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Water in the Coordination Sphere of Metallocarbonic Anhydrases: A Solvent Proton Longitudinal Relaxation Study at Several Frequencies

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The proton longitudinal relaxation rates of water solutions of carbonic anhydrase derivatives in which the native zinc(II) ion has been substituted with the paramagnetic metal ions cobalt(II), copper(II) and manganese(II) have been measured at magnetic resonance frequencies between 4 and 60 MHz. The investigation was extended to a number of systems containining inhibitors of the metal enzyme. The rate enhancements due to coupling between resonating protons and unpaired electrons vary significantly with magnetic field. For the cobalt and copper derivatives, correlation times have been estimated and structural information on the water in the enzymatic cavity have been obtained. The electronic relaxation times for the cobalt systems are related to the stereochemistry, four and five coordinated, of the metal ion. For the manganese derivative the electronic correlation times depend on the magnetic field. Such dependence has been analyzed in terms of structural data and the correlation time of the modulation of the zero field splitting.

Introduction

In recent years kinetic [1] and spectroscopic [2] studies on native and metal substituted carbonic anhydrase have added a harvest of information to the overall knowledge of the enzyme, but also required models of increasing complexity in the attempt to fit the data into a general scheme. As far as the enzymatic activity is concerned, the role of exchangeable protons within the active site cavity becomes more and more intriguing: an internal proton transfer between two basic groups during the catalytic cycle [3, 4] and a buffer mediated proton transfer to the bulk solution [5, 6] seem to be required, both processes at least as fast as the turnover number.

On the other hand, electronic [7–10] and ESR [11] spectroscopic studies on the catalytically active cobalt(II) substituted enzyme and its inhibited derivatives have suggested the occurrence in some cases of five coordination around the metal ion. The availability of two more coordination sites on the metal, besides the three coordination positions occupied by the histidine nitrogen donors from the protein, could be of importance along the catalytic pathway [12], either allowing an associative mechanism of product removal or facilitating the proton transfer within the active site cavity.

Measurements of solvent water proton relaxation times in solutions of metalloenzymes substituted with paramagnetic metal ions have proved to be among the most direct methods of detecting the presence of exchangeable protons close to the metal in the active site [13–16]. Indeed, in the case of copper(II) [17] and manganese(II) [18] substituted carbonic anhydrase such measurements performed at 80 MHz have shown that the paramagnetic effect on the water proton is drastically reduced by reacting the enzyme with the strong inhibitors *p*-toluene-sulfonamide or oxalate, while addition of monoanionic inhibitors like the azide ion was almost without effect on the coupling between exchangeable protons and the paramagnetic center. Accordingly, a water molecule was proposed to remain bound to the metal ion in the latter kind of adducts [17, 18], again suggesting five coordinate chromophores. In the case of the active cobalt enzyme [9], the paramagnetic effect on the water protons is always reduced upon addition of inhibitors to the water solutions although on the ground of electronic [7–10] and ESR [11] spectra the occurrence of both four and five coordinated species has been proposed, the latter stereochemistry being reached through water coordination. In both cases the ^1H T_1^{-1} values are about 20–30% of the values of the non inhibited enzyme [9]. A major question is therefore which protons give rise to the observed residual relaxivity in the case in which the coordinated water has been removed and why, when the water is the fifth ligand, the relaxation is so low.

As a matter of fact, the actual paramagnetic effect measured on the ^1H nuclei, besides being related to the nucleus–electron distance, depends among other factors on the strength of the magnetic field and on a correlation time for the dipolar interaction

between electronic and nuclear spins [19]. A comparison of the water proton relaxation times of two different derivatives, to obtain information on the amount of exchangeable protons interacting with the paramagnetic center, is only meaningful under the assumption that the correlation times are equal. As will be shown, such assumption is not fulfilled in some cases. ^1H T_1^{-1} measurements at different magnetic fields may provide information on the correlation times involved, and in principle, yield more quantitative information on the amount and nature of the exchanging protons interacting with the metal ions [13–16, 20]. Indeed, from such measurements performed by different investigators on cobalt(II) [21, 22], copper(II) [23], and manganese(II) [24] substituted carbonic anhydrases independent sets of information have been obtained on each system, which provided a detailed analysis of the field dependence of the nuclear relaxation and its relation to the correlation times.

Furthermore, by assuming a value of the proton–metal distance, the number of exchangeable protons has also been tentatively proposed. However, the analysis was limited in every case to the non inhibited enzymes and the inhibitor adducts were simply used as a blank, their effects being subtracted from those measured for the non inhibited derivatives.

We felt that a general picture of the exchangeable protons in the active site of carbonic anhydrase could only be reached through a systematic investigation of a large number of enzyme derivatives at several magnetic fields. The data, if properly analyzed, could provide structural parameters which can be meaningfully compared among the inhibitor derivatives of the same metalloprotein and among the various metal systems. The data will be particularly meaningful in the cobalt system, which is as active as the native enzyme [25]; in this case the obtained information can be directly transferred on the native enzyme with some confidence. With this in mind we have reinvestigated the cobalt(II), copper(II), and manganese(II) substituted bovine carbonic anhydrase B (Co, Cu, MnBCAB hereafter), as well as a number of properly chosen inhibitor derivatives, at magnetic resonance frequencies between 4 and 60 MHz.

Experimental

Bovine carbonic anhydrase was purchased from Sigma. All the chemicals were of analytical grade. The isoenzyme B [26], apoenzyme [27, 28], and metal derivatives [9, 17, 18], were prepared and checked according to the usual procedures. The metal enzymes in unbuffered solutions were concentrated by ultradialysis up to $1–3 \times 10^{-3}$ *M*; the inhibitors were added in saturating amounts as judged from the known values of their affinity constants for the various metalloenzymes. The pH of each NMR sample was measured using a microelectrode.

The model complex {Co[tris(3,5-dimethyl-1-pyrazolylmethyl)amine]H_2O}$^{2+}$(Co(TPyMA)H_2O^{2+}) was prepared as previously reported [29].

The electronic spectra in the visible and UV regions were recorded on a Cary 17D spectrophotometer.

The nuclear magnetic resonance measurements were performed at 25 °C with a Bruker CXP 100 spectrometer consolle attached to a variable field Varian DA 60 electromagnet, in the magnetic field range 0.094–1.41 T, corresponding to a proton Larmor precession frequency range of 4–60 MHz.

The longitudinal relaxation rates, T_1^{-1}, were measured by the inversion recovery method, using an appropriate nonlinear least-squares fitting program.

Analysis of the T_1^{-1} Results

The nuclear longitudinal relaxation rate enhancements due to the coupling between resonating nuclei and unpaired electrons have been quantitatively expressed by the well known Solomon, Bloembergen, and Morgan equation for the simplified case of a magnetically isotropic system and in absence of zero field splitting [19, 30]. A reasonably concise form for the paramagnetic contribution to T_1^{-1}, T_{1p}^{-1}, is the following:

$$T_{1p}^{-1} = \frac{[E]}{111}\left[\frac{2}{15}S(S+1)\gamma_I^2 g^2 \beta^2 \times \left(\Sigma_i \frac{n_i}{r_i^6}\right)\left(\frac{3\tau_c}{1+\omega_I^2\tau_c^2} + \frac{7\tau_c}{1+\omega_s^2\tau_c^2}\right) + \frac{2}{3}S(S+1)\left(\frac{A}{\hbar}\right)^2 \frac{\tau_e}{1+\omega_s^2\tau_e^2}\right] \quad (1)$$

where E is the enzyme derivative concentration and n_i is the number of protons interacting with the paramagnetic center at a distance r_i; τ_e is the electronic correlation time, and τ_c is given by

$$\tau_c^{-1} = \tau_e^{-1} + \tau_r^{-1} + \tau_M^{-1} \quad (2)$$

τ_r being one-third the rotational correlation time and τ_M^{-1} the proton exchange rate. The other symbols have the usual meaning. The first term, which is dipolar in origin, describes the coupling between the unpaired electrons resident on the metal and the resonating nucleus, and the latter is the contact contribution. The electronic correlation times, τ_e, depend on

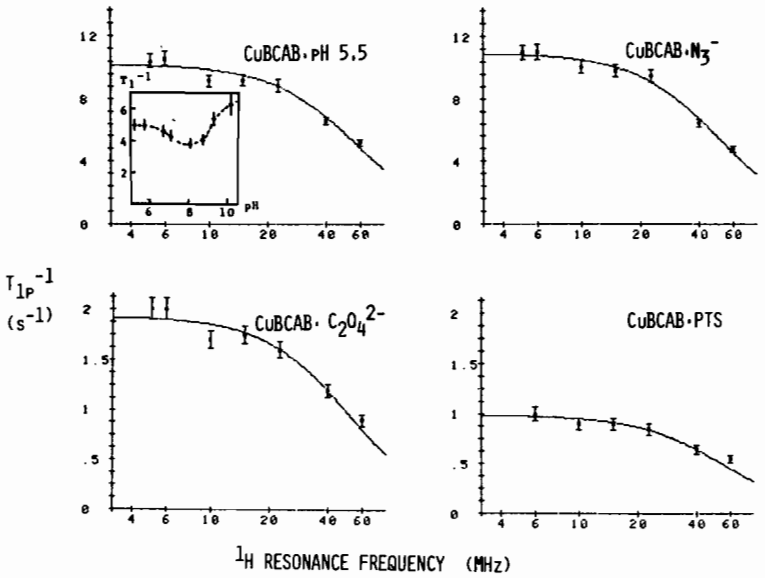

Fig. 1. ^1H T_{1p}^{-1} values as a function of frequency for 1.07×10^{-3} *M* water solutions of Copper(II) Bovine Carbonic Anhydrase B at pH 5.5, of its azide and oxalate derivatives at pH 7, and of the *p*-toluene sulfonamide derivative at pH 9. The lines represent the best fitting curves according to eqn. 3. The inset shows the pH dependence of T_{1p}^{-1} for the pure copper enzyme measured at 80 MHz.

the nature of the metal ion and to a lesser extent on the geometry of the chromophore; however, they are shorter than 10^{-8} s, which is about the rotational correlation time for macromolecules of the size of carbonic anhydrase [31]. The exchange time for a bound water molecule is hardly that short and therefore τ_M^{-1} negligibly contributes to the overall τ_c^{-1}. It may be concluded that τ_c^{-1} is in general mostly controlled by τ_e^{-1} [13–15].

The contact term has been proved to be negligible for protons in aqua systems as long as τ_c is dominated by τ_e [13–16], although the possibility that delocalized unpaired electrons on the oxygen atom contribute to the overall nuclear relaxation cannot be ruled out [32].

However, the most severe limitation to a quantitative use of this equation is the magnetic anisotropy of the system, which is expected to be quite large in cobalt(II) chromophores. In the absence of more adequate theoretical tools we have analyzed the data of the CoBCAB systems as though they were magnetically isotropic.

For simplicity, the dipolar part of eqn. 1 will be expressed as:

$$T_{1p}^{-1} = KG \left(\frac{3\tau_c}{1 + \omega_I^2 \tau_c^2} + \frac{7\tau_c}{1 + \omega_s^2 \tau_c^2} \right) \qquad (3)$$

where K is a product of known physical constants, and G is the geometrical part of the equation including the number of protons and their distance from the paramagnetic center.

For most of the systems investigated sizeable variations of T_{1p}^{-1} in the range 4–60 MHz were measured; values obtained are suitable for determining the geometrical G factor and τ_c from the least squares fitting of the data to eqn. 3. The standard deviations in G and τ_c were in every case acceptable and the quality of the analysis is represented by the agreement between the calculated and experimental values, which were within the experimental error. A mapping of $\Sigma(T_{1p\,obs}^{-1} - T_{1p\,calc}^{-1})^2$ over an extended range of G and τ_c values was also performed in order to rule out the occurrence of false minima. For the assumption of negligible contact contribution to the nuclear relaxation mechanism, magnetic anisotropy affects mostly the G factor, whereas the presence of zero field splitting may change the coefficients of τ_c [33]. Therefore the calculated G and τ_c parameters may not be the actual ones, but, as will be shown, still quite meaningful within this series of compounds.

The above type of analysis is not possible for manganese systems [34], as was already shown [24], since τ_e and hence τ_c are not constant in the range of the magnetic field investigated. It was therefore assumed that τ_e depends on magnetic field according to the following equation:

$$\tau_e^{-1} = B \left(\frac{\tau_v}{1 + \omega_s^2 \tau_v^2} + \frac{4\tau_v}{1 + 4\omega_s^2 \tau_v^2} \right) \qquad (4)$$

where B is related to the magnitude of the zero field splitting and τ_v is the zero field splitting modulation

TABLE I. Best Fitting Values of the Geometrical Factor (G) and of the Correlation Time (τ_c) for Metal Substituted Carbonic Anhydrase B Derivatives. Standard Deviations in Brackets.

	G (pm^{-6})		τ_c (s)	
Cu BCAB pH 5.5	5.6×10^{-15}	(±11%)	2.7×10^{-9}	(±10%)
CuBCAB·N_3^-	5.3×10^{-15}	(±9%)	3.1×10^{-9}	(±7%)
CuBCAB·$C_2O_4^{2-}$	0.93×10^{-15}	(±5%)	3.1×10^{-9}	(±5%)
CuBCAB·PTS	0.52×10^{-15}	(±2%)	2.8×10^{-9}	(±1%)
CoBCAB pH 6.0	3.3×10^{-15}	(±17%)	3.3×10^{-11}	(±26%)
CoBCAB pH 9.7	4.0×10^{-15}	(±17%)	3.2×10^{-11}	(±25%)
CoBCAB·NCO^-	1.4×10^{-15}	(±7%)	3.1×10^{-11}	(±4%)
CoBCAB·PTS	0.82×10^{-15}	(±3%)	4.4×10^{-11}	(±2%)
CoBCAB·$Au(CN)_2^-$	3.6×10^{-15}	(±4%)	4.2×10^{-12}	(±3%)
CoBCAB·NO_3^-	4.1×10^{-15}	(±6%)	6.0×10^{-12}	(±5%)
CoBCAB·NCS^-	2.4×10^{-15}	(±2%)	5.6×10^{-12}	(±2%)
CoBCAB·CH_3COO^-	5.5×10^{-15}	(±4%)	4.2×10^{-12}	(±3%)
CoBCAB·$C_2O_4^{2-}$	0.97×10^{-15}	(±1%)	5.3×10^{-12}	(±1%)
Co(TPyMA)H_2O^{2+} [a]	5.7×10^{-15}	(±50%)	5.3×10^{-12}	(±45%)
	G(pm^{-6}) [b]	B ($rad/s)^2$ [b]	τ_v (s) [b]	
MnBCAB pH 8.9	3.3×10^{-15} (±7%)	4.7×10^{19} (±12%)	3.2×10^{-12}	(±13%)
MnBCAB·N_3^-	3.6×10^{-15} (±4%)	6.9×10^{19} (±5%)	4.7×10^{-12}	(±5%)

[a] Co Tris(3,5-dimethyl-1-pyrazolylmethyl)amine H_2O^{2+}. [b] Values obtained from simultaneous fitting to equations 3 and 4.

correlation time [30, 35]. The data were successfully [24] analyzed in terms of the parameters G, B, and τ_v, by substituting in eqn. 3 the expression for τ_e given in equation 4 and using $\tau_r = 1.2 \times 10^{-8}$ s [31].

Results

The ^1H T_1^{-1} values of solutions containing metal substituted carbonic anhydrases and their inhibitor derivatives have been measured as a function of the magnetic field. In every case the corresponding value of the diamagnetic enzyme has been subtracted ($T_1^{-1} - T_{1dia}^{-1} = T_{1p}^{-1}$). In each experiment the same solution of apoprotein was used in order to reduce the uncertainty due to concentration differences. To such solutions slightly less than the stoichiometric amount of the appropriate metal ion was added in order to reconstitute the metalloprotein.

The T_{1p}^{-1} values at various frequencies for CuBCAB solutions have been measured at pH from 5.5 to 10. The values at pH 5.5 are reported in Fig. 1, together with the best fitting curve calculated on the basis of eqn. 3. In the inset the dependence of T_1^{-1} on pH at 80 MHz [17] is also reported. Almost the same pattern, with a minimum around pH 8, is present at every frequency investigated. Among the inhibitors the following have been investigated: the azide ion, which is known to bind the metal without affecting the ^1H T_1^{-1} value at 80 MHz, and oxalate and *p*-toluenesulfonamide, which reduce the ^1H T_1^{-1} values [17]. The T_{1p}^{-1} dependence on magnetic field for these derivatives is also shown in Fig. 1. The best fitting values for G and τ_c are reported in Table I. The small standard deviations on τ_c (Table I) indicate that the latter are substantially field independent in the magnetic field range investigated. These values and this statement are consistent with the previous investigation by Koenig and coworkers on the human copper(II) substituted isoenzyme, although they found that τ_c is field dependent when the investigation is extended down to 2.35×10^{-4} T [23]. The geometrical factor is about 5.5×10^{-15} pm^{-6} for the pure copper enzyme and the azide adduct, and below 1×10^{-15} pm^{-6} for the sulfonamide and oxalate derivatives (Table I).

In the case of the cobalt(II) derivative the reliability of the determination of the T_{1p}^{-1} values has been carefully checked since the absolute values, even for concentrated enzyme solutions, are close to the values of the diamagnetic zinc enzyme. This

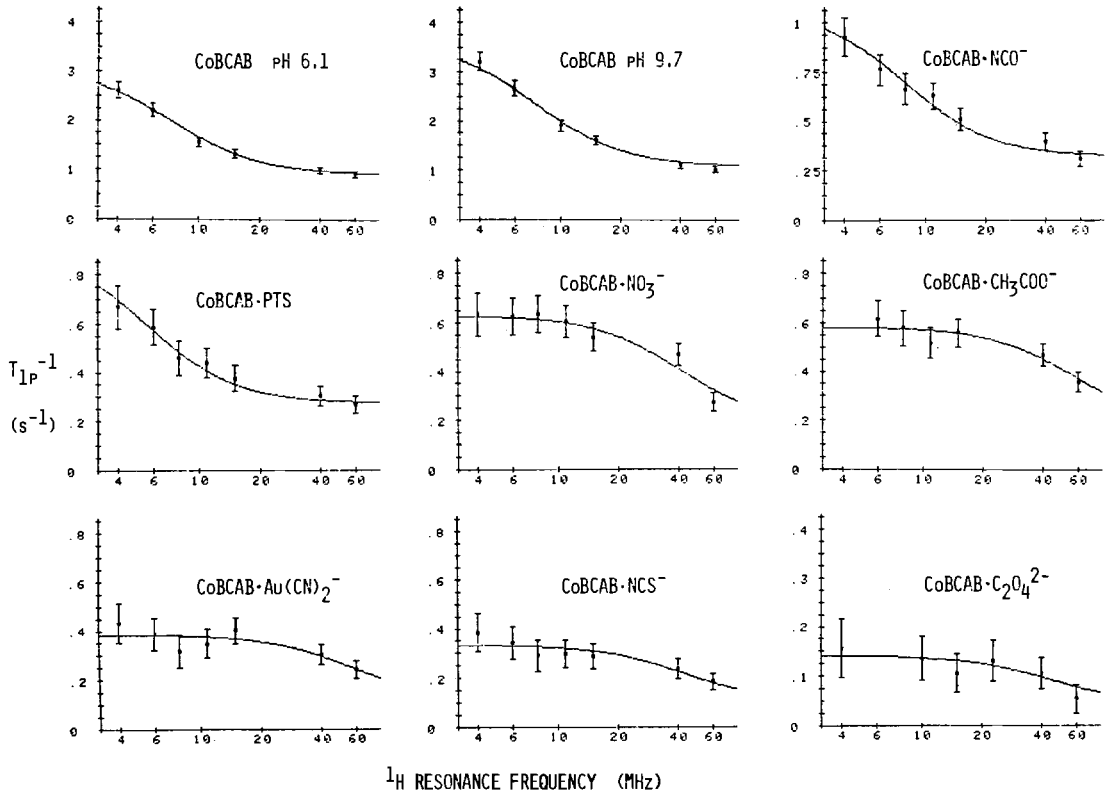

Fig. 2. ^1H T_{1p}^{-1} values as a function of frequency for 2.5×10^{-3} *M* water solutions of Cobalt(II) Bovine Carbonic Anhydrase B at pH 6.1 and 9.7 and of its derivatives with the following inhibitors: cyanate (2.3×10^{-3} *M*), nitrate (2.3×10^{-3} *M*), acetate (2.3×10^{-3} *M*) dicyanoaurate (2.3×10^{-3} *M*), thiocyanate (2.3×10^{-3} *M*) and oxalate (2.5×10^{-3} *M*), at pH 7; *p*-toluene sulfonamide (2.3×10^{-3} *M*) at pH 9. The lines represent the best fitting curves according to eqn. 3.

aspect is now well understood and is related to the intrinsic electronic relaxation times of the cobalt(II) ion [36]. However, there is a relatively large spectrum of possible τ_c values related to the magnitude of the zero field splitting which in turn depends on the coordination number and geometry of the chromophore. On sets of measurements referred to the same solution of apoenzyme the standard deviation has been tested to be as low as 4%, whereas the error on T_{1p}^{-1} is estimated through error propagation to range between 6 and 12%. The experimental T_{1p}^{-1} values have been fitted within the above standard deviation. Sets of measurements on different protein samples have shown that the overall uncertainty of T_{1p}^{-1} can be as high as ±20%. Despite this uncertainty the systems investigated can be grouped into two classes, one with τ_c of the order of $4-6 \times 10^{-12}$ s and the other with τ_c of the order of $3-4 \times 10^{-11}$ s, well outside the experimental uncertainty. The existence of two ranges of τ_c is clearly shown by the best fitting curves reported in Fig. 2: derivatives with $\tau_c < 10^{-11}$ s have inflection points at high fields, and the others at low fields. This analysis of the T_1^{-1} data indicates that the procedure of subtracting from the observed T_1^{-1} the residual T_1^{-1} measured on an inhibited enzyme is indeed rather hazardous, unless it is established that τ_c is the same for both systems: in principle, only G values can possibly be subtracted.

The G values span a range whose limits are about those of the copper systems. Besides the thiocyanate derivative which has an intermediate value of 2.4×10^{-15} pm^{-6}, the derivatives can be grouped into two classes, whose extreme values are 3.3–5.5 and $0.97-1.4 \times 10^{-15}$ pm^{-6}.

As previously noted [24], the T_{1p}^{-1} dependence on frequency for the manganese derivatives (Fig. 3) could not be fitted to equation 3, in the assumption of constant τ_c. The reported curves are calculated as discussed in the previous section by simultaneously fitting the experimental data in terms of the parameters G, B, and τ_v (Table I). The T_{1p}^{-1} for the *p*-toluenesulfonamide and oxalate derivatives are almost frequency independent and therefore no computer treatment was attempted.

Discussion

The low standard deviations for the calculated G and τ_c values give one confidence to discuss them

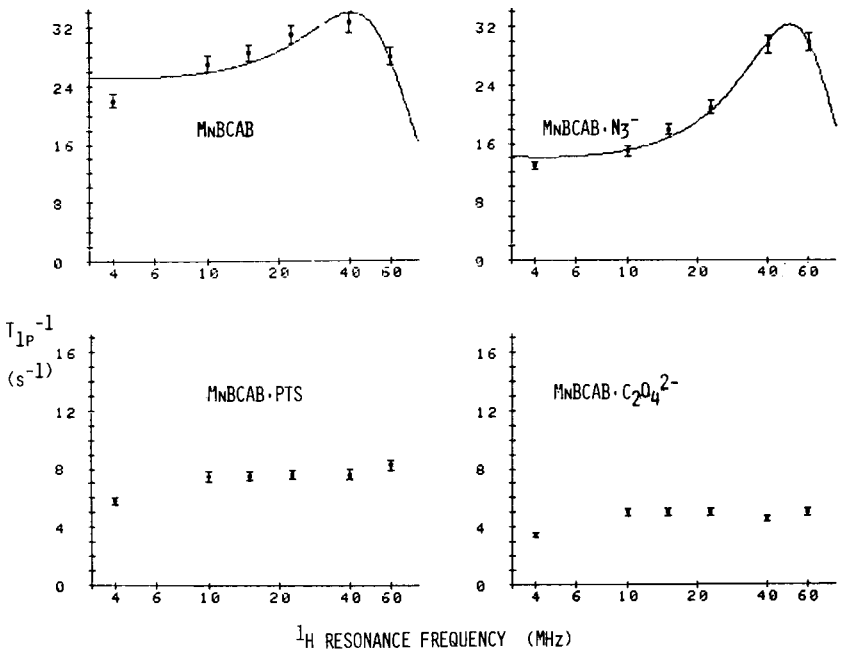

Fig. 3. ^1H T_{1p}^{-1} values as a function of frequency for 8.2×10^{-4} *M* solutions of manganese(II) Bovine Carbonic Anhydrase B at pH 9.0 and of its azide, oxalate, and *p*-toluene sulfonamide derivatives at pH 9.0. The lines represent the best fitting curves according to equations 3 and 4.

in terms of structural properties. The τ_c values for the investigated copper systems are constant and in a range typical of that ion [36–43]; the absolute G values are directly related to the presence or absence of coordinated water, and, to a lesser extent, to the contribution of the other protons. The two derivatives with low T_{1p}^{-1} values display a G factor indicating the presence of exchangeable protons at a distance larger than the usual coordination distance. For example, a G factor of 0.93×10^{-15} pm^{-6}, if due to water, corresponds to a Cu–H distance of 360 pm and to a Cu–O distance of about 290 pm. On the other hand, a geometrical factor of 5.5×10^{-15} pm^{-6}, as found for the system showing the largest T_{1p}^{-1} values, may either mean a single water molecule bound to the paramagnetic center with a Cu–H distance of 267 pm (Cu–O ≅ 200 pm) [44], or a coordinated water molecule with a Cu–H distance of 276 pm (Cu–O ≅ 210 pm) plus a non coordinated water molecule in the same position as in the previous case.

The calculated τ_c values for the cobalt systems fall in two ranges differing by an order of magnitude; within the frame of eqn. 1 they are the actual τ_e values of the chromophores. The electronic relaxation times of paramagnetic metal ions decrease with increasing number of low lying excited levels [45, 46]. In pseudooctahedral cobalt(II) complexes the splitting at zero field of the $^4T_2$ ground level provides the low lying excited states [47]. Accordingly, six coordinate cobalt(II) complexes are

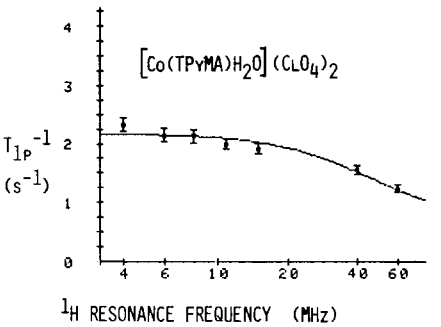

Fig. 4. ^1H T_{1p}^{-1} values as a function of frequency for 6.6×10^{-3} *M* solutions of {Co[tris(3,5-dimethyl-1-pyrazolylmethylamine)]H_2O}$^{2+}$ at pH 8.0. The line represents the best fitting curve according to eqn. 3.

expected to display τ_e values as short as 5×10^{-13} s [48].

In the tetrahedral case the ground level is not degenerate, the first excited level being above 3000 cm^{-1} [47]; therefore larger τ_e values are expected. To our knowledge there are no τ_e values determined on tetrahedral complexes except for the non-inhibited CoBCAB system [21], for which a τ_e of 3.3×10^{-11} s is estimated in the present work. Indeed, it is well known that octahedral cobalt(II) compounds give rise to sharper NMR signals than pseudotetrahedral compounds [49]. One might expect that five coordinate complexes display intermediate τ_e values: actually, in the trigonal bipyramidal case,

five orbital energy levels are within 5000 cm^{-1} from the ground level [50], and in the case of the square pyramid the ground A_2 level and the first excited E level are so near that often both of them are populated at room temperature [51]. Consistently, the five-coordinate model complex {Co-[tris(3,5-dimethyl-1-pyrazolylmethyl)amine]H_2O}$^{2+}$ [29, 52] shows a τ_c value of the order of 5×10^{-12} s (Table I and Fig. 4). Derivatives with $\tau_c < 10^{-11}$ s may be thus tentatively considered as five coordinate. It should be pointed out that the very same derivatives were assigned a five coordinate stereochemistry on the basis of electronic spectra [9, 10] and an essentially square pyramidal geometry was proposed on the basis of the ESR spectra [11].

Both classes of derivatives grouped according to the values of τ_c may have G values either in the range 3.3–5.5 or 0.82–1.4 $\times 10^{-15}$ pm^{-6}, with the exception of the thiocyanate containing system. The derivatives falling into the former range are the pure enzyme and the five coordinate species with monodentate inhibitors like nitrate, acetate, and dicyanoaurate(I). Thus, all of them have a water molecule in the donor set. Indeed, there is a general agreement on this point for the pure enzyme [1, 25] and $Au(CN)_2^-$ has been found through X-ray techniques to be present within the cavity in a different site with respect to the water site [53] and through spectroscopic techniques to be bound to the metal [10]. The derivatives with $G \leqslant 1.4 \times 10^{-15}$ pm^{-6} probably do not have any water coordinated to the metal ion. Indeed, sulfonamides and cyanate are generally believed to be pseudotetrahedral with three histidines and the inhibitor as ligand [54, 55]. The oxalate derivative is five coordinated ($\tau_c < 10^{-11}$ s) with the inhibitor acting as bidentate [8] ($G = 0.97 \times 10^{-15}$ pm^{-6}). The thiocyanate derivative, whose assignment as five coordinate [9, 11] is confirmed by its low τ_c value, probably also contains a water molecule in the coordination sphere: its G value is 2.4×10^{-15} pm^{-6}, which is somewhat smaller than the G values for the other CoBCAB systems containing a coordinated water molecule. The spread of the G values among the various systems containing coordinated water may be attributed to variations of magnetic anisotropy; however, if some allowance is made on the absolute figures, the combined G and τ_c values do provide a criterion for understanding the chemical behavior of the systems.

The understanding of the origin of the residual T_{1p}^{-1}, *i.e.* of the nature of protons causing a G value $\leqslant 1.4 \times 10^{-15}$ pm^{-6} in both cobalt and copper systems in absence of coordinated water is a relevant problem. A possibility would be that the imino protons of the coordinated histidines contribute to the residual G values. However, they exchange slowly on the NMR time scale, as observed in the diamagnetic zinc enzyme [56]. The OH group of the active site residue threonine 199 is reported to be hydrogen bonded to the coordinated water molecule [57], but alcoholic protons display exchange times around 10^{-2} s [58], *i.e.* more than one order of magnitude longer than required to fulfil the fast exchange conditions in the present systems. Unless an unusual exchange mechanism is operative for the latter proton, two protons at a distance of 330–350 pm would account for the residual G values, indicating the presence of a second site for water within the cavity, as suggested by Clementi *et al.* on the ground of Monte Carlo calculations [59]. Since both five and four coordinated chromophores show about the same residual G values, the site is probably not very specific. The pH dependence of T_1^{-1} of CuBCAB solutions may also indicate that variable amount of aspecific water is present in the cavity. In fact, since the minimum of the curve at pH 8 is present at every frequency, G and not τ_c is responsible for the observed pattern. Finally, the solvation of the bound inhibitor may provide a further pathway for protons to relax, owing to the coupling with electrons delocalized onto the ligand. Again, this contribution would be different for each compound.

A final comment is due to the pH dependence of the T_{1p}^{-1} values around the pK_a of the acidic group controlling the affinity of the inhibitors [9, 17, 60–62]. Under the conditions at which the present experiments were performed, the electronic spectra of CoBCA and CuBCA vary according to pK_a values of approximately 6.6 and 8.5, respectively; only the acidic forms would be complexed with the inhibitors of the native enzyme. Among various candidates the coordinated water has been proposed as dissociating group [65]. The present data do not help in this respect: the G values for CoBCAB are almost constant over the entire pH range investigated; those of CuBCAB are also constant (about 4×10^{-15} pm^{-6}) within two pH units from the above pK_a. Therefore if there is a dissociation of the water at high pH values, the loss of a proton would be compensated by a shorter metal–proton distance. Such distance is indeed shorter in inorganic hydroxocomplexes by 10–20% than in aqua complexes [66]. A 14% difference would account for the pH independence of the data*.

Regarding the manganese(II) system a further problem arises from the magnetic field dependence

*According to Koenig *et al.* [67] the pK_a of the acidic group would be much lower than the above values in non inhibited enzyme solutions. Such hypothesis, which could not be verified by us, would be consistent with the presence of a water molecule coordinated to the metal all over the pH range investigated in a unique enzymatically active form.

of τ_e. In this case a successful analysis was already performed by treating τ_e as dependent from the zero field splitting modulation correlation time [24]. However, the T_{1p}^{-1} values used in this analysis were those reduced for the residual relaxivity of a sulfonamide derivative [24]. From the present T_{1p}^{-1} data a G value of 3.3×10^{-15} pm^{-6} is obtained (Table I) which satisfactorily compared with the other systems having a coordinated water molecule. The set of values of the parameters G, B, and τ_v are consistent with the values previously proposed [24]. Even in the case of the azide derivative a similar analysis provides a G value of 3.6×10^{-15} pm^{-6}, confirming the presence of the water molecule in the adduct. It is noteworthy that in this case T_{1p}^{-1} decreases with decreasing frequency half a way towards the residual relaxation values (Fig. 3); a field dependence of the τ_e values allows to fully account for the observed data. Although the above analysis could not be performed in the case of the *p*-toluenesulfonamide and oxalate derivatives, the large difference in T_{1p}^{-1} values for the latter systems as compared to the non-inhibited enzyme and azide derivative all over the frequency range investigated suggests that in both cases water is removed from the metal coordination sphere, analogously to what found for the cobalt and copper systems.

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