paramagnetic relaxivity of

[2] It is noted that the difference in relaxivity between monoalkylated cobalt(II) carbonic anhydrase and the enzyme–acetazolamide reversible complex indicates that the acetylthiadiazole group of acetazolamide occupies different positions in the active site of the covalently and reversibly inhibited enzymes. Such a possibility has been raised previously (Kandel et al., 1974).

Table I: Parameters Describing the Paramagnetic Relaxivity of Cobalt(II) Bovine Carbonic Anhydrase B[a]

ν (MHz)	pK_p'[b]	pK_p''[b]	R_p'[b] (mM^{-1} s^{-1})	R_p''[b] (mM^{-1} s^{-1})	R_r[d] (mM^{-1} s^{-1})	$f_{7.3}R_p''$[e] (mM^{-1} s^{-1})	$f_{8.7}R_p''$[e] (mM^{-1} s^{-1})
0.02	6.20 ± 0.02		1.031 ± 0.007	[c]			
20	6.14 ± 0.02		0.689 ± 0.005	[c]			
0.02	6.13 ± 0.02	8.40 ± 0.24	0.948 ± 0.021	0.114 ± 0.023	0.166 ± 0.005 (11)	0.009	0.076
20	6.08 ± 0.02	8.60 ± 0.28	0.646 ± 0.013	0.072 ± 0.016	0.070 ± 0.002 (8)	0.003	0.040

[a] In all cases, diamagnetic relaxivity was taken as equal to R_t for the zinc(II) enzyme, smoothed according to eq 3. [b] Values of pK_p', pK_p'', R_p', and R_p'' were obtained by fitting eq 4 to the data illustrated in Figure 3. [c] R_p'' set equal to zero for the expression describing a single ionization. [d] Mean values of R_r were obtained as described in the text using cobalt(II) bovine carbonic anhydrase plus excess Ethoxzolamide between pH 7.30 and 8.74 (seven samples) or excess acetazolamide at pH 8.70 (four samples at 0.02 MHz, one sample at 20 MHz). [e] Values of the relaxivity at pH 7.3 and pH 8.7 calculated according to the expression $fR_p'' = R_p''K_p''/([H] + K_p'')$.

cobalt(II) bovine carbonic anhydrase beyond the likelihood that the pK_a of 6.1 reflects the same ionization monitored by previous investigators, but for which a range of pK_a has been reported. The early work of Fabry et al. (1970) indicated that inhibitable paramagnetic relaxivity required the basic form of a single group exhibiting a pK_a of 7.0 ± 0.2, later revised upward to approximately 7.2 (Koenig & Brown, 1972). More recently, however, Bertini et al. (1977b, 1978a,b) have reported that under conditions of low ionic strength both cobalt(II) and manganese(II) bovine carbonic anhydrase cause a paramagnetic enhancement of T_1^{-1} that is constant over the pH range between approximately 5.8 and 9.2. By measuring the optical density at 640 nm, Jacob et al. (1978) subsequently have shown that the amount of enzyme in basic form correlates well with relaxivity at every pH irrespective of ionic strength. They conclude that the apparent pH independence of T_1^{-1} in the absence of buffer reflects a shift in the controlling pK_a to values below 5. The pK_a of 6.1 found in the present investigation behaves in accord with the results and interpretation of Fabry et al. (1970) and Jacob et al. (1978). It is reduced from the value of 7.2 (Koenig & Brown, 1972) owing to the precautions taken to preclude chloride leakage from the salt bridge of the pH electrode. Repetition of the original titration of Fabry et al. (1970) using a standard KCl electrode yielded a pK_a of 7.88 ± 0.33 (Wells, 1975). Exceedingly low values of pK_a comparable to those of Bertini et al. (1977b, 1978b) and Jacob et al. (1978) were not obtained, presumably owing to the presence of 25 mM Na_2SO_4.

In attempting to assign the pK_a of 8.5 to a group or groups in the protein, the problem is similar to that encountered in identifying the activity-linked ionization. Several authors have noted the scarcity of amino acid residues with pK_as above neutrality in the active site of high activity carbonic anhydrases (cf. Campbell et al., 1977; Fabry, 1978). Since bicarbonate derived from atmospheric CO_2 is reported to affect relaxivity in the cobalt(II) human enzyme (Fabry, 1978), a similar interaction may account for the transition observed at pH 8.5.

The present data imply that solvent molecules exchange at more than one position within the region of the paramagnetic center in cobalt(II) bovine carbonic anhydrase. In view of the evidence for distortions from tetrahedral geometry, one might speculate that a pentacoordinated metal ion binds two solvent ligands: one at the fourth coordination position revealed by X-ray analysis (Lindskog et al., 1971), and the second at a more distant, fifth position similar to that occupied by imidazole in human carbonic anhydrase B (Kannan et al., 1977a; Wolpert et al., 1977) or to that found for various other inhibitors in human isoenzymes B and C (Kannan et al., 1977b; cf. Wyeth & Prince, 1977). With regard to the nature of the exchanging solvent protons at high pH, the proposals of Fabry (1978) and of Bertini et al. (1977b, 1978a) imply that H^+ dissociates rapidly and independently from metal-coordinated water. It has been pointed out by Taylor et al. (1971), however, that ^{17}O and ^1H show essentially identical exchange rates in the case of water coordinated to d transition metals in octahedral aquometal complexes (Swift & Connick, 1962; Pearson et al., 1960; Pearson & Lanier, 1964). If the enzymic exchange proceeds in a manner similar to that in model complexes, the ligand responsible for inhibitable paramagnetic relaxivity is likely to be molecular water as exchanging OH^- can be ruled out on kinetic grounds (Jacob et al., 1978).

While clearly a diamagnetic effect, the origin of the upward drift in relaxivity above pH 9 remains uncertain. Models based on the ionization of a single group such as bulk water can be ruled out, however, by the poor agreement between fitted curves and the experimental data. The distribution expression of Bruni et al. (1976) was used primarily to smooth the data for subsequent manipulations. It nevertheless is noteworthy that this expression provides a better approximation of the data with five parameters than does, for example, the sum of three Henderson–Hasselbach expressions with six. High pH effects on diamagnetic relaxivity thus appear to reflect the ionization of several amino acid side chains such as those of lysine and arginine, the pK_as of which are rather broadly distributed. Increased relaxivity could arise from an increase in the number of water molecules associated with charged groups on the surface of the protein.

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