Temperature and Frequency Dependence of Solvent Proton Relaxation Rates in Solutions of Manganese(II) Carbonic Anhydrase†

Amos Lanir,‡ Sonia Gradstajn,§ and Gil Navon*

ABSTRACT: Longitudinal and transverse proton relaxation rates of water in solutions of manganese(II) bovine carbonic anhydrase have been measured by pulsed nuclear magnetic resonance spectrometry as a function of temperature (2–35°), frequency (5–100 MHz) and pH. The pH dependence of the longitudinal relaxation rate was fitted to a sigmoidal curve with a pK value at 7.8, while the esterase activity of the manganese(II) enzyme in the hydrolysis of p-nitrophenyl acetate revealed an inflection point at pK = 8.2. The hydration number of manganese(II) carbonic anhydrase could be derived using either the frequency dependence of T_{1p} or the T_{1p}/T_{2p} ratio at only one (high) frequency. Both treatments are in agreement with a model in which one water molecule is bound to the metal at high pH. At low pH the relaxation data imply that no H_2O exists in the first coordination sphere of the manganese ion. The various parameters which are responsible for the proton relaxation mechanisms have been evaluated and are compared to other manganese(II) enzyme systems. The pH dependence of the binding constant of manganese to apocarbonic anhydrase is also reported.

Carbonic anhydrase (carbonate hydrolyase EC 4.2.1.1) is a zinc containing enzyme. It catalyzes the reversible hydration of carbon dioxide, as well as other reactions like hydrolysis of esters and hydration of aldehydes. The zinc atom can be replaced by various divalent metal ions and the various metallocarbonic anhydrases can be used to probe the immediate environment of the catalytic center. It has been shown (see Lindskog *et al.*, 1971) that a basic form of a group closely linked to the metal ion and having a pK_a near 7 is critically involved in catalysis. There are two main proposals as to the nature of this group. The pK_a may be assigned to an ionizable basic amino acid side chain such as a histidine residue (Pocker and Stone, 1967; Pocker and Storm, 1968; Appleton and Sarkar, 1974). However, most investigators believe that the mechanism of the enzyme involves a proton dissociation from a zinc-coordinated water molecule to form a zinc-coordinated hydroxide ion which is active in the hydration and esterase reactions. Their arguments are summarized in a very recent paper by Lindskog and Coleman (1973). Nevertheless, no direct evidence for the zinc hydroxide mechanism has been presented. The nuclear magnetic relaxation technique is a valuable tool for the observation of water molecules bound either directly or very close to the paramagnetic metal ions in metalloenzymes (Mildvan and Cohn, 1970; Dwek, 1973). Fabry *et al.* (1970) and Lanir *et al.* (1973) followed the pH dependence of solvent proton relaxation in the presence of the Co(II) or Mn(II) carbonic anhydrases. It was suggested (Koenig and Brown, 1972; Lanir *et al.*, 1973) that a water molecule rather than a hydroxide ion is directly linked to the metal ion at high pH values, while probably no rapidly exchanging water molecule is bound at low pH values. This indicates that the ionizing group on the enzyme with a pK near 7 that is involved in the catalysis is a basic amino acid side chain.

In this paper we further characterized the manganese-substituted carbonic anhydrase using the temperature and frequency dependencies of the proton relaxation rates of the water molecules in the first solvation shell of the manganese(II) ion. The physical mechanisms which govern the relaxation process were investigated in an attempt to substantiate our previous conclusions concerning the water molecule in the coordination sphere of the manganese atom in Mn(II) carbonic anhydrase (Lanir *et al.*, 1973).

Experimental Section

Materials. Bovine carbonic anhydrase B prepared and purified by the method of Lindskog (1960) was obtained from Seravac. Acetazolamide (5-acetamido-1,3,4-thiadiazole-2-sulfonamide), p-toluenesulfonamide, and p-nitrophenyl acetate were purchased from Koch-Light Labs. All other compounds were reagent grade.

Enzyme Preparation. The procedure for preparation of manganese carbonic anhydrase from the native zinc enzyme was described in a previous publication (Lanir and Navon, 1972). The residual zinc content was determined either by atomic absorption (Varian spectrometer Type AA-5) or by selective inhibition of the manganese and zinc enzymes mixture by acetazolamide (Lanir and Navon, 1972). The protein concentration was determined from measurements of the optical density at 280 nm taking a molar absorptivity of 5.7×10^4 cm^{-1} M^{-1} and a molecular weight of 30,000 (Nyman and Lindskog, 1964).

Enzymatic activity was determined using p-nitrophenyl acetate as a substrate by following the absorbance at 348 nm. This wavelength is the isosbestic point for p-nitrophenol and p-nitrophenolate ion, with $\epsilon = 5.4 \times 10^3$ M^{-1} cm^{-1} (Armstrong *et al.*, 1966).

† From Department of Chemistry, Tel-Aviv University, Ramat-Aviv, Tel-Aviv, Israel. *Received July 18, 1974.* Part of this work was presented at the 42nd Conference of the Israel Chemical Society (Lanir *et al.*, 1972).

‡ Present address: Department of Biochemistry, George S. Wise Center for Life Sciences, Tel-Aviv University, Ramat-Aviv, Tel-Aviv, Israel.

§ Deceased June 1, 1973.

Electron Paramagnetic Resonance Spectra and Determination of Free Mn(II). Concentrations of free Mn(II) were determined from the peak to peak heights of the electron paramagnetic resonance (epr) spectra obtained on a Varian V-4500 spectrometer. This is possible because complexes of Mn(II) have extremely broad epr spectra in the X-band frequency region.[1] The sample, approximately 0.02 ml in volume, was held in a quartz capillary tube positioned in the cavity. The equilibrium constant at each pH value is the average of three measurements obtained with different manganese concentrations. The amplitudes of each sample containing the enzyme was compared to that of a standard $MnCl_2$ solution.

Nuclear Magnetic Resonance (nmr) Experiments. Longitudinal relaxation times at 5–60 MHz were measured with a Bruker Type B-KR-322 pulsed spectrometer, and at 100 MHz using a spin-echo attachment to a high resolution nmr spectrometer (Ginsburg *et al.*, 1970), by the 180–90° null method. Values of T_2 were obtained at 100 MHz from the spectral line width, using the expression $1/T_2 = \pi\Delta\nu$, where $\Delta\nu$ is the full line width at half-maximum peak height.

Theoretical Background

The theory for the proton magnetic relaxation rates of water in solutions of manganese-containing macromolecules is now well documented (Peacock *et al.*, 1969; Mildvan and Cohn, 1970; Navon, 1970; Reuben and Cohn, 1970; Danchin and Guéron, 1970; Reed *et al.*, 1972; Dwek, 1973). Hence, only the essential relationships and some comments are presented here. The net proton relaxation rates for solutions of manganese complexes are given by

$$\frac{1}{T_{ip}} = \frac{Nq}{55.6}\frac{1}{T_{iM} + \tau_M} \quad i = 1,2 \qquad (1)$$

where N is the molar concentration of bound manganese ions; q is their hydration number; T_{1M} and T_{2M} are the longitudinal and transverse relaxation times of the coordinated water molecules, and τ_M is their residence lifetime. The proton relaxation rates of the coordinated water molecules of manganese bound to a macromolecule, where $\omega_s^2\tau_c^2 \gg 1$, are given by the simplified Solomon-Bloembergen equations (Solomon 1955; Bloembergen, 1957; Navon *et al.*, 1968; Peacock *et al.*, 1969). In eq 2–4, S is the electronic spin quantum number; γ_I is the nuclear magnetogyric ratio, g is the electronic g factor; β is the Bohr magneton; r is the distance between the nucleus and the paramagnetic ion; $A/\hbar$ is the electron-nuclear hyperfine coupling constant in radians sec^{-1}; ω_I and ω_s are the nuclear and electronic Larmor angular frequencies, respectively; τ_r is the rotational correlation time, and τ_s the longitudinal electron spin relaxation time. Later in the paper we shall use the notation in eq 5.

$$\frac{1}{T_{1M}} = \frac{6}{15}\frac{S(S+1)\gamma_I^2 g^2\beta^2}{r^6}\left[\frac{\tau_c}{1 + \omega_I^2\tau_c^2}\right] \qquad (2)$$

$$\frac{1}{T_{2M}} = \frac{1}{15}\frac{S(S+1)\gamma_I^2 g^2\beta^2}{r^6}\left[4\tau_c + \frac{3\tau_c}{1 + \omega_I^2\tau_c^2}\right] + \frac{1}{3}\frac{S(S+1)A^2}{\hbar^2}\tau_e \qquad (3)$$

$$\tau_c^{-1} = \tau_r^{-1} + \tau_M^{-1} + \tau_s^{-1}$$
$$\tau_e^{-1} = \tau_M^{-1} + \tau_s^{-1} \qquad (4)$$

$$D = \frac{6}{15}\frac{S(S+1)\gamma_I^2 g^2\beta^2}{r^6} \qquad (5)$$

The following assumptions are made in the derivations of eq 2–4. (1) The complete Solomon-Bloembergen equations include terms of the form $\tau_{c2}/(1 + \omega_s^2\tau_{c2}^2)$ and $\tau_{e2}/(1 + \omega_s^2\tau_{e2}^2)$ where τ_{c2} and τ_{e2} are correlation times defined by eq 6 and in which τ_{s2} is the transverse electron spin relaxation time (Abragam, 1961; Connick and Fiat, 1966; Reuben *et al.*, 1970). These terms can be usually neglected for manganese ions bound to macromolecules which either satisfy the conditions $\omega_s^2\tau_{c2}^2, \omega_s^2\tau_{e2}^2 \gg 1$, or $\tau_{c2} \ll \tau_{c1}$ and $\tau_{e2} \ll \tau_{e1}$. (2) The rotation of the manganese(II) enzyme complex is assumed to be isotropic. (3) The Zeeman interaction of the electronic spin with the external magnetic field is greater than the zero field splitting energy. Since there is no valid theory for the intermediate range, where these two energies have comparable magnitudes, the use of eq 2–4 for explaining the frequency dependence of the relaxation times should be restricted to high frequencies only. Koenig *et al.*, (1971) gave approximate expressions for the relaxation rates which included the effect of the zero field splitting for the region of very low fields, where the interaction with H_0 is much smaller than the zero field splitting. However, these expressions should be used with caution, even for that range since, as was pointed out in the addendum of their paper, the expressions do not have the correct limit for $H_0 = 0$. (4) The effect due to an anisotropic rotation of the coordinated water molecules about their Mn–O axis is neglected. However, this effect was shown (Dwek, 1972) to bring about a small reduction of less than 20% in the dipolar interaction (see Discussion).

$$\tau_{c2}^{-1} = \tau_r^{-1} + \tau_M^{-1} + \tau_{s2}^{-1}$$
$$\tau_{e2}^{-1} = \tau_M^{-1} + \tau_{s2}^{-1} \qquad (6)$$

Of all the correlation times in eq 4 only τ_s is frequency dependent and given by (Bloembergen and Morgen, 1961)

$$\frac{1}{\tau_s} = B\left[\frac{\tau_v}{1 + \omega_s^2\tau_v^2} + \frac{4\tau_v}{1 + 4\omega_s^2\tau_v^2}\right] \qquad (7)$$

τ_v is the correlation time of the modulation of the zero field splitting and B is a constant, related to the value of the zero field splitting parameters. Expression 7 has been shown to give identical numerical results to other calculations based on a general mechanism of modulation of the zero field splitting of the manganese ion (Rubinstein *et al.*, 1971).

It has been demonstrated (Navon, 1970) that the hydration number, q, for manganese ions bound to macromolecules can be evaluated from the net relaxation times at only one (high) frequency, provided that the system is in the fast exchange limit, *i.e.*, $\tau_M \ll T_{1M}, T_{2M}$. This was done using eq 8 which was derived from eq 1–3 assuming the same ratio of the contact to the dipolar interaction as in hexaaquomanganese ion.[2] In most Mn(II)–protein systems τ_e and

$$\frac{T_{1p}}{T_{2p}} = \frac{1}{2} + \left(\frac{2}{3} + 0.022\frac{\tau_e}{\tau_c}\right)(1 + \omega_I^2\tau_c^2) \qquad (8)$$

[1] The Mn(II) carbonic anhydrase complex still retains 27% of the amplitude of free Mn^{2+} in the same concentration, due to the $-\frac{1}{2} \leftrightarrow \frac{1}{2}$ transition (E. Meirovitch and A. Lanir, unpublished results).

[2] Insensitivity of the manganese–water contact interaction in various manganese complexes was shown recently by ^{17}O nmr (Zetter *et al.*, (1972)).

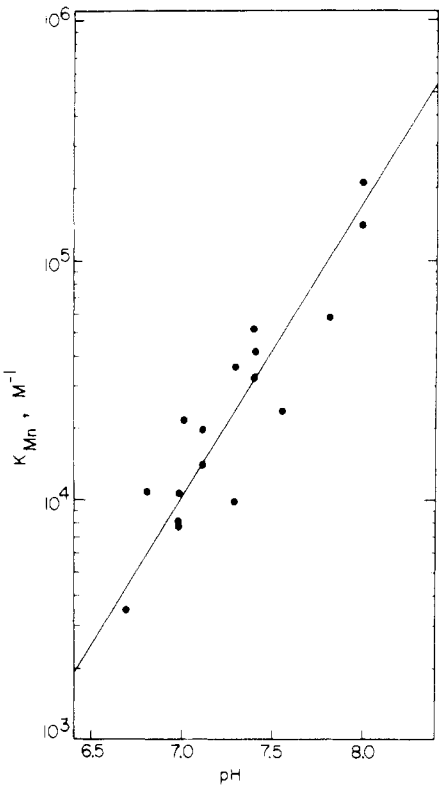

FIGURE 1: Apparent stability constants of Mn(II) bovine carbonic anhydrase B complex as a function of pH, measured by the electron paramagnetic resonance method at 25°. All solutions contained 1.56×10^{-4} M apoenzyme in Tris sulfate buffer (0.01 M) and different concentrations of manganese ion.

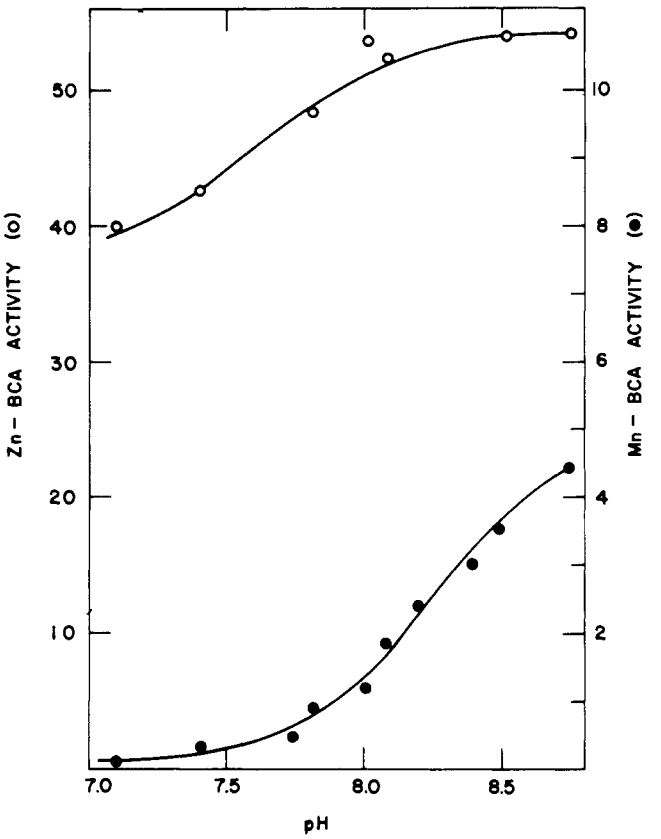

FIGURE 2: Zinc(II) (O) and manganese(II) (●) carbonic anhydrases catalyzed hydrolysis of *p*-nitrophenyl acetate as a function of pH. The activity is presented as moles of substrate hydrolyzed by mole of enzyme per minute. The assay medium contained Tris sulfate buffer (0.05 M), 3.3×10^{-4} M *p*-nitrophenyl acetate, and 3×10^{-4} M $MnCl_2$. Temperature, 25°. The concentration of the manganese enzyme was corrected for incomplete binding of the manganese to the apoenzyme according to Figure 1.

τ_c are equal since they are governed by either τ_S^{-1} or τ_M^{-1} hence

$$T_{1p}/T_{2p} = 1.19 + 0.69\omega_I^2\tau_c^2 \quad (9)$$

Using the free ion value for D and substituting τ_c in eq 2, the values of q can be evaluated (Navon, 1970). This equation is adequate only at high frequencies such that $\omega_I^2\tau_c^2 > 1$ and therefore $T_{1p}/T_{2p} > 1.19$.

$$q = 3.26 \times 10^{-14} \frac{(T_{1p}/T_{2p}) - 0.5}{[(T_{1p}/T_{2p}) - 1.19]^{1/2}} \frac{\omega_I}{NT_{1p}} \quad (10)$$

Results

Binding of Manganese Ions to Apocarbonic Anhydrase. The equilibrium constant at pH 7.0 of the manganese ion-apocarbonic anhydrase complex was previously estimated. (Lanir and Navon, 1972). Since binding of metal ions to the apoenzyme was found to be highly pH dependent (Nyman and Lindskog, 1964; Henkens and Sturtevant, 1968), we examined the pH dependence of the Mn(II)-apoenzyme binding constant (Figure 1).

The value for log K_{Mn} at pH 7 is 4.0 ± 0.2. This value is higher by 0.6 pK unit than previously reported (Lanir and Navon, 1972), but still is lower than that obtained for the bovine B form by Wilkins and Williams (1974) (pK = 4.8).

Activity of Mn(II) Carbonic Anhydrase. The activity of the Mn(II) carbonic anhydrase was obtained using selective inhibition of the manganese and zinc carbonic anhydrases mixture by acetazolamide, as previously described (Lanir and Navon, 1972). Figure 2 shows a plot of the observed rates of hydrolysis of *p*-nitrophenyl acetate at substrate concentration of 3.3×10^{-4} M as a function of pH, by Zn(II) and Mn(II) carbonic anhydrases. The activity of the manganese enzyme was found to be pH dependent with an apparent pK of 8.2. It appears that replacing the naturally occurring zinc ion by manganase ion shifts the pK of the group that controls the activity of the enzyme by about 1 pH unit, and reduces the esterase activity to about 8% of that of the native enzyme at alkaline pH, and to less than 2% at acidic pH values.

pH Dependence of the Relaxivity. Figure 3 presents the pH dependence of T_{1p}^{-1} at 100 MHz for Mn(II) carbonic anhydrase solutions as well as the effect of some sulfonamide inhibitors on the proton relaxation rates. The relaxation rates were corrected according to the binding constant of Mn(II) to apocarbonic anhydrase at each pH value (Figure 1). The relaxation data were fitted to a sigmoidal curve with a pK value of 7.8. This value, 0.4 pH unit less than the apparent pK for the activity of the Mn(II) enzyme, calls into question whether the groups controlling the relaxation and the activity of Mn(II) carbonic anhydrase are the same. However, the experimental errors do not allow definite conclusions concerning this point.

It is seen from Figure 3 that while the unsubstituted sulfonamides acetazolamide and *p*-toluenesulfonamide markedly reduce the relaxivity, N^1-acetylsulfanilamide behaves like an anion (Lanir *et al.*, 1973), *i.e.*, does not affect the relaxivity. It may be noted that N^1-acetylsulfanilamide has a pK of 5.35 (Lanir and Navon, 1971) and therefore exists in its anionic form in the pH range used in our experiments.

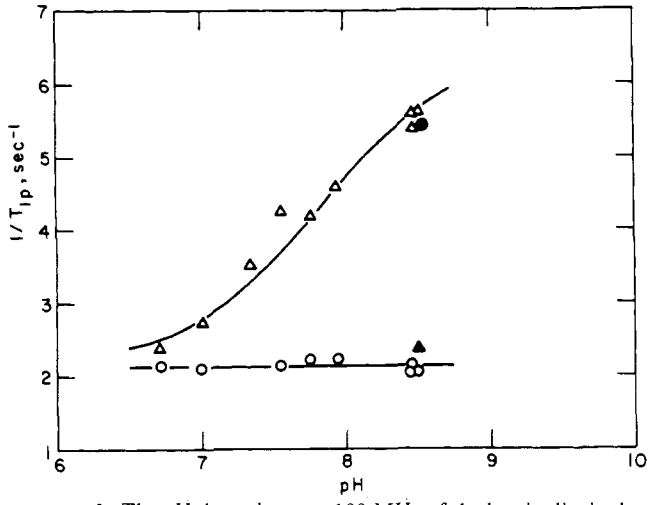

FIGURE 3: The pH dependence at 100 MHz of the longitudinal relaxation rate for 1.9×10^{-4} M bovine manganese(II) carbonic anhydrase in the absence (△) and in the presence of saturating amounts of: (○) *p*-toluenesulfonamide; (▲) acetazolamide; (●) N^1-acetylsulfanilamide.

Temperature Dependence of Relaxation Rates. Longitudinal and transverse relaxation rates of Mn(II) carbonic anhydrase (0.34 mM) measured in the temperature range of 2–35° at pH 8.0 and 100 MHz are plotted in Figure 4. The values of $1/T_{2p}$ decrease monotonically with increasing temperature. This trend of temperature dependence indicates that $T_{2M} \gg \tau_M$ in eq 1, *i.e.*, the transverse relaxation rate T_{2p}^{-1}, is in the fast exchange limit. The value of T_{1p}^{-1} appears to be insensitive to the temperature variation in the above range. Such behavior can occur in principle if τ_M is of the same order of magnitude as T_{1M}. However, since always $T_{1M} > T_{2M}$ it is clear that $T_{1M} \gg \tau_M$. The fact that $T_{1p}/T_{2p} > 1$ (see below) also indicates that T_{1p} is in the fast exchange limit.

T_{1p}/T_{2p} Ratios and Determination of Hydration Number. T_{1p}/T_{2p} ratios at 100 MHz in the absence and in the presence of saturating amounts of the inhibitor *p*-toluenesulfonamide were measured at three pH values (Table I). The limiting value of $1/T_{1p}$ at alkaline pH for 1.9×10^{-4} M Mn(II) carbonic anhydrase was extrapolated to 6.45 sec^{-1} using pK = 7.8 and the data of Figure 3. After subtracting the residual $1/T_{1p}$ that remained in the presence of *p*-toluenesulfonamide, or in acidic pH values, the relaxivity that could be titrated was 4.4 sec^{-1}. Substituting this value together with the averaged T_{1p}/T_{2p} ratio of 3.4 ± 0.3 in eq 10 yields an hydration number $q = 0.96 \pm 0.05$. An average value for the correlation time τ_c calculated from eq 9 is $\tau_c = (2.9 \pm 0.3) \times 10^{-9}$ sec. The fact that τ_c was not found to vary either upon changing the pH or upon addition of a sulfonamide inhibitor (Table I) indicates that both sulfonamide binding and the pH titration of the relaxivity are linked to replacement of a water molecule and not to a change in the relaxation mechanism.

Magnetic Field Dependence of T_{1p}^{-1}. The magnetic field dependence of the observed spin-lattice relaxation rate with and without a sulfonamide inhibitor in solutions of Mn(II) carbonic anhydrase is shown in Figure 5. The results for the titratable water molecules were fitted according to eq 1–7 for the following four parameters: $NqD/55.6$, B, τ_v, and τ_M. τ_r was taken as the rotational correlation time of the whole enzyme molecule, 1.2×10^{-8} sec (Lanir and Navon, 1971). The least-squares procedure yielded two minima (see Figure 6) and the corresponding parameters

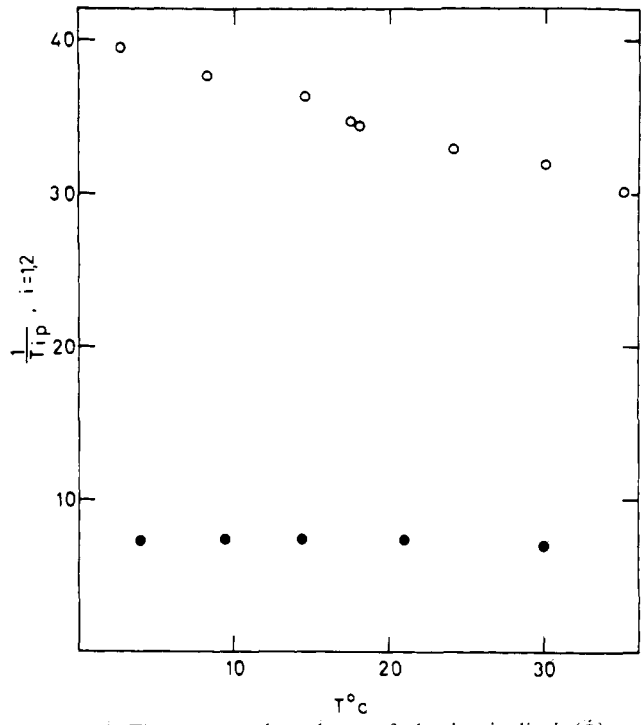

FIGURE 4: Temperature dependence of the longitudinal (●) and transverse (○) relaxation rates at 100 MHz. The solution contained 3.4×10^{-4} M Mn(II) carbonic anhydrase in Tris sulfate buffer (0.01 M, pH 8.0) and 5×10^{-4} M $MnCl_2$.

are listed in Table II. It can be seen that while for the first minimum $\tau_M \ll T_{2M}$, *i.e.*, the fast exchange limit holds, in the second minimum $\tau_M > T_{2M}$. Since the temperature dependence of T_{2p} (Figure 4) indicates that $T_{2M} > \tau_M$, only the first minimum should be considered. Identical calculated curves for the frequency dpendence of T_{1p}^{-1} are obtained using the fitted parameters corresponding to the two minima. These are reproduced in Figure 5 as the continuous curve. From the best fitted parameters it was possible to calculate also the correlation time τ_c and the T_{1p}/T_{2p} expected for a frequency of 100 MHz (see Table II and Figure 6). It can be seen that the calculated T_{1p}/T_{2p} ratio does not agree with our experimental value of $T_{1p}/T_{2p} = 3.4$. Moreover, this ratio does not exceed the value of 2.5 even for the other best fitted parameters, with different τ_M values (see Discussion). However, the value of the hydra-

Table I: Determination of τ_c from the T_{1p}/T_{2p} Ratio at 100 MHz, 30°.[a]

pH	7.00		7.78		8.46	
Mn(II)–CA Concn	1.75×10^{-4}		1.63×10^{-4}		1.59×10^{-4}	
p-TSA	−	+	−	+	−	+
T_{1p}^{-1} (sec^{-1})	2.8	2.12	3.58	2.08	4.23	1.90
T_{2p}^{-1} (sec^{-1})	9.7	7.4	13.2	6.6	12.85	6.9
T_{1p}/T_{2p}	3.46	3.50	3.67	3.20	3.04	3.69
τ_c (nsec)	2.9		3.1		2.6	

[a] The enzyme solutions contained Tris sulfate buffer 0.01 M and 4×10^{-4} M $MnCl_2$. The solution with *p*-toluenesulfonamide (*p*-TSA) contained 1×10^{-3} M inhibitor.

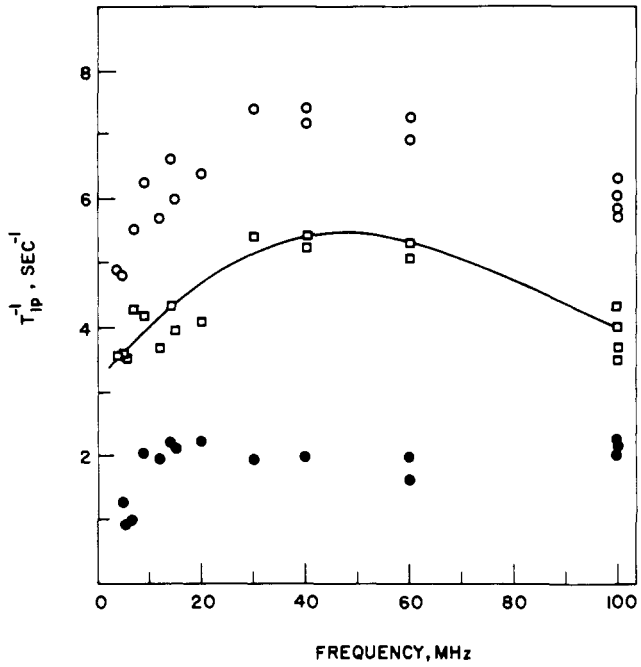

FIGURE 5: Longitudinal relaxation rate T_{1p}^{-1} plotted against proton Larmor frequency for solution of uninhibited 1.9×10^{-4} M bovine manganese(II) carbonic anhydrase (○), *p*-toluenesulfonamide-inhibited manganese(II) enzyme (●), and the titratable part of the relaxation (□). The solutions contained 0.02 M Tris sulfate buffer (pH 8.3) and 1.3×10^{-4} M $MnCl_2$. The curve was calculated from the best fit parameters listed in either in columns 2 or 3 of Table II.

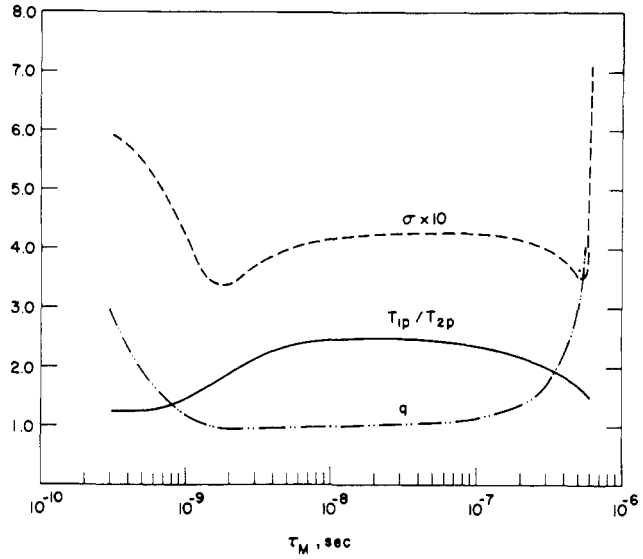

FIGURE 6: Results from the least-squares curve fitting of the frequency dependence of T_{1p} (for experimental points see Figure 5) as a function of the assumed τ_M values. (- - -) Standard deviation; (—) T_{1p}/T_{2p} ratio calculated from the best fitted parameters; (- · · -) calculated hydration number, *q*, from the best fitted parameter $NqD/55.6$, extrapolated to maximal relaxation rates, at basic pH values. The scale on the ordinate is the same for the three variables.

tion number $q = 0.96$ found for the first minimum is in agreement with the value found above using the T_{1p}/T_{2p} ratio at 100 MHz alone. This is reasonable since according to eq 7 *q* is relatively insensitive to the value of T_{1p}/T_{2p} in the region of $1.4 < T_{1p}/T_{2p} < 3.4$.

Discussion

The pH–rate profiles for the catalytic hydration and hydrolysis reactions of carbonic anhydrase are sigmoid with an inflection point around neutrality. It appears that an ionizing group on the enzyme with a pK_a near 7 is involved in catalysis. Several attempts have been made to identify this group. Khalifah (1973), Lindskog and Coleman (1973), and Prince and Woolley (1973) have summarized the presently known data on carbonic anhydrase and suggested models for the catalytic activity of the enzyme. In their preferred mechanism the activity-linked p*K* represents the dissociation of a zinc(II) coordinated H_2O molecule above pH 7:

$$E\text{–}Zn\text{–}OH_2 \rightleftharpoons E\text{–}Zn\text{–}OH + H^+$$

However, the nuclear magnetic resonance results seem to be in disagreement with the zinc hydroxide mechanism. The pH dependence of the relaxation rates of the water molecule bound to the Co(II) and Mn(II) carbonic anhydrases suggests that one water molecule is bound to the metal ion at alkaline pH values, while no H_2O is directly linked to the low pH form of the enzyme (Koenig and Brown, 1972; Lanir *et al.*, 1973, and the present work).

Table II: Fitted Parameters for Water in the First Coordination Sphere of Manganese(II) Bound to Enzymes, and Free Manganese(II) Ions.

Parameters	Mn(II) Carbonic anhydrase I[a]	Mn(II) Carbonic anhydrase II[a]	Mn(II) Pyruvate Kinase		Mn(II) Carboxypeptidase A[b]	Free Mn(II)[d]
			b	*c*		
τ_M (sec)	1.9×10^{-8}	5.6×10^{-7}	4×10^{-8}	5×10^{-8}	2.5×10^{-8}	2.5×10^{-8}
q	0.96	3.9	2.04	3[e]	1.08	6
B (rad/sec)^2	2.76×10^{10}	7.6×10^{10}	0.8×10^{10}	1.46×10^{10}	3.1×10^{10}	1×10^{10}
τ_v (sec)	5.4×10^{-12}	6.6×10^{-12}	14×10^{-12}	6×10^{-12}	7×10^{-12}	2×10^{-12}
T_{1M}^f (sec)	9.5×10^{-7}	3.8×10^{-7}	9.1×10^{-7}		6.5×10^{-7}	1.6×10^{-7}
T_{2M}^f (sec)	5.3×10^{-7}	0.5×10^{-7}	1.72×10^{-7}		2.55×10^{-7}	1.3×10^{-8}
T_{1p}^f/T_{2p}^f (theoretical)	1.8	1.54	5.3		2.55	
T_{1p}^f/T_{2p}^f (experimental)	3.4	3.4	5.5		2.61	12

[a] I and II are related to the two minima in the least-squares fitting procedure of the frequency dependence of the relaxation rates to eq 1–7 in the text. [b] Taken from Navon (1970). [c] Taken from Reuben and Cohn (1970). [d] Taken from Bloembergen and Morgan (1961). [e] Assuming $r = 2.9$Å. [f] Calculated for a Larmor frequency of 100 MHz.

Our calculation of the number of titratable water molecules within the pH range of 9.0–6.7 or that displaced by sulfonamides is based on the assumption that the distance between the metal ion and the water protons is the same as in hexaaquo manganese(II), *i.e.*, an effective distance of about 2.73 Å. If a hydroxide ion is bound to the metal at alkaline pH the metal–proton distance would be 2.43 Å which seems to be too short.[3] Pocker and coworkers (Pocker and Storm, 1968; Pocker and Stone, 1968; Pocker and Watamori, 1973) found a second inflection point around pH 11–11.5 in the pH–activity profiles. They ascribed this inflection point to the formation of a more reactive zinc hydroxide complex. This explanation is compatible with the single H_2O molecules observed around pH 8–9 by the nmr relaxation data.

The lack of relaxation effect in acid pH values can be explained by a low pH form of the enzyme to which either no water molecule is bound or by a very slow rate of H_2O exchanges from the coordination sphere of the metal ion (*i.e.*, $>2 \times 10^{-4}$ sec and $>1.2 \times 10^{-6}$ sec for the Co(II) or Mn(II) carbonic anhydrases, respectively). The second explanation is improbable since the rate of exchange of water molecules bound to manganese ions located at the active center of macromolecules found in the range of 10^{-6}–10^{-9} sec (Navon, 1970; Reuben and Cohn, 1970; Meirovitch and Kalb, 1973). Furthermore, the rate of exchange of Cl^- ligands bound to the metal at the active site of the Co(II) enzyme at pH 6.46 is 1×10^{-6} sec (Ward and Cull, 1972), implying a shorter exchange lifetime of the water molecule which is replaced by the Cl^- ion (Fabry *et al.*, 1970; Koenig and Brown, 1972). Hence, the failure to observe a water molecule at low pH is probably not due to an increase in the lifetime of the metal-bound water molecule. It appears that, at acidic pH, the fourth coordination position of the metal ion in carbonic anhydrase is either empty or is occupied by another side chain ligand which can be displaced by an anion but not by water.

One of the most striking arguments against the basic amino acid side chain mechanism was that although several modifications of histidine residues have been attempted, none of them caused complete destruction of enzymic CO_2 hydration activity (Khalifah and Edsall, 1972). However, Pocker and Watamori (1973) found that alkylation of a specific histidine residue located at or near the active site cavity of bovine carbonic anhydrase abolishes the esterase activity of the enzyme at physiological pH. Wong *et al.* (1972) also found that dialkylated human carbonic anhydrase B in which both 1 and 3 nitrogen positions of a histidine residue are modified was almost completely inactive. This raises the possibility that a histidine residue plays an important role in the catalysis and might be responsible for its pH dependence; 70 ± 5% of the proton relaxation rate due to manganese(II) carbonic anhydrase could be abolished either by addition of a sulfonamide inhibitor or by pH titration (Figure 3). This effect was attributed to the displacement of a water molecule from the first coordination sphere of the manganese ion. The residual relaxivity probably arises from contributions of water molecules outside the first coordination sphere. Another contribution to the residual relaxivity may come from protons belonging either to an amino acid side chain or to the inhibitor protons exchanging with the bulk water.

The hydration number q of the manganese ion in Mn(II) carbonic anhydrase was calculated in this paper either from the T_{1p}/T_{2p} ratio, or from the frequency dependence of T_{1p}. The first method should be considered as more reliable since: (a) the frequency dependence is not always sensitive to all four parameters; (b) the frequency dependence relies on data taken at high as well as low magnetic fields, where the use of the Solomon–Bloembergen equations (eq 2 and 3) is not always justified (These equations for the relaxation rates induced by manganese(II) are strictly valid only at high magnetic fields where the Zeeman energy is much greater than the zero field splitting (see theoretical background section). Therefore it is preferable to use the T_{1p}/T_{2p} method which is done at high frequencies only.); (c) the Bloembergen–Morgan expression for τ_s (eq 7) which is being used for the fitting to the frequency dependence is an approximation valid for free manganese ions (Rubinstein *et al.*, 1971), but not necessarily for the slowly rotating manganese–macromolecule complexes. On the other hand, the detailed frequency dependence of τ_s is not important for the calculation of q by the T_{1p}/T_{2p} method. One limitation of the T_{1p}/T_{2p} method is that it can be used only when the fast exchange limit is valid. This can be checked by the temperature dependence of T_{2p} which, since it is always shorter than T_{1p}, is a more sensitive criterion for the exchange condition. In cases where τ_M is comparable to T_{1M} or T_{2M} the hydration number q can be estimated only from the frequency dependence of T_{1p}, preferably with a measurement of T_{2p} as well.

Rapid internal motion of the coordinated water molecules, about the metal–oxygen axis, may lead to a reduction of about 20% in the dipolar interaction between the water protons and paramagnetic center (Dwek, 1972). Such a reduction will occur if the internal rotation is faster than the correlation time. Thus, since our hydration numbers were derived by comparison of the proton–manganese dipolar interaction with that of free manganese, such a reduction will affect the calculated hydration number only in a case where the internal motion is effective in reducing the dipolar interaction in the manganese–protein complex and not in free manganese. In such a case the calculated hydration number will be 20% less than the true value.

Our method for the calculation of q involves a comparison of the dipolar interaction with that of free manganese assuming an hydration number of 6 for the latter. It is assumed that the manganese–water distances are the same for the hexaaquo manganese ion and the manganese bound to the protein. This procedure avoids possible errors in the interpretation of the dipolar interaction, such as deviations from the point-dipole approximation assumed in the Solomon–Bloembergen equations (Navon *et al.*, 1968; Waysbort and Navon, 1973). Such deviations can be caused by spin delocalization onto the ligand orbitals, thus leading to an enhanced electron–nucleus dipolar interaction. On using the Solomon–Bloembergen formulas this would appear either as a smaller apparent manganese–proton distance or as a greater hydration number.

It is interesting to note that although the environments of the manganese ions bound to various enzymes are expected to be totally different from each other and particularly from that of free manganese(II) ion, the parameters affecting the electronic longitudinal relaxation time, τ_s, are similar

[3] For example, the dipolar interaction between the metal and the hydroxide proton in $Fe^{III}EDTA \cdot OH$ was found to be very close to that in $Fe^{III}EDTA \cdot OH_2$, implying that the metal–proton distance is essentially the same for coordinated hydroxide and water molecules (J. Bloch and G. Navon, unpublished data).

(Table II). B in the Bloembergen–Morgan expression (eq 7) is related to the zero field splitting of the manganese ion which should be large for an asymmetric complex. Also τ_v which is the modulation time of the interaction responsible for the manganese electronic relaxation may be expected to be longer for the slowly rotating manganese–protein complexes. However, the electronic longitudinal relaxation time is mostly affected by processes with correlation times which are not too far from the electronic Larmor frequencies, ω_S. Therefore it is possible that the large zero field splittings which are expected to be present in the manganese–protein complexes are not effective in the longitudinal electronic relaxation, since their modulation times are too slow (Navon, 1970). On the other hand, τ_s is governed by weaker interactions with shorter correlation times, such as transient distortions in the microenvironment of the manganese bound to the proteins. The strong, but slow modulated zero field splitting is, however, expected to affect the electronic transversal relaxation rates and should therefore lead to a broad epr spectrum.

References

Abragam, A. (1961), The Principles of Nuclear Magnetism, London, Oxford University Press.

Appleton, D. W., and Sarkar, B. (1974), *Proc. Nat. Acad. Sci., U. S. 71,* 1686.

Armstrong, J. McD., Myers, D. V., Verpoorte,, J. A., and Edsall, J. T. (1966), *J. Biol. Chem. 241,* 5137.

Bloembergen, N. (1957), *J. Chem. Phys. 27,* 572.

Bloembergen, N., and Morgan, L. O. (1961), *J. Chem. Phys. 34,* 842.

Connick, R. E., and Fiat, D. (1966), *J. Chem. Phys. 44,* 4103.

Danchin, A., and Guéron, M. (1970), *J. Chem. Phys. 53,* 3599.

Dwek, R. A (1972), *Advan. Mol. Relax. Proc. 4,* 1.

Dwek, R. A. (1973), Nuclear Magnetic Resonance in Biochemistry: Application to Enzyme Systems, Oxford, Clarendon Press.

Fabry, M. E., Koenig, S. H., and Schillinger, W. E. (1970), *J. Biol. Chem. 245,* 4256.

Ginsburg, A., Lipman, A., and Navon, G. (1970), *J. Phys. E 3,* 699.

Henkens, R. W., and Sturtevant, J. M. (1968), *J. Amer. Chem. Soc. 90,* 2669.

Khalifah, R. G. (1973), *Proc. Nat. Acad. Sci. U. S. 70,* 1986.

Khalifah, R. G., and Edsall, J. T. (1972), *Proc. Nat. Acad. Sci. U. S. 69,* 172.

Koenig, S. H., and Brown, R. D. (1972), *Proc. Nat. Acad. Sci. U. S. 69,* 2422.

Koenig, S. H., Brown, R. D., and Studebaker, J. (1971), *Cold Spring Harbor Symp. Quant. Biol. 36,* 551.

Lanir, A., Gradstajn, S., and Navon, G. (1972), Proceedings of the 42nd Meeting of the Israel Chemical Society, p. 130.

Lanir, A., Gradstajn, S., and Navon, G. (1973), *FEBS Lett. 30,* 351.

Lanir, A., and Navon, G. (1971), *Biochemistry 10,* 1024.

Lanir, A., and Navon, G. (1972), *Biochemistry 11,* 3536.

Liljas, A., Kannan, K. K., Bergstén, P. C., Waara, I., Fridborg, K., Strandberg, B., Carlbom, U., Jarup, L., Lövgren, S., and Petef, M. (1972), *Nature (London), New Biol. 235,* 131.

Lindskog, S. (1960), *Biochim. Biophys. Acta 39,* 218.

Lindskog, S., and Coleman, J. E. (1973), *Proc. Nat. Acad. Sci. U. S. 70,* 2505.

Lindskog, S., Henderson, L. E., Kannan, K. K., Liljas, A., Nyman, P. O., and Strandberg, B. (1971), *Enzymes, 3rd Ed. 5,* 587.

Meirovitch, E., and Kalb, A. J. (1973), *Biochim. Biophys. Acta 303,* 258.

Mildvan, A. S., and Cohn, M. (1970), *Advan. Enzymol. 33,* 1.

Navon, G. (1970), *Chem. Phys. Lett. 7,* 390.

Navon, G., Shulman, R. G., Wyluda, B. J., and Yamane, T. (1968), *Proc. Nat. Acad. Sci. U. S. 60,* 86.

Nyman, P. D., and Lindskog, S. (1964), *Biochim. Biophys. Acta 85,* 141.

Peacock, A. R., Richards, R. E., and Sheard, B. (1969), *Mol. Phys. 16,* 177.

Pocker, Y., and Stone, J. T. (1967), *Biochemistry 6,* 668.

Pocker, Y., and Stone, J. T. (1968), *Biochemistry 7,* 4139.

Pocker, Y., and Storm, D. R. (1968), *Biochemistry 7,* 1902.

Pocker, Y., and Watamori, N. (1973), *Biochemistry 12,* 2475.

Prince, R. H., and Woolley, R. P. (1973), *Bioorg. Chem. 2,* 337.

Reed, G. H., Diffenbach, H., and Cohn, M. (1972), *J. Biol. Chem 247,* 3066.

Reuben, J., and Cohn, M. (1970), *J. Biol. Chem. 245,* 6539.

Reuben, J., Reed, G. M., and Cohn, M. (1970), *J. Chem. Phys. 52,* 1617.

Rubinstein, M., Baram, A., and Luz, Z. (1971), *Mol. Phys. 20,* 67.

Solomon, I. (1955), *Phys. Rev. 99,* 559.

Ward, R. L., and Cull, M. D. (1972), *Arch. Biochem. Biophys. 150,* 436.

Waysbort, D., and Navon, G. (1973), *J. Chem. Phys. 59,* 5685.

Wilkins, R. G., and Williams, K. R. (1974), *J. Amer. Chem. Soc. 96,* 2241.

Wong, S.-C. C., Kandel, S. I., Kandel, M., and Gornall, A. G. (1972), *J. Biol. Chem. 247,* 3810.

Zetter, M. S., Grand, M. W., Wood, E. J., Dodgen, H. W., and Hunt, J. P. (1972), *Inorg. Chem. 11,* 2701.