Nuclear Magnetic Relaxation Dispersion in Protein Solutions

IV. PROTON RELAXATION AT THE ACTIVE SITE OF CARBONIC ANHYDRASE*

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SUMMARY

Bovine and human B erythrocyte carbonic anhydrases substituted with Co** cause an enhancement of the nuclear magnetic relaxation rate of solvent water protons (T1-1) at high pH that is decreased by the addition of carbonic anhydrase inhibitors such as azide and Ethoxzolamide. The part of T1-1 which can be inhibited by Ethoxzolamide is due to exchangeable protons located at the active site of the enzyme. This inhibitable part of T1-1 is pH-dependent with a pK of 7.0 ± 0.2 for the bovine cobalt enzyme and a pK of 8.2 ± 0.2 for the human B cobalt enzyme. From the magnetic field dependence of the inhibitable part of T1-1, a correlation time for the dipolar interaction of a proton with a cobalt electronic spin of 10^{-12} sec, a proton-cobalt distance of 2.5 to 2.9 A, and for the bovine enzyme a proton residence time, τH, small compared to 10^{-3} sec are calculated. We conclude from the pH dependence and the proton-cobalt distance that the proton which contributes to the inhibitable part of T1-1 is located either on a hydroxide ion bound to the Co** at the active site or on a water molecule with 1 proton hydrogen bonded to a nearby residue. In contrast to the similarity of the inhibitable part of T1-1 for the bovine and human B cobalt enzymes, there is a non-inhibitable, pH-independent contribution resulting from the cobalt substitution, relatively large for the bovine enzyme and very small for the human B enzyme, that is not understood. The contributions of the (diamagnetic) native zinc enzymes to T1-1 have also been measured. As in the case of apotransferrin (KOENIG, S. H., AND SCHILLINGER, W. E., J. Biol. Chem., 244, 3283 (1969)), the results agree qualitatively but not quantitatively with theory.

At high pH, the carbonic anhydrases (EC 4.2.1.1) catalyze the hydration of CO2. The enzymes have 1 zinc atom per molecule, removal of the metal abolishes enzyme activity. The enzyme activity is pH-dependent. The base-catalyzed inorganic reaction may be written as CO2 + OH- → HCO3-. It has been suggested by a number of workers (2-5) that the enzyme-catalyzed reaction proceeds by the attack of a metal-bound hydroxide ion on a CO2 molecule bound near the metal ion. Several investigators have attempted (4, 6) to show the presence of the water molecule (or hydroxide ion) involved in this mechanism. Their results are compatible with, but do not definitely prove, the presence of the water molecule in question.

The nuclear magnetic relaxation rate (T1^{-1}) of solvent water protons is enhanced under certain conditions by the presence of paramagnetic metal ions and is extremely sensitive to the accessibility of water protons to the metal ion. The zinc in carbonic anhydrase can be replaced by cobalt, yielding an enzyme with about 45% of the activity of the zinc enzyme with respect to CO2 hydration at high pH when saturated with CO2. Since the cobalt enzyme is paramagnetic (8), measurements of T1 should provide a sensitive method for determining the presence or absence of the water molecule or hydroxide in question.

Previous papers in this series (9, 10) describe measurements of T1^{-1} as a function of magnetic field (nuclear magnetic relaxation dispersion) and indicate how this technique is capable of giving both qualitative and quantitative information about the protons involved in relaxation. The present series of experiments uses nuclear magnetic relaxation dispersion measurements to show the presence of an exchangeable proton on a ligand of the cobalt in both human B and bovine cobalt carbonic anhydrase.

EXPERIMENTAL PROCEDURE

Materials—Bovine carbonic anhydrase was prepared from blood by the chloroform-ethanol denaturation method described by Armstrong et al. (11). The procedure was modified in that, after dialysis to remove the chloroform and ethanol, the crude enzyme was dialyzed against 0.025 M Tris-HCl (pH 8.7) and run directly onto a DEAE-Septadex column. The column was then eluted with 0.1 Tris-HCl (pH 8.5). Human carbonic anhydrase B was also prepared by the chloroform-ethanol method described by Armstrong et al. with no modifications. The crude enzyme was chromatographed twice on DEAE-Septadex to ensure complete removal of carbonic anhydrase C. Cobalt carbonic anhydrase was prepared from the native enzyme by a method similar to that of Lindskog and Malmstrom (7). The preparation differed in that the orthophenanthroline concentration was raised to 10^{-2} M. In addition, the Co** was added to the apo-enzyme by dialyzing overnight against a 5-fold molar excess of Co^{2+}. The Co^{2+} which was not bound to enzyme was removed.
by extensive dialysis against distilled water. The Ethoxzolamide used was contributed by Upjohn and Company (Lot 494-P). All other chemicals were reagent grade.

**Carbonic Anhydrase Activity**—The enzyme purity was determined by the esterase activity toward p-nitrophenyl acetate as a measure of activity. Ethoxzolamide (6-ethoxybenzothiazole-2-sulfonamide) was used to “titrate” the enzyme: enzyme activity in arbitrary units was plotted against Ethoxzolamide concentration in moles per liter. This results in a linear plot due to the low dissociation constant (7 × 10^-10 M) of Ethoxzolamide with carbonic anhydrase (12). The intercept of the plot with the concentration axis gives the Ethoxzolamide concentration needed to produce zero activity. This concentration is equal to the active enzyme concentration in the assay mixture. The concentration of active enzyme was then compared to the concentration as determined by optical density, with a molar extinction coefficient at 280 nm of 5.6 × 10^4 for the bovine enzyme (13) and a value of 4.9 × 10^4 for the human enzyme (14). The native zinc enzymes, both human and bovine, typically gave values of 95 to 100% active enzyme by this assay. The reactivated cobalt enzymes gave values of 70% active enzyme for the bovine and 60% active enzyme for the human.

**Metal Determination**—The zinc and cobalt concentrations were determined with a Perkin-Elmer 303 atomic absorption apparatus with a scale expander and recorder.

**Enzyme Solutions** Solutions were prepared by adding 50 to 60 mg of lyophilized material to 0.5 ml of chilled buffer to give a final absorbance at 280 nm of 100 to 170 units for the bovine enzyme and an absorbance at 280 nm of 150 to 160 units for the human enzyme.

**Buffers**—The buffers used in the relaxation experiments were 0.1 M in Tris and 0.1 M in SO_4^-2 unless otherwise stated. The pH of the Tris was adjusted with H_2SO_4 and Na_2SO_4, was then added to maintain a constant SO_4^-2 concentration. Sulfate was chosen as a negative ion since the observations of Kernohan (14) and Lindskog (15) indicate that SO_4^-2 causes minimal shifts in the pH dependence of both the enzyme activity and the optical spectrum. Independent observations were also made on the visible spectrum of the bovine cobalt enzyme at pH 6.5 (cf. “Results”).

**Titration**—The titrations in the relaxation experiments were done by preparing two enzyme solutions of equal concentration (as determined by the absorbance at 280 nm) but different pH. The low pH solution had a pH of 5.9, and the high pH solution a pH of 8.0. Aliquots of 20 to 50 µl of the high pH solution were mixed with the low pH solution and vice versa. This method avoids variations in enzyme concentration, ionic strength, and anion concentration during the titration runs.

**Visible Spectra**—Visible spectra were run and recorded with a Cary model 14 spectrophotometer.

**Relaxation Measurements**—The procedures for measuring T_1^-1 are identical with those described in Reference 9. All T_1^-1 experiments were done in equilibrium with atmospheric CO_2. T_1^-1 data are expressed as relaxivity, R:

\[ R = (1 - V)(T_1^-1 - T_1^-0)/N \]  

where \( T_1^-1 \) is the observed relaxation rate of solvent protons in the protein solution, \( V \) is the volume fraction occupied by the protein, \( N \) is the molar concentration of protein, and \( T_1^-0 \) is the relaxation rate of the buffer.

** RESULTS **

**Frequency and pH Dependence**—When Ethoxzolamide reacts with carbonic anhydrase, a 1:1 complex is formed which is enzymatically inactive. Fig. 1 shows R plotted against \( \nu \) at 25° for the uninhibited bovine cobalt enzyme at pH 8.86, the inhibited bovine cobalt enzyme, and the bovine zinc enzyme. Also shown for the zinc enzyme are data taken at 9°, scaled in amplitude, and shifted in frequency by the relative values of the ratio of solvent viscosity to absolute temperature at 25° and 9°, according to the procedures outlined in Reference 9. For both the inhibited cobalt enzyme and the zinc enzyme, the data are independent of pH (over the approximate range pH 6 to pH 9) at all frequencies. R for the native zinc enzyme and the Ethoxzolamide-inhibited zinc enzyme is indistinguishable under all conditions.

![Image of the figure](http://www.jbc.org/)
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BOVINE CARBONIC ANHYDRASE in 0.1 M Tris O.IM SO4 pH 8.66 25°C

Fig. 2. The inhibitable part $R_{IN}$ and residual part $R_{RES}$ of the relaxivity of bovine cobalt carbonic anhydrase plotted against proton Larmor frequency. The solid lines are Equations 5 and 6 of the text, obtained by a least squares fit to $R_{IN}$ and $R_{RES}$, respectively. Each symbol represents a separate enzyme preparation.

MAGNETIC FIELD (OERSTEDS)

HUMAN CARBONIC ANHYDRASE in 0.1 M Tris O.IM SO4 pH 8.45 25°C

Fig. 3. Relaxivity plotted against proton Larmor frequency for the uninhibited human B cobalt enzyme (Co), the Ethoxzolamide-inhibited cobalt enzyme (Im Co), and the native human B zinc enzyme (Zn).

The solid curve through the zinc data, Fig. 1, is the function

$$42 + 367 \left(1 + \frac{\nu}{2.54}\right)^{-4}$$

This is an empirically chosen functional form which was found to represent the data quite well, and was used to smooth the data for subsequent manipulations.

The difference in $R$ between the uninhibited cobalt enzyme and the inhibited (pH-independent) cobalt enzyme is referred to as the inhibitable part of the relaxivity $R_{IN}$, the difference between the inhibited cobalt enzyme and the zinc enzyme is referred to as the residual part of the relaxivity $R_{RES}$. Both $R_{IN}$ at high pH and $R_{RES}$ for the bovine cobalt enzyme are plotted in Fig. 2.

The solid line through $R_{IN}$ is the function (cf. Equation 3)

$$151 + 388 \left(1 + \frac{\nu}{20.4}\right)^{-1}$$

This functional form is that anticipated from theory (cf. Equation 10 below). The line through $R_{RES}$ is the function

$$173 + 553 \left(1 + \frac{\nu}{2.54}\right)^{-1}$$

In this case, $\nu_0$ was fixed at the value found in Equation 4. In Equations 4 to 6, $\nu$ and $\nu_0$ are in megahertz.

Fig. 3 shows data for human B carbonic anhydrase analogous to the bovine data in Fig. 1. Although the data for the bovine and human enzyme are qualitatively similar, there is additional structure in the dispersion curves for the human enzyme, which is particularly noticeable in the results for the high pH cobalt enzyme. We believe that this structure, which disappears at low temperatures, is an artifact which can be explained by contamination of about $10^{-4}$ M Fe$^{3+}$ or Mn$^{2+}$. However, the presence of Fe$^{3+}$ and Mn$^{2+}$ has not been experimentally shown.

When the smoothed curve through the data for the human B zinc enzyme is subtracted from the high pH cobalt data, the upper curve in Fig. 4 is generated. $R_{RES}$ for the human B cobalt enzyme is the lower curve in Fig. 4. Qualitatively the results are quite similar to the analogous data for the bovine enzyme in Fig. 2. The major difference is that $R_{RES}$ for the human enzyme is very small.

Fig. 5 shows the pH dependence at 25° of the relaxivity in the limit of low frequency, $R(\nu = 0)$, for both the bovine and human enzymes. The solid lines in each case are a least squares fit to the data to a titration curve; the pK so derived is 7.0 ± 0.2 for the bovine cobalt enzyme and 8.2 ± 0.2 for the human B cobalt enzyme. The bovine result is to be compared to the apparent pK of 7.0 for the bovine zinc enzyme in the presence of 80 mM Cr$^{3+}$ (14) and the pK of 7.1 obtained by spectrophotometric titration of the bovine cobalt enzyme with ICl (15). For the human cobalt enzyme, an optical spectrum was run for each point on the relaxivity titration curve; the variation in absorbance at 640 nm parallels the relaxivity data. Bradbury (16) surveyed five pH titrations of the human B zinc enzyme and found
pH values between 7.3 and 7.4 reported. Coleman (4) reported a pK of 8.1 for both the zinc and cobalt human B enzyme.

**Temperature Dependence**—Fig. 6 shows the temperature dependence of \( R \) (\( \nu = 0 \)) for the uninhibited bovine cobalt enzyme at pH 8.86 (measured at room temperature), the inhibited cobalt enzyme, and the zinc enzyme (obtained at 16 kHz). The pH of Tris buffer increases as the temperature decreases; however, since the baseline for the bovine enzyme is pH-independent above pH 8.5, the results shown in Fig. 6 should not be affected. The temperature dependence of \( R_{\text{IN}} \) (\( \nu = 0 \)) is seen to be quite different from the temperature dependence of the \( R_{\text{RES}} \) (\( \nu = 0 \)). Both \( R_{\text{RES}} \) (\( \nu = 0 \)) and \( R \) (\( \nu = 0 \)) for the zinc enzyme have a temperature dependence directly proportional to the ratio of solvent viscosity to absolute temperature.

Analogous data for the human B enzyme are similar. At pH 8.86, this enzyme is about 80% titrated, and the results would be somewhat sensitive to pH changes of the buffer. However, the variations in \( R \) (\( \nu = 0 \)) were much greater than those anticipated from the pH variation.

**Inhibition**—Qualitatively the addition of both \( \text{NO}_3^- \) and \( \text{N}_3^- \) results in a decrease in \( R \). When the enzyme is saturated with azide (as judged from the optical spectrum), \( R \) is the same in magnitude and frequency dependence as that of the Ethoxzolamide-inhibited enzyme. When the Ethoxzolamide concentration is varied such that the enzyme is partially inhibited, one finds a 1:1 correlation between the percentage residual enzyme activity and the percentage decrease of \( R_{\text{IN}} \) (cf. Table I). These experiments were only performed on the bovine enzyme.

**Effect of Sulfate**—Na\(\text{SO}_4 \) was added incrementally, to a final concentration of 0.25 \( \text{m} \), to a solution of bovine cobalt carbonic anhydrase in distilled water with an initial and final pH of 6.5. Spectra were taken at \( \text{SO}_4^{2-} \) concentrations of 0.0, 0.05, 0.1, 0.15, and 0.25 \( \text{m} \). No changes in the visible spectrum were detected.

**Metal Content**—When the zinc enzyme was assayed for zinc, it was found to contain 0.95 to 1.05 zinc atom per enzyme molecule. However, when the bovine cobalt enzyme was assayed for cobalt per mole of active enzyme it was found to contain about 2 cobalt atoms per molecule. The extra cobalt was not removable by long term dialysis against distilled water. Extensive efforts were made to remove the extra cobalt. Conditions capable of removing the additional cobalt tended to remove cobalt from the active site. DEAE-Sephadex chromatography of the cobalt enzyme resulted in a single symmetrical peak containing carbonic anhydrase with two cobalts per molecule. Binding of zinc to the bovine apoenzyme in excess of the zinc required to restore enzyme activity has also been reported (17). Coleman has also observed the presence of a second strong binding site for cobalt in both the cobalt and the zinc enzymes.2

The native zinc bovine enzyme was also dialyzed against cobalt. After dialysis to remove excess cobalt it was found to contain 1 zinc atom and about 1 cobalt atom per molecule. The zinc-cobalt enzyme is pale orange in color (the zinc enzyme is colorless) with a very broad absorption spectrum in the visible, in marked contrast to the violet color of the cobalt enzyme. The rate of \( p \)-nitrophenyl acetate hydrolysis, the percentage of

\(^2\) J. E. Coleman, personal communication.
active enzyme per absorbance unit of the zinc-cobalt enzyme, and \( R \) for the zinc-cobalt enzyme were the same as for the native enzyme. It is therefore presumed that the excess cobalt does not affect \( R \) of the cobalt enzyme either.

A similar situation was found for the human B cobalt enzyme.

**INTERPRETATION AND DISCUSSION**

The general expression for \( R \) may be written as the sum of a diamagnetic contribution, \( R_d \), which for an approximately spherical protein like carbonic anhydrase should be of the form \( (10) \)

\[
R_d = A + B(1 + \langle \omega^2 \rangle)^{-1}
\]

with \( A \ll B \), and a paramagnetic contribution \( R_p \) given by (cf. Reference 11)

\[
R_p = R_0 + \sum_i R_i
\]

\[
R_i = \frac{n_i}{2N_w} \left[ G_i^{(0)} + \tau_{m,w} \right]^{-1}
\]

where \( N_w \) is the molarity of water, \( n_i \) is the number of occupied proton binding sites of the \( i \)th class on the protein, \( T_i^{(p)} \) is the relaxation time of a proton in the \( i \)th class site, and \( \tau_{m,w} \) the residence time of a proton in that class. The summation is over all the different sites in which \( T_i^{(p)} \) is due to an interaction with the paramagnetic ion. The contribution \( R_0 \) arises from solvent protons which diffuse freely, and come sufficiently close to the paramagnetic centers to be relaxed; it is generally referred to as the "outer sphere" relaxation.

For the case of \( Co^{2+} \) specifically bound to protein, for which the electron spin relaxation time \( \tau_S \) is expected to be quite short (18) (in Reference 18 (Erratum)), the authors quote \( \tau_S < 5 \times 10^{-11} \) sec for octahedrally coordinated \( Co^{2+} \), but speculate on the existence of tetrahedrally coordinated \( Co^{2+} \) (as in carbonic anhydrase) (8) at high temperature with a longer \( \tau_S \), the relaxation rate \( T_0^{-1} \), for any class of site should be given by \( (10, 19, 20) \)

\[
T_0^{-1} = \frac{2}{15} \frac{\gamma_S}{\gamma_R} \frac{2}{3} S(S+1) \tau_S \left( \frac{3+7}{1+\omega_S^2 \tau_S^2} \right)
+ \frac{2}{15} \frac{\gamma_S}{\gamma_R} \frac{2}{3} S(S+1) \tau_S \left( \frac{1}{1+\omega_S^2 \tau_S^2} \right)
\]

where \( S \) is the electronic spin of the \( Co^{2+} \), \( \omega_S \) is its Larmor frequency in radians per sec, \( r \) is its distance from the proton, and \( A \) is the strength of the isotropic interaction. \( \gamma_S \) and \( \gamma_R \) are the respective gyromagnetic ratios of the electronic moment and the proton moment. The two bracketed contributions in Equation 10 are the dipolar and isotropic (contact) terms, respectively. The usual assumptions that \( \omega_S > 2\pi \) and that \( \tau_S \) is the correlation time for both the dipolar and isotropic interactions are included. Depending on the relative magnitudes of the dipolar and isotropic interactions, \( T_0^{-1} \), will decrease at high fields to between 0 and 30% of its low field value.

**Inhibitable Part of Relaxivity**—Only that portion of the relaxivity which is inhibited by sulfonamides is relevant to the enzyme mechanism. This portion, which we refer to throughout as \( R_{IN} \), is here considered separately. The interpretation of the present results presumes that the anion binding site is the metal atom at the active site. Three separate techniques have given evidence of this. The earliest is Lindskog's work on the bovine cobalt enzyme (15). It was found that, for each anion and for the sulfonamide inhibitor acetazolamide, the cobalt enzyme had a distinct visible spectrum with maxima located at wave lengths characteristic of the anion. The infrared studies of Riepe and Wang (5) indicated that azide is bound to the metal in both the zinc and the cobalt enzyme and that the azide can be displaced by Ethoxzolamide, another sulfonamide. Recently, Ward (21) has used the quadrupole relaxation of \( ^{35}Cl \) by bovine zinc carbonic anhydrase to demonstrate that chloride ion binds to the zinc. He also found that in the presence of a sulfonamide (acetazolamide, in this case) access to the zinc by chloride was blocked. Lindskog's (17) studies of the visible spectrum of the cobalt enzyme when inhibited with sulfonamides combined with the x-ray studies of Fridborg et al. (22) also identify the sulfonamide binding site with the metal atom.

Above pH 8.5, for bovine cobalt carbonic anhydrase solutions, \( R \) at 16 kHz decreases from \( 1.75 \times 10^4 \) mole\(^{-1}\) sec\(^{-1}\) to 1.12 \( \times 10^4 \) mole\(^{-1}\) sec\(^{-1}\) when excess Ethoxzolamide inhibitor is added. If the enzyme is partially inhibited, there is a 1:1 relation (Table I) between the percentage residual esterase activity and the percentage inhibitable relaxivity remaining. This indicates that 1 or more of the protons involved in the relaxation at high pH are located within the active site. A similar effect could be produced by a conformation change outside of the active site, but there is no evidence that the binding of sulfonamides produces such a change.

Ethoxzolamide is a useful probe of the active site because of its low dissociation constant; however, it is very large and could conceivably displace molecules far from the metal ion. Azide is much smaller. When enough azide is added to inhibit the enzyme totally (as judged from the visible spectrum), \( R \) decreases to the value observed for the Ethoxzolamide-inhibited enzyme. The most plausible explanation is that the azide, known to be bound to the cobalt, physically displaces the molecule previously bound to the cobalt on which the exchangeable proton was located. Further support of this hypothesis comes from the magnitude and frequency dependence of \( R_{IN} \). Assume that there is one class of site which contributes to \( R_{IN} \). The entire frequency dependence of \( R_{IN} \) is then contained in the expression for \( T_0^{-1} \). It is quite clear that the functional form of the data (cf. Equation 5) is described by Equation 10. If one assumes \( \tau_M < T_0 \), and 1 exchanging water molecule per cobalt atom at the active site then one can calculate, with Equations 5 and 10, that, at high pH, \( T_0 \approx 1.7 \times 10^8 \) sec. The reasons for assuming only 1 water molecule are several fold: the magnetic susceptibility studies (8) and the visible extinction coefficient of the cobalt enzyme (7) indicate that the cobalt is tetrahedrally coordinated, the x-ray studies indicate that 3 amino acid residues are coordinated with the metal (22), and anion-binding studies indicate that there is only one anion and hence only one water-binding site on the metal (4, 6, 15, 21). Comparing Equations 9 and 10, one obtains \( \tau_M = 1.2 \times 10^{-11} \) sec. From this correlation time and the value of \( A \) (23) for a proton in a water of hydration bound to a \( Co^{2+} \) ion, one finds that the contribution to \( R_{IN} \) of the isotropic interaction is about 20 mole\(^{-1}\) sec\(^{-1}\) and therefore negligible. In addition, an isotropic interaction between the rapidly exchanging proton involved in \( R_{IN} \) and the cobalt would be easily detectable by the shift of the water resonance in a high resolution NMR experiment. No such shift was detected when concentrated bovine cobalt carbonic anhydrase
solutions were examined at 220 MHz. Therefore, the dipolar interaction is responsible for the frequency dependence of $R_{IN}$. Since $R_{IN}$ decreases at high frequencies to $\sim 30\%$ of its low field value, it must be that $\tau_M < T_{1pr}$, as assumed. From $\tau_S$, $R_{2H}$, and the magnetic susceptibility of cobalt in carbonic anhydrase (8) (which gives a value of $\gamma_S^{N}(S + 1)$), we compute $r = 2.85$ Å. If 1 proton on a hydroxide is involved, $r = 2.5$ Å. The former distance corresponds very well to the distance of 2.8 Å which one would expect between the cobalt and a proton on a water molecule to the cobalt (see Reference 24, Table VI-4).

It has been suggested that a histidine residue could be coordinated to the metal ion. However, the distance of 2.5 Å for 1 proton is too small to be compatible with the distance of about 5 Å (25) estimated for the cobalt-proton distance in the case of a proton located on the imidazole moiety of a histidine residue. Therefore, we believe that the relaxivity data imply that the exchangeable proton is located on a water molecule or hydroxide ion directly liganded to the cobalt.

For the human B enzyme, the values for $\tau_S$ and the proton-cobalt distance are essentially the same as for the bovine enzyme.

It would be desirable at this point to evolve a mechanism which can account for both the pH dependence of $R_{IN}$ and for the rate of proton exchange. There are two general mechanisms for exchanging protons between the metal ligand and the bulk solution. One is exchange of the proton while the oxygen atom remains bonded to the metal; the other is exchange of the entire hydroxide ion or water molecule. Neither the known rate nor pH dependence of proton exchange is compatible with the involvement of hydroxide ions from the solution in explaining the small size of $\tau_M$ (cf. Reference 10). Therefore, proton or ligand exchange must be mediated by water molecules from the solution phase. The pH dependence of $R_{IN}$ has the form of a titration curve with its maximum value at high pH and a pK of 7.0 + 0.2 for the bovine cobalt enzyme, and a pK of 8.2 ± 0.2 for the human B enzyme. The pK of 8.2 for the human B enzyme does not seem to be an artifact due to contamination by inhibitory anions, since the visible spectrum of the undiluted low pH samples showed no evidence of anion binding. To explain the pH dependence we assume that, since the low pH value of $R_{IN}$ is essentially that of the inhibited enzyme, the pH dependence of $R_{IN}$ simply reflects a variation in the concentration of a high pH form, and that at low pH either the protons are not relaxed or that the exchange of the protons becomes very slow. The pH dependence cannot be due to a difference in the paramagnetic susceptibility of the two forms of the enzyme since the results of Lindskog and Ehrenberg show that the susceptibility is quite insensitive to the cobalt ligand (8). The conclusion is that the high pH form has a ligand to the metal which is either a hydroxide ion or a water molecule, with 1 proton hydrogen bonded to a nearby imidazole. The low pH form would contain a slowly exchanging water molecule or, conceivably, have no fourth metal ligand at all. The rate of exchange of a proton on a hydroxide ion bound to the cobalt (in the high pH case) could be faster than the rate of exchange of protons on a water molecule similarly bound (in the low pH case) if one assumes that there is a simultaneous exchange of the hydroxide proton with a proton on an incoming water molecule.

This type of exchange would probably proceed more slowly or not at all when a water molecule is bound to the cobalt.

Relaxivity of Native Enzyme—In all aspects, the variation of the relaxivity of the native bovine enzyme solutions with temperature and frequency is analogous to apotransferrin solutions, as discussed in detail in Reference 9. One concludes that the present data can be explained, qualitatively, by a model in which a small, temperature-independent, number of water molecules (about 10) bind to each enzyme molecule in such a manner that they sense its rotatory Brownian motion. The residence time of these exchangeable water molecules must lie within the interval $10^{-8}$ to $10^{-6}$ sec.

For apotransferrin, it was noted that the functional form of the relaxation dispersion could not be explained quantitatively. The theoretical situation in that case was complicated because apotransferrin is a highly protate molecule. The molecules of bovine zinc carbonic anhydrase are close to spherical, as determined from x-ray (22) and viscosity (12) data. Therefore, the theoretical expectation is that the frequency-dependent part of the relaxivity of the zinc enzyme should be describable by Equation 3, rather than Equation 2. This difference between theory and experiment is not as yet understood.

Residual Relaxivity—The difference between the relaxivity of the inhibited cobalt enzyme and the native zinc enzyme also is not understood. The difference is quite small for the human B case, but is substantial for the bovine case, for which a comparison of Equations 4 and 6 shows that $R_{RES}$ can be regarded as an increase in $R_{H}$ by a factor of about 2. It would appear that replacement of zinc by cobalt in the bovine enzyme roughly doubles the number of water molecules bound by the protein. It is difficult, however, to understand how this could occur. The additional cobalt atom bound per active cobalt enzyme molecule does not appear to play a role in $R_{RES}$ since (a) it binds to the bovine zinc enzyme and produces no contribution to $R$ and (b) the magnitude of the $R_{RES}$ is so different in the human and bovine enzymes.

REFERENCES
