^{35}Cl Nuclear Magnetic Resonance Studies of a Zinc Metalloenzyme Carbonic Anhydrase*

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ABSTRACT: The interaction of chloride ions with the Zn(II) metal of bovine carbonic anhydrase has been studied by ^{35}Cl nuclear magnetic resonance spectroscopy. It has been shown that there exists one zinc coordination site available for chloride interaction. This zinc–chloride interaction can be inhibited by cyanide and acetazolamide. The zinc–chloride interaction is highly pH dependent decreasing to zero above pH 10. The apparent pK_a of the chloride broadening is chloride concentration dependent and measurements at several chloride concentrations yield, on extrapolation to zero chloride, a value of 7.0 ± 0.1. The ^{35}Cl nuclear magnetic resonance spectrum has been observed in the presence of the apoenzyme. The apoenzyme is approximately 25 times less effective in broadening the chloride line than carbonic anhydrase. Carbonic anhydrase is approximately a 1000-fold more effective than aquo Zn(II) ions in broadening the chloride line. The effectiveness of carbonic anhydrase, as compared to aquo Zn(II) ions, in changing the ^{35}Cl relaxation rate is attributed to the fact that both the chloride exchange process and the molecular rotational interactions have correlation times of the order of 10^{-8} sec, whereas for aquo Zn(II) ions the molecular rotational process dominates the relaxation mechanism with a correlation time of the order of 10^{-11} sec.

Recent work (Ward and Happe, 1967) has shown that ^{35}Cl nuclear magnetic resonance, introduced by Stengle and Baldeschwieler (1966, 1967) in a study of mercury complexes of macromolecules, can be used to study small zinc complexes. We now wish to report on a ^{35}Cl nuclear magnetic resonance investigation of the zinc-containing enzyme carbonic anhydrase.

Erythrocyte carbonic anhydrases are highly compact zinc metalloenzymes with molecular weights of the order of 30,000. These enzymes catalyze the physiologically important reaction, the reversible hydration of carbon dioxide to form bicarbonate. The zinc atom is firmly bound to the protein and appears to be located at the active site. An excellent discussion of the properties and other reactions catalyzed by carbonic anhydrase is given in the review by Edsall (1968).

Evidence will be presented in this paper that chloride ions interact with the zinc atom of carbonic anhydrase and that this interaction, as measured by ^{35}Cl nuclear magnetic resonance, correlates well with the observed pK_a for enzymic activity, with cyanide inhibition, and with the known binding of the potent sulfonamide inhibitor, acetazolamide.

Experimental Section

Materials. Bovine carbonic anhydrase was obtained from Worthington Biochemical Corp. All samples were thoroughly dialyzed against EDTA or EDTA–1,10-phenanthroline mixtures and Tris buffer (pH 8). They were then lyophilized and stored in the cold. Apoenzyme was prepared as reported by Henkens and Sturtevant (1968). Acetazolamide was purchased from K & K. All other chemicals were analytical reagent grade.

Nuclear Magnetic Resonance Measurements. The ^{35}Cl spectra were obtained at 5.88 MHz with a Varian V-4311 fixed radiofrequency unit. A PAR Model HR 8 lock-in amplifier was used for field modulation and phase sensitive detection. The spectrometer was equipped with a field-frequency lock system. The radiofrequency field level was low enough so that saturation effects were absent. The ^{35}Cl line widths were measured from recorded spectra as the full width at half-maximum amplitude. The spectra were calibrated by changing the frequency of the external lock signal. The reported line widths are the average of at least five spectra and are reproducible to about 5% for the narrowest lines and to about 10% for the broadest lines. The resolution was such that 0.5 M aqueous NaCl exhibited a line width, $\Delta\nu_{Cl^-}$, of 12.5 ± 1 Hz. Relaxation rate and line widths are related by the expression, $\pi\Delta\nu = 1/T_2$. All spectra were obtained at the probe temperature, 32°.

Theory

The ^{35}Cl nucleus has a spin of $^3/_2$ and an electric quadrupole moment Q. Interactions between the electric quadrupole moment and fluctuating electric field gradients at the nucleus provide the dominant nuclear magnetic relaxation mechanism for chlorine nuclei in solution. Aqueous chloride ions, however, exhibit a fairly narrow, 12.5 ± 1 Hz, ^{35}Cl nuclear magnetic resonance absorption because of symmetrical hydration by water

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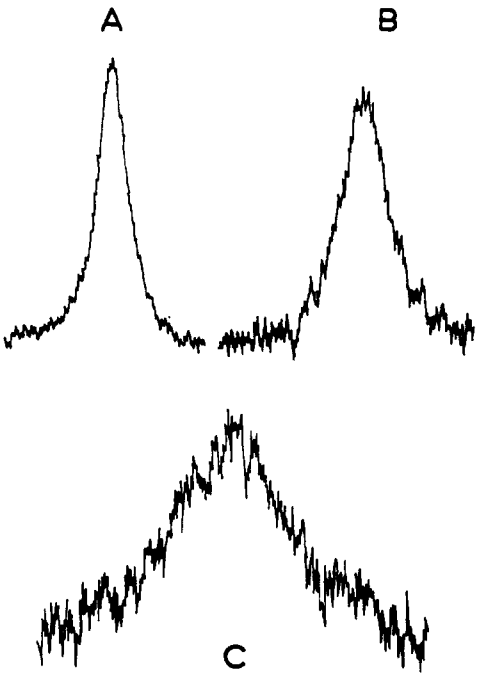

FIGURE 1: ^{35}Cl nuclear magnetic resonance absorption of 0.5 M NaCl solution. (A) no protein, $\Delta\nu_{Cl^-} = 12.5$ Hz. (B) 3.2×10^{-5} M apocarbonic anhydrase in 0.01 M acetate buffer (pH 4.8), $\Delta\nu - \Delta\nu_{Cl^-} = 7.0$ Hz. (C) 4.1×10^{-6} M carbonic anhydrase in 0.01 M Tris buffer (pH 8.2), $\Delta\nu - \Delta\nu_{Cl^-} = 49.4$ Hz.

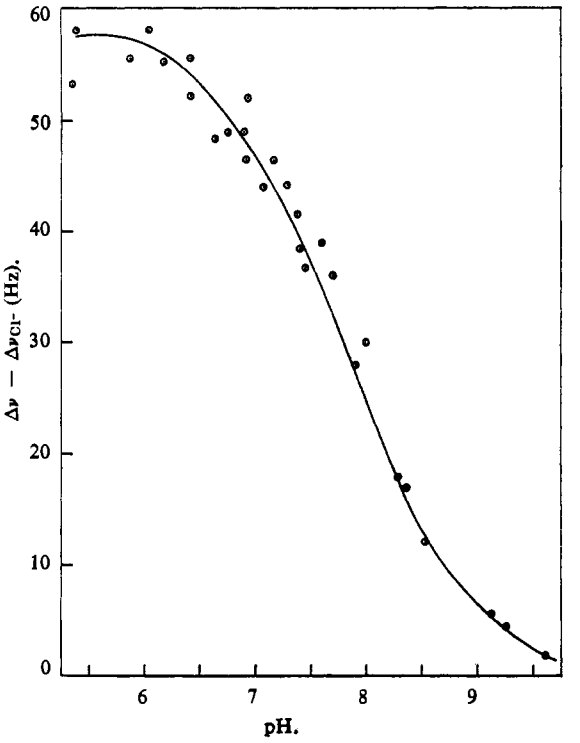

FIGURE 2: The increase in ^{35}Cl line width, $\Delta\nu - \Delta\nu_{Cl^-}$ (Hz), *vs.* pH for a solution containing 0.5 M NaCl and 2.29×10^{-5} M carbonic anhydrase.

molecules. For those environments in which chlorine nuclei experience electric field gradients the line width is given by (Abragam and Pound, 1953)

$$\Delta\nu = \left(\frac{2\pi}{5}\right)[e^2qQ]^2\tau \qquad (1)$$

where $\Delta\nu$ is the full width at half-maximum amplitude, q is the electric field gradient at the nucleus, and τ is a correlation time which describes the random molecular motions responsible for the time-varying electric field gradients.

For those systems in which chloride ions can exist at various sites, such as bound to Zn(II) or as free aqueous chloride ions, and for which the exchange rate of the chloride ion among these sites is rapid compared with the width of the broadest line, a composite signal is observed (Ward and Happe, 1967). This composite signal can be described by the expression $\Delta\nu = \Sigma(\Delta\nu_i)P_i$, where P_i is the probability that chloride ions are at site i.

In order to compare the effectiveness of zinc ions in various environments in altering the relaxation rate of chloride ions we define the quadrupolar enhancement parameter, ϵ_q, as

$$\epsilon_q = \frac{\Delta\nu^* - \Delta\nu^*(0)}{\Delta\nu - \Delta\nu(0)} \qquad (2)$$

where $\Delta\nu$ is the observed line width, $\Delta\nu(0)$ the line width in the absence of zinc ions, and the asterisk indicates the presence of a chelating agent. It has been previously reported (Ward and Happe, 1967) that $\Delta\nu - \Delta\nu(0)$ for zinc ions in 0.5 M NaCl is equal to 2×10^3 Hz mole^{-1} l. [Zn(II)]. We have observed enhancements of less than one for Zn(II)–EDTA, between 2 and 15 for Zn(II) nucleotide di- and triphosphate complexes, and approximately 1000 for carbonic anhydrase.

Results

Carbonic Anhydrase Chloride Interactions. The ^{35}Cl nuclear magnetic resonance spectra of 0.5 M NaCl solutions containing carbonic anhydrase (4.1×10^{-5} M, pH 8.2), apocarbonic anhydrase (3.2×10^{-5} M, pH 4.8), and no protein are presented in Figure 1. The ^{35}Cl nuclear magnetic resonance line width in the presence of carbonic anhydrase varies linearly with protein concentration over the range measured, 0 to $\sim 4.8 \times 10^{-5}$ M. Although the protein dependence of the line width for the apoenzyme has not been measured, it too is expected to be linear over the range of interest. The chloride broadening at a fixed concentration of carbonic anhydrase is strongly pH dependent (Figure 2) and it is important in comparing the chloride line widths in Figure 1 to note the pH difference of the two protein solutions. The ^{35}Cl nuclear magnetic resonance measurement in the presence of carbonic anhydrase was made at pH 8.2, whereas the pH of the apoenzyme solution was 4.8. From Figure 2 it can be estimated that the ^{35}Cl line width in the presence of carbonic anhydrase, at the same pH as the apoenzyme, would be at least three times larger than that shown in

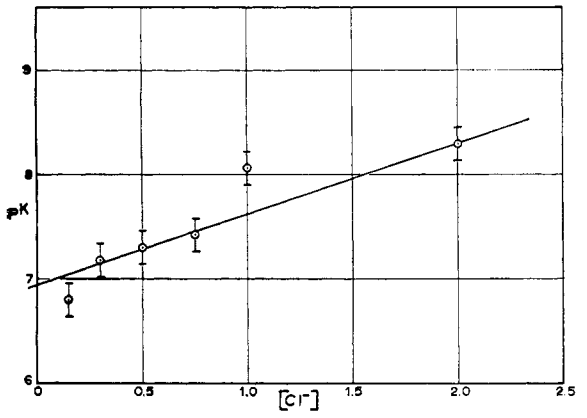

FIGURE 3. Apparent pK_a for bovine carbonic anhydrase as a function of total chloride concentration.

Figure 1. The increase in the ^{35}Cl line width for comparable protein concentrations (3.22×10^{-5} M) at pH 5 in 0.5 M NaCl would thus be ~130 Hz for carbonic anhydrase and 7 Hz for the apoenzyme. This 7-Hz broadening in the presence of apocarbonic anhydrase is readily explained in terms of the presence of ~5% residual zinc and the nonspecific broadening by the protein.

The pH dependence of the ^{35}Cl nuclear magnetic resonance broadening produced by the apoenzyme was difficult to obtain due to the instability of the apoenzyme at the temperature of the nuclear magnetic resonance probe, ~32°. The points that were obtained showed a decrease in broadening as the pH was increased, with the broadening approaching zero about pH 10. The apoenzyme exhibited less than 5% of the normal activity of our bovine carbonic anhydrase preparation, as measured by the hydrolysis of *p*-nitrophenylacetate, and full activity was regained upon addition of Zn(II) ions. We observed no effect on the stability of carbonic anhydrase after a number of hours at 32° at concentrations of 1–2 mg/ml.

These data demonstrate the effect of the presence of zinc in carbonic anhydrase in changing the relaxation rate of chloride ions. In terms of the enhancement factor, ϵ_q, carbonic anhydrase is approximately 1000 times more effective than Zn(II) ions in relaxing chloride ions. Although the electric field gradient at the chloride site is probably somewhat different in carbonic anhydrase as compared with aqueous zinc chloride, it appears that the correlation time for the quadrupolar interaction is the dominant factor in the large enhancement (see Discussion section).

The pH dependence of the ^{35}Cl broadening (Figure 2) in the presence of carbonic anhydrase, as will be shown, can be correlated with proposed modes of action of carbonic anhydrase. We have observed that the apparent pK_a is a function of the total chloride ion concentration (Figure 3) but that an estimate of the pK_a for the enzyme site can be made by extrapolating to zero chloride. This value is 7.0 ± 0.1. This pK_a agrees quite well with that reported for enzymic activity as measured by the hydrolysis of *o*- and *p*-nitrophenylacetate (Verpoorte *et al.*, 1967) and from the pH *vs.* activity profile of the bovine enzyme using CO_2 as the substrate. Recently

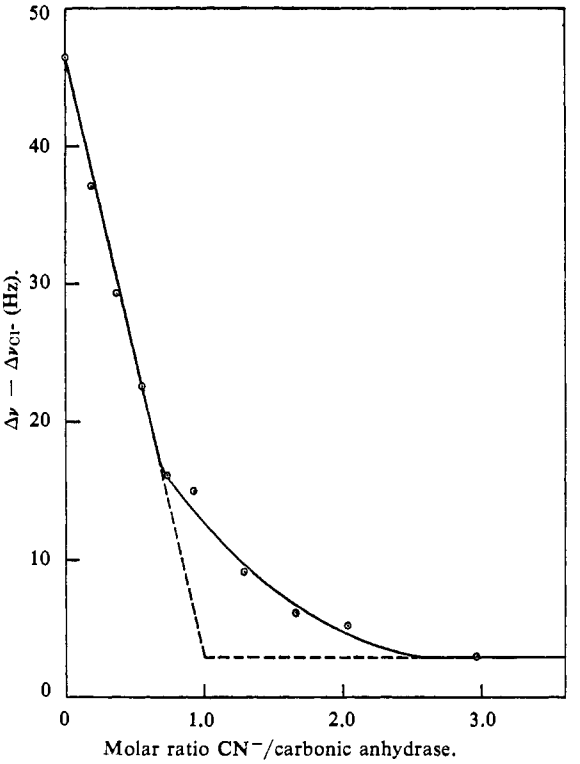

FIGURE 4: $\Delta\nu - \Delta\nu_{Cl}$ (Hz) *vs.* molar ratio of CN^-/carbonic anhydrase for a solution containing 0.5 M NaCl, 3.8×10^{-5} M carbonic anhydrase, and 0.05 M Tris buffer (pH 8.2).

Pocker and Stone (1968) have obtained a value of pK_a ~ 7.5 in a study of the inhibition of bovine carbonic anhydrase by chloride ion.

Available Zinc Coordination Sites. Cyanide ion binds at the zinc site of carbonic anhydrase and strongly inhibits the enzymatic activity. We have titrated a solution of carbonic anhydrase (3.8×10^{-5} M) in 0.5 M NaCl (pH 8) with cyanide ion using the ^{35}Cl nuclear magnetic resonance line width as the measured parameter (Figure 4). The addition of stoichiometric amounts of CN^- reduces the chloride line width to nearly that observed for free chloride. Extrapolation of the linear portion of the curve to the base line yields a ratio of carbonic anhydrase to CN^- of 1:1. This stoichiometry and the almost complete removal of broadening clearly show that Cl^- is interacting with the zinc atom of carbonic anhydrase and, moreover, that there is only one zinc coordination site available for Cl^-, and thus presumably for CN^- or H_2O binding.

Sulfonamide Inhibition. In 1940, Keilin and Mann reported the discovery that sulfonamides were highly specific and potent inhibitors of carbonic anhydrase. The addition of acetazolamide, in stoichiometric amounts, to a solution of carbonic anhydrase reduces the ^{35}Cl broadening to that of the cyanide-inhibited enzyme (Figure 5). The end point is sharp at a molar ratio of carbonic anhydrase to acetazolamide of 1:1. This stoichiometry agrees with that reported by Lindskog (1963).

The fact that approximately 95% of the chloride broadening produced by carbonic anhydrase is removed

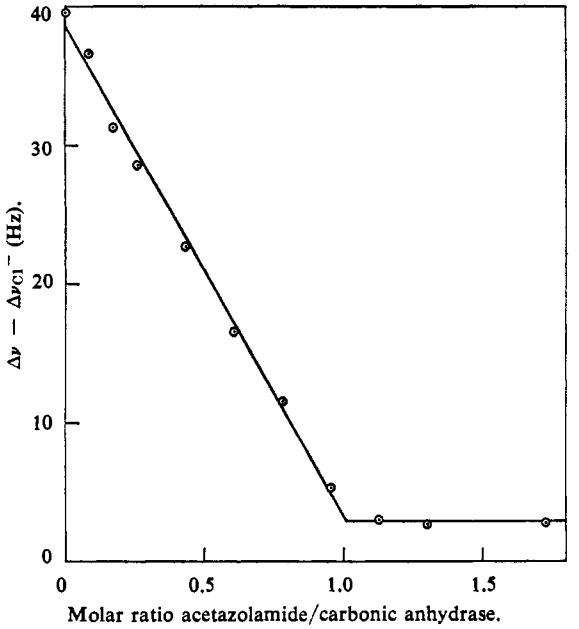

FIGURE 5: $\Delta\nu - \Delta\nu_{Cl^-}$ (Hz) *vs.* molar ratio of acetazolamide/carbonic anhydrase for a solution containing 0.5 M NaCl, 4.05×10^{-5} M carbonic anhydrase, and 0.05 M Tris buffer (pH 8.05).

by the addition of 1 equiv of acetazolamide implies that the chloride ion probe is interacting near or at the active site of carbonic anhydrase. The remaining 5% of broadening is due to viscosity or nonspecific structural effects on the symmetrical solvation of chloride ions.

Discussion

It has been demonstrated that the Zn(II) site of carbonic anhydrase is the dominant site for increasing the relaxation rate of chloride ions. Cyanide–chloride competition has been used to show that there exists only one zinc coordination site for chloride interaction. Further, the addition of the potent inhibitor acetazolamide reduces the chloride nuclear magnetic resonance broadening to that of the cyanide-inhibited enzyme. The pH profile of the chloride broadening also indicates that chloride ion at high pH either does not bind to the zinc atom or that chloride exchange is prevented.

Roughton and Booth in 1946 reported that carbonic anhydrase was inhibited by anions. Using CO_2 as a substrate at pH 7.4 they showed that studies at two concentrations of NaCl revealed that the binding of a single chloride ion was sufficient to abolish enzyme activity. They also observed that at high pH the affinity of the enzyme for all anions decreased markedly. Kernohan (1964) confirmed these observations for the bovine enzyme and in addition noted that the disappearance of anion inhibition at high pH suggested a relationship between the pH dependence of activity and the pH dependence of anion binding.

Lindskog (1966) and Lindskog and Thorslund (1968) studied the effect of anions on the pK_a of the group controlling activity. Their data showed that anions that bind more tightly increased the pK_a more than less inhibitory species. We have preliminary data on the broadening of the nuclear magnetic resonance absorption of ^{81}Br nuclei by bovine carbonic anhydrase which indicates that the pK_a in 0.5 M NaBr solutions is about 8.5. This is to be compared to the less strongly bound chloride ion (Roughton and Booth, 1946) which has a pK_a in 0.5 M NaCl of ~7.3. Furthermore, they showed that an increase in the concentration of any given anion caused a corresponding increase in the pK_a of the group governing both hydrase and esterase activity. This observation is in agreement with the ^{35}Cl nuclear magnetic resonance observations (Figure 3).

Mann and Keilin in 1940 suggested that the inhibitory action of sulfonamides was a result of their interaction with the zinc atom. Lindskog (1963) showed by equilibrium dialysis experiments that equimolar amounts of acetazolamide were bound to the Zn(II) and Co(II) bovine enzyme with K_I values of 0.009 and 0.008 μM, respectively, and that the metal-free enzyme bound essentially no inhibitor. Whitney *et al.* (1967) have also shown that sulfanilamide and chloride act as competitive inhibitors of human carbonic anhydrase B which indicates that they bind at the same site or have part of their binding sites in common. The crystallographic study (Fridborg *et al.*, 1967) of the human carbonic anhydrase C–sulfonamide complex also shows that the sulfonamide group is in the vicinity of the zinc atom.

Our ^{35}Cl nuclear magnetic resonance data reveal that chloride ion must bind directly to the Zn(II) ion of carbonic anhydrase. Although the cyanide titration data demonstrate cyanide–chloride competition for the zinc atom, the acetazolamide nuclear magnetic resonance data can be interpreted in either of two ways. Acetazolamide can either occupy the available zinc coordination site or it can bind to the enzyme in such a manner as to prevent chloride exchange. Most of the available data, however, does indicate the close proximity of the zinc site and the acetazolamide binding site.

The pH dependence of the chloride broadening in the presence of carbonic anhydrase (Figure 2) and the dependence of the observed pK_a on chloride concentration appear to correlate with the pH dependence of the reactions catalyzed by carbonic anhydrase. This pH dependence suggests that the basic form of an ionizing group in the enzyme with a pK_a near 7 is involved in the hydration and esterase reactions. Davis (1959) suggested that the function of the zinc was to donate a bound hydroxyl group to CO_2 in the hydration reaction, thus forming bicarbonate ion. The reverse process would take place in the dehydration reaction. The ionization of the zinc–enzyme complex (E–Zn–OH_2) could be written as

$$\text{E–Zn–OH}_2 \rightleftharpoons \text{E–Zn–OH}^- + \text{H}^+ \qquad (3)$$

and E–Zn–OH^- would be the reactive form in the hydration reaction. The assumption of a pK_a value near 7 for this ionization is debatable. The corresponding pK_a value for aquo zinc complexes is near 9 (Chaberek *et al.*, 1952), but it is possible that the environment in the enzyme molecule could conceivably shift the pK_a to lower values (Koshland and Neet, 1968).

The observed apparent pK_a value near 7, however, also suggests that the basic form of an imidazole group in a histidine residue of the enzyme could be involved. The imidazole group could bind at the zinc site and prevent the zinc–chloride interaction. Certainly Edsall's group has obtained evidence that a histidine residue in human carbonic anhydrase B is closely associated with the active site (Edsall, 1968). R. L. Ward and J. T. Edsall (to be published), however, have obtained a pK_a of ~9.3 for human carbonic anhydrase in 0.5 M NaCl. This value would tend to disprove the idea of an imidazole group being the cause of a reduction of the zinc–chloride interaction.

The early results of Roughton and Booth (1946) revealed that the reversible binding of a single chloride ion was sufficient to abolish enzyme activity. The studies presented here and the known effects of inhibitors and pH on the enzymic activity indicate that this single chloride binding site, which abolishes activity, is the zinc atom of carbonic anhydrase.

Verpoorte *et al.* (1967), however, have demonstrated that human carbonic anhydrase B binds approximately six chloride ions in 0.1 M KCl and only one chloride ion in 0.005 M KCl. The exact number of chloride binding sites is not known for human carbonic anhydrase C or the bovine enzyme. Because we have observed similar ^{35}Cl nuclear magnetic resonance effects (except for the different pK_a's mentioned earlier) for the three enzymes, a comment is in order concerning the possible effects of this additional chloride binding on the ^{35}Cl nuclear magnetic resonance observations. Approximately 6% of the initial broadening produced by carbonic anhydrase remains after the addition of the inhibitors cyanide or acetazolamide. This residual broadening can be attributed to viscosity effects, nonspecific chloride binding, or any mechanism whereby the symmetrical water environment about the chloride ion is altered. Viscosity effects have been noted by Marshall (1968) who reported a linear increase in chloride line width with added sucrose up to 1 M sucrose. Nonspecific chloride binding sites, which do not produce a reasonable electric field gradient at the chlorine nucleus, will produce little broadening.

Although the electric field gradient at the chloride ion is probably somewhat different in the aquo zinc chloride complex from that in the carbonic anhydrase complex, it is not expected to be an order of magnitude different (unpublished results on zinc complexes). It thus appears that the correlation time for the quadrupolar interaction must be the primary cause of the large enhancement of ~1000 for carbonic anhydrase. Chen and Kernohan (1967) have estimated the rotational relaxation time of carbonic anhydrase from depolarization of fluorescence data and obtained a value of about 3×10^{-8} sec, which is consistent with a low degree of molecular asymmetry for the molecule. Eigen and Wilkins (1965) have reported that the chloride exchange time for aqueous zinc chloride is also of the order of 10^{-8} sec. If this chloride exchange time is not affected greatly by the protein structure, then the effective correlation time for chloride interaction with carbonic anhydrase should be determined by a combination of these two processes. Thus the large quadrupolar enhancement exhibited by carbonic anhydrase is a result of the much longer effective correlation time, ~10^{-8} sec, compared with the shorter rotational correlation time, 10^{-11} sec, of the aquo zinc chloride complex.

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