Carbon-13 Longitudinal Relaxation Times of Acetate Ion in the Presence of Metal-substituted Bovine Carbonic Anhydrases

By **Ivano Bertini,* Claudio Luchinat,** and **Andrea Scozzafava,** Cattedra di Chimica Generale ed Inorganica dell'Istituto di Chimica Farmaceutica dell'Università di Firenze, and Laboratorio per lo Studio dei Composti di Coordinazione del C.N.R., Via G. Capponi, 7-50121 Firenze, Italy

Longitudinal relaxation times T_1 have been determined for both carbons of ^{13}C-enriched acetate in the presence of bovine apo-, zinc(II), cobalt(II), and manganese(II) carbonic anhydrase, and the factors affecting them have been analyzed. The existence of a further interaction besides the binding at the metal atom has been confirmed by measuring T_1 in the presence of excess of a strong inhibitor like toluene-*p*-sulphonamide. By use of the Solomon–Bloembergen analysis, distances and orientations of acetate with respect to the metal ion have been estimated both at the metal binding site and when unco-ordinated.

LANIR AND NAVON showed that acetate ion binds zinc [1] and manganese [2] carbonic anhydrase through both the metal ion and the protein part of the enzyme. By means of ^1H n.m.r. linewidth measurements they proposed a

[1] A. Lanir and G. Navon, *Biochim. Biophys. Acta*, 1974, **341**, 65.

[2] A. Lanir and G. Navon, *Biochim. Biophys. Acta*, 1974, **341**, 75.

model in which a second binding site different from that at the metal was characterized. More recently evidence was found also for interactions between acetate ion and the protein part of cobalt carbonic anhydrase.[3]

Longitudinal relaxation times T_1 for ^{13}C in small molecules are relatively long and are expected to be very sensitive to interactions with macromolecules [4] and/or with a paramagnetic centre,[5-7] although there are few literature references. In the case of paramagnetic centres, analysis by use of the Solomon–Bloembergen equation may provide useful structural information on the type of interaction.[8-10] With this in mind, the T_1 values have been measured for both carbons of ^{13}C-enriched acetate in presence of apo-, zinc(II), manganese(II), and cobalt(II) carbonic anhydrase.

EXPERIMENTAL

Bovine carbonic anhydrase (carbonate dehydratase, E.C. 4.2.1.1.) was obtained as lyophilized material from Sigma Chemical Co. Sodium [$^{13}C_1$]- and [$^{13}C_2$]-acetate were obtained from Prochem B.O.C. Ltd. Sodium azide, toluene-*p*-sulphonamide, and all the other materials were of analytical grade.

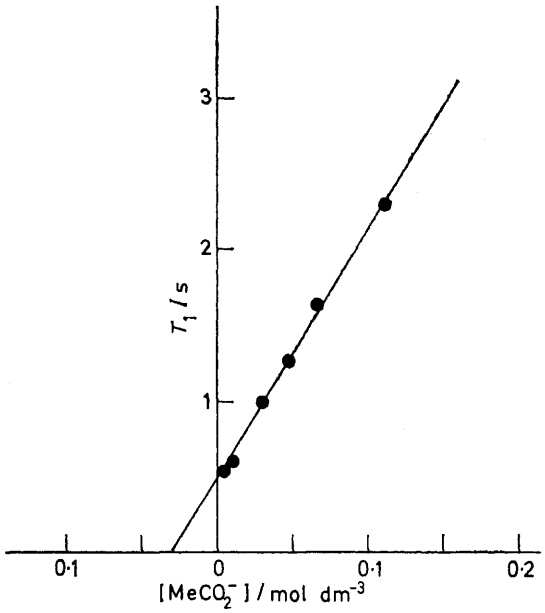
Protein concentrations were determined from the absorbance at 280 nm, using a molar absorption coefficient of 5.7×10^4 dm^3 mol^{-1} cm^{-1}.[11] Esterase-activity measurements were made using *p*-nitrophenyl acetate as substrate at 22 °C.[12]

Apocarbonic anhydrase was prepared from the commercial enzyme by extensive dialysis against 0.01 mol dm^{-3} 1,10-phenanthroline in 0.1 mol dm^{-3} acetate buffer (pH 5.2), to a residual activity of $<5\%$. Samples of the zinc, cobalt, and manganese enzymes were prepared by dialysis of apocarbonic anhydrase against the corresponding 10^{-3} mol dm^{-3} metal sulphate, in aminotris(hydroxymethyl)methane–sulphate buffered solutions (pH 7.5), as previously reported.[11,13] Free zinc(II) and cobalt(II) ions were dialyzed from the respective enzyme solutions using several changes of buffer. The manganese enzyme solution contained 100% excess of manganese ions, in order to prevent dissociation of the metal–enzyme complex.[2,11,13]

Samples for n.m.r. examination were prepared by adding ^{13}C-enriched Na[O_2CMe] to the buffered stock solutions of metalloenzyme; a small amount of acetone was added as reference. The final pH was 7.5. The diamagnetic samples were degassed in order to exclude paramagnetic interferences. Blank samples were prepared using the final dialysis solutions. Concentrations were *ca.* 10^{-4} mol dm^{-3} for manganese(II) carbonic anhydrase and 0.5×10^{-3}—1×10^{-3} mol dm^{-3} for the other enzymes, whereas acetate concentrations ranged between 0.2 and 0.3 mol dm^{-3}, unless otherwise stated. N.m.r. spectra were recorded on a Varian CFT 20 spectrometer operating at 15 °C, using ^2H_2O as lock signal. Chemical shifts were measured from the acetone CO signal; T_2 values were obtained from the relation $T_2 = 1/\pi\Delta\nu$ where $\Delta\nu$ is the signal linewidth at half-peak height. Signals for T_2 measurements were obtained from Fourier transform of the free induction decay without any weighting function. Values of T_1 were obtained using the sequence 180° pulse–τ–90° pulse–delay.

RESULTS

Longitudinal relaxation times T_1 for acetate ion were determined in buffered blank solutions as well as in presence of apo-, zinc(II), cobalt(II), and manganese(II) bovine carbonic anhydrase. The drastic reduction of the T_1 values observed with respect to those of the reference samples, independently of the presence of the paramagnetic centre and of the metal itself, shows that the acetate ion is bound to the macromolecule according to a dipolar relaxation mechanism between the resonating nuclei and the nuclei of the protein part. The paramagnetic ion causes



Concentration dependence of the longitudinal relaxation time T_1 of the $^{13}C^1$ carbon of acetate in the presence of 4.3×10^{-4} mol dm^{-3} cobalt(II) carbonic anhydrase, toluene-*p*-sulphonamide, and 10^{-1} mol dm^{-3} phosphate buffer (pH 7.5)

such values to be further reduced: in the case of the manganese enzyme the T_1 values were two orders of magnitude smaller than for the other enzyme derivatives. The strong enzyme inhibitor toluene-*p*-sulphonamide (ts) removes almost quantitatively the acetate co-ordinated to the metal ion and gives a sulphonamide adduct. However, on addition of ts in a 10% or more excess, the T_1 values increased only slightly for the zinc and cobalt derivatives; T_1 values appropriate for free acetate were obtained only after addition of a large excess of $[N_3]^-$. It is meaningful that the linewidth of the n.m.r. signal of the cobalt enzyme

[3] I. Bertini, C. Luchinat, and A. Scozzafava, *Biochim. Biophys. Acta*, 1976, **452**, 239.
[4] A. Allerhand and D. Doddrell, *J. Amer. Chem. Soc.*, 1971, **93**, 2777.
[5] G. C. Levy and R. A. Komoroski, *J. Amer. Chem. Soc.*, 1974, **96**, 678.
[6] P. L. Yeagle, C. H. Lochmuller, and R. W. Henkens, *Proc. Nat. Acad. Sci. U.S.A.*, 1975, **72**, 454.
[7] I. Bertini, C. Luchinat, and A. Scozzafava, *Inorg. Chim. Acta*, 1976, **2**, 201.
[8] I. Solomon, *Phys. Rev.*, 1955, **99**, 559.
[9] N. Bloembergen, *J. Chem. Phys.*, 1957, **27**, 572.
[10] A. S. Mildvan and M. Cohn, *Adv. Enzymol.*, 1970, **33**, 1; A. S. Mildvan and J. L. Engle, *Methods Enzymol.*, 1972, **26**, 654.
[11] S. Lindskog and P. O. Nyman, *Biochim. Biophys. Acta*, 1964, **85**, 462.
[12] R. W. Henkens and J. M. Sturtevant, *J. Amer. Chem. Soc.*, 1968, **90**, 2669.
[13] A. Lanir and G. Navon, *Biochemistry*, 1972, **11**, 3536.

is much reduced by the addition of ts,[3] indicating that the T_2 values are drastically affected by the binding to the metal.

For the manganese enzyme the difference in T_1 in the presence and absence of ts was within the experimental error, even when the acetate concentration was increased from 2.6×10^{-1} to 1.4 mol dm^{-3} and the ratio of manganese enzyme to acetate was kept constant. This is presumably due to a low occupancy of the binding site at the metal, the affinity constant for this site being very low, probably even lower than the value of 0.8 dm^3 mol^{-1} determined by inhibition measurements.[2]

A typical set of T_1 values is reported in the Table. The

T_1 values(s) [a] for $^{13}C^1$ and $^{13}C^2$ of the acetate ion [b] in the presence of various bovine carbonic anhydrase derivatives at pH 7.5

			Inhibitor			
			ts		ts + $[N_3]^-$	
Enzyme	C^1	C^2	C^1	C^2	C^1	C^2
c	34	10			31	9.0
Apo	2.8	4.5	3.0	4.0	36	10
Zinc	5.1	7.1	7.3	7.9	21	10
Cobalt	1.3	2.3	2.9	4.5	25	9.5
Manganese	0.10	0.23	0.11	0.26	3.6	5.5
	(4.4)	(7.6)			(10)	(10)

[a] Estimated accuracy is ±10% except for long T_1 values, which also depend on small amounts of paramagnetic impurities. [b] Acetate : enzyme ratio = 3.3×10^{-2} : 1 except for the manganese derivative. In the latter case the ratio was 3.3×10^3 : 1. Values for the manganese-containing blank solutions are given in parentheses. [c] Blank solution containing only 2.4×10^{-1} mol dm^{-3} acetate.

values are reproducible to within 10%, the largest variations occurring for the systems containing ts, and are proportional to the molar fraction of the bound acetate. For the cobalt enzyme in the presence of ts, T_1 measurements with increasing acetate concentration (5×10^{-3}—10^{-1} mol dm^{-3}) were made (Figure). A solution 7.2×10^{-4} mol dm^{-3} in cobalt enzyme and 2.4×10^{-1} mol dm^{-3} in acetate showed an isotropic shift of C^1 and C^2 of 10 Hz. This shift was titrated by adding ts.[3] In the case of manganese no shift was detected in the range of concentrations suitable for observing well shaped signals.

DISCUSSION

The data obtained with the apoenzyme show that acetate ion strongly interacts with the protein part of the enzyme; as expected, the longitudinal relaxation times T_1 are strongly affected by the interactions of acetate with the macromolecule which represents a slowly rotating system.[4,14] The fact that the T_1 values are shorter for the apo- than for the zinc enzyme is also interesting. Indeed, the affinity of acetate for zinc in the zinc enzyme has been estimated through inhibition measurements to be quite low, *i.e.* of the order of 10 dm^3 mol^{-1} at pH 7.5.[1] Removal of the zinc ion provides some more binding sites for the acetate in the metal-free cavity itself. The T_1 values of the system apoenzyme–acetate are not affected by addition of ts, although sulphonamides have been found to bind also the apoenzyme.[15] This indicates that acetate and ts do not compete for the same binding sites of the apoenzyme and that the binding sites of acetate are positive residues at which only negative ions may compete.[16]

The short T_1 values of acetate ion in the presence of manganese carbonic anhydrase and ts are diagnostic of strong dipolar coupling between the manganese ion and the resonating nuclei. Since such coupling effects depend on r^{-6},[8,9] interactions of acetate with sites not very close to the metal ion may be neglected. Therefore it is concluded that one or more acetate ions remain in close proximity to the paramagnetic centre. The same conclusion had been reached from ^{1}H n.m.r. linewidth measurements and the existence of a second specific binding site was proposed which differed from that on the metal.[2] Also, in the case of the cobalt enzyme, if it is assumed that the zinc and cobalt enzymes are similar, acetate ion in the presence of ts experiences the close proximity of the paramagnetic centre. A plot of T_1 against the concentration of free acetate gives a straight line from whose intercept a value for the affinity constant of *ca.* 40 dm^3 mol^{-1} is obtained (Figure). A similar value was obtained for the manganese enzyme in the presence of ts.[2] As previously proposed, this constant and the relative shortening of T_1 are associated with a second site which is to be distinguished from the binding site at the metal which is referred as the first site. Other information on these sites follows, from a further analysis of the T_1 values.

Structural Information.—The availability of relaxation times for the two carbons of acetate may provide some information on the orientation of the inhibitor with respect to the metal at each binding site. In particular, the data relative to the first site of the cobalt enzyme and to the second site of the manganese enzyme appear to be intrinsically the least affected by error and more meaningful with respect to the Solomon–Bloembergen analysis.[8,9] The Solomon–Bloembergen equation is as in (1) where T_{1M}

$$\frac{1}{T_{1M}} = \frac{2}{15} \cdot \frac{S(S+1)\gamma_I^2 g^2 \beta^2}{r^6} \times \left(\frac{3\tau_c}{1+\omega_I^2\tau_c^2} + \frac{7\tau_c}{1+\omega_s^2\tau_c^2} \right) + \frac{2}{3} \cdot \frac{S(S+1)A^2}{\hbar^2} \cdot \frac{\tau_e}{1+\omega_s^2\tau_e^2} \quad (1)$$

is the longitudinal relaxation time of acetate bound to the metal enzyme, r is the distance from the metal to the resonating nucleus, and all the other symbols have the usual meaning. The contact term, which is independent of r, can be neglected because it is zero by definition in the case of the second site and because it is small in the case of the first site owing to the small hyperfine coupling constant, A.[17]

[14] A. Allerhand, D. Doddrell V. Glushko, D. W. Cochran, E. Wenkert, P. J. Lawson, and F. R. N. Kurd, *J. Amer. Chem. Soc.*, 1971, **93**, 544.

[15] J. E. Coleman, *Nature*, 1967, **214**, 193.

[16] J. E. Verpoorte, S. Mehta, and J. T. Edsall, *J. Biol. Chem.*, 1967, **242**, 4221.

[17] T. J. Swift and R. E. Connick, *J. Chem. Phys.*, 1962, **37**, 307.

The longitudinal relaxation time T_{1M} is related to the paramagnetic contribution to the observed relaxation rate, T_{1p},[18] by equation (2), where x is the molar fraction of the

$$(xT_{1p})^{-1} = (T_{1M} + \tau_M)^{-1} \quad (2)$$

acetate bound to the enzyme and τ_M is the residence time in the co-ordination sphere. Finally, T_{1p} is related to the measured T_1 by equation (3) where T_{1p}^{-1} includes all the

$$T_{1p}^{-1} = T_1^{-1} - \Sigma_i T_{1i}^{-1} \quad (3)$$

paramagnetic contributions due to other binding sites and the diamagnetic contributions measured for the zinc sample.

Information on the first site. As previously noted, only data relative to the cobalt compound are meaningful in this respect. Values of T_{1i} in equation (3) are T_1 of the second site in the cobalt derivative and T_1 of the zinc derivative at the first site.

Values of xT_{1p} for C^1 and C^2 were determined as 7.7×10^{-3} and 1.7×10^{-2} s respectively. In order to estimate τ_M, values of T_2 were determined from linewidth measurements. In a solution containing acetate (2.4×10^{-1} mol dm^{-3}) and enzyme (7.2×10^{-4} mol dm^{-3}) linewidths for C^1 and C^2 were 18 and 21 Hz, decreasing to 1.0 and 2.4 Hz in the presence of ts. The calculated values of xT_{2p} for C^1 and C^2 are 5.9×10^{-5} and 5.5×10^{-5} s respectively. Since $(xT_{1p})^{-1} \ll (xT_{2p})^{-1}$, the linewidths cannot be dominated by T_{2M}, the transverse relaxation time, but they must depend on $1/\tau_M$ or $\tau_M\Delta\omega^2$, where $\Delta\omega$ is the isotropic shift.[3,17] The values of τ_M obtained from the two relations are similar and in each case may be neglected with respect to xT_{1p}, so that it can be reasonably assumed that $xT_{1p} = T_{1M}$. Therefore, taking into account equation (1), the ratio between the experimental xT_{1p} values for the two carbon atoms, $T_{1p(C^1)}$: $T_{1p(C^2)}$, is equal to the sixth power of the distance ratio $Co–C^1$: $Co–C^2$. This ratio, equal to 0.87 : 1, shows that the $C^1–C^2$ line is almost orthogonal to the $Co–C^1$ line. This picture of the acetate as a ligand rules out the possibility of bidentate behaviour of the acetate. Since the acetate adduct has been assigned five-co-ordinate stereochemistry,[2,18] a further donor atom is present in the cavity.

If the correlation time τ_c were known the absolute distance could be determined through the Solomon–Bloembergen equation. However, τ_c is generally believed to be dominated by the electron relaxation time, τ_e, and to be therefore in the range 10^{-11}—5×10^{-13} s.[19] As an example, if a value of 10^{-12} s is assumed for τ_c, the estimated $Co–C^1$ distance is 3.05 Å, which is consistent with a Co–O distance of 2.1 Å, a C^1–O distance of 1.3 Å, and a $Co–O–C^1$ angle of *ca.* 130°.[20]

* By neglecting τ_M with respect to T_{1M}, as in the case of the first site, and using again $\tau = 10^{-12}$ s, a $Co–C^1$ distance of 3.4 Å is obtained together with a $Co–C^1$: $Co–C^2$ distance ratio of 0.88 : 1.

[18] I. Bertini, C. Luchinat, and A. Scozzafava, *J. Amer. Chem. Soc.*, 1977, **99**, 581.

Information on the second site. Assuming that the observed T_1 values for the manganese enzyme in the presence of ts are due to a single specific binding site, analysis using the Solomon–Bloembergen equation is again fruitful. From the values in the Table and from the affinity constant of 40 dm^3 mol^{-1} at 15 °C,[2] xT_{1p} values of 3.4×10^{-5} and 7.8×10^{-5} s are obtained for C^1 and C^2 respectively. In this case, however, τ_M has been shown to be of the same order of magnitude as T_{1p} and therefore their difference is affected by a large error. If the previously used [2] values of $\tau_M = 1.6 \times 10^{-5}$ s and $\tau_c = 1.0 \times 10^{-8}$ s are taken, a $Mn–C^1$ distance of 3.9 Å and a $Mn–C^1$: $Mn–C^2$ distance ratio of 0.9 : 1 are found. These data are quite consistent with those obtained for the proton from a linewidth analysis.[2] They are also substantially consistent with the experimental data in the Table relative to the second site of the cobalt enzyme,* although in this case a further source of error arises from T_{1Zn} being of the same order of magnitude as the experimental T_1. Undoubtedly such a $M–C^1$ distance is very short; however, the electronic spectrum of the ts adduct of the cobalt enzyme is insensitive to the presence of acetate. Therefore interactions, if any, between the metal ion and acetate at the second site do not involve changes in the electronic levels of the metal atom.

An alternative explanation of the experimental longitudinal relaxation times should be mentioned; *i.e.* similarly to the water molecules in the crevice of the native enzyme,[21] several acetate ions are present within the active site crevice. One acetate ion is directly bound to the metal ion, whereas the others simulate a molecule binding to a single ' second site ' closer to the metal ion than any real molecule. For example, the data in the Table for the second site of the manganese derivative could be accounted for by assuming the presence of two acetate ions with $Mn–C^1$ distances of 4.4 Å or three ions with distances of 4.7 Å.

However, the apparent association constants for the second site are the same (*ca.* 40 dm^3 mol^{-1}) for the manganese [2] and cobalt and zinc derivatives.[1] In the case of the paramagnetic species the constants are relative to acetate ions interacting with the macromolecule within *ca.* 5 Å of the paramagnetic centre, whereas for zinc carbonic anhydrase all the interactions over the whole molecule are included. Therefore the suggestion of the existence of a single second binding site, whose effects may be distinguished from non-specific binding to many sites, seems reasonable.

We thank Professor L. Sacconi for discussion, and Professor P. Papini for encouragement.

[7/091 *Received*, 18*th January*, 1977]

[19] Z. Luz and S. Meiboon, *J. Chem. Phys.*, 1964, **40**, 1058, 2686.
[20] J. N. Van Niekerk and F. R. L. Schoening, *Acta Cryst.*, 1953, **6**, 609.
[21] K. K. Kannan, B. Notstrand, K. Fridborg, S. Lovgren, A. Ohlsson, and M. Petef, *Proc. Nat. Acad. Sci. U.S.A.*, 1975, **72**, 51.