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^{17}O NMR Investigation of Copper(II) Substituted Carbonic Anhydrases

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The water ^{17}O longitudinal and transverse relaxation rates have been measured in solutions of copper substituted bovine and human carbonic anhydrases B and of some of their inhibitor derivatives, as well as in solutions of the corresponding native zinc enzymes.

The sizeable transverse relaxation rate enhancement observed when the paramagnetic copper(II) ion is substituted for zinc(II) is ascribed to contact electron–nuclear coupling, typical of water coordinated to a copper(II) chromophore in equatorial position.

Exchangeable $H_2{}^{17}O$ is present in the metal coordination sphere all over the pH range 5–10, as well as in the adducts with the monodentate anionic inhibitors, while it is removed in the adducts with the bidentate oxalate and with p*-toluene-sulfonamide. For other sulfonamide derivatives the lack of paramagnetic effect on $H_2{}^{17}O$ is ascribed either to a more axial position of the coordinated water or to a slower exchange rate with the bulk solution.*

Introduction

The essential role of water in the catalytic mechanism of carbonic anhydrase has been thoroughly investigated [1, 2]. There is general consensus on a water molecule being coordinated at the zinc(II) ion, at least at acidic pH values. Its behaviour, with respect to 1) the acid–base properties, 2) the interaction with substrates and 3) the substitution by inhibitors, can be guessed from several kinds of experimental measurements. In principle ^1H, ^2H, and ^{17}O NMR experiments can directly monitor the nuclei of the water molecule. However, the molar fraction of coordinated water can be only as high as $3–4 \times 10^{-3}/55.5$. Therefore, any effect on the NMR parameters of the coordinated water is decreased by a factor at least equal to the above molar fraction.

When the native zinc(II) ion is substituted by paramagnetic metal ions, a sizeable effect is measured on the ^1H NMR relaxation rates of the water solutions [3–6]. Such measurements have allowed to establish that exchangeable protons are bound to the donor atoms of the coordination sphere at every pH in the range 5.6–11. The nuclear relaxation rates increase through nucleus-unpaired electron(s) coupling which occurs *via* dipolar and contact interactions [7]. The former contribution depends on the sixth power of the distance from the paramagnetic center and the latter from spin delocalization effects. In the case of ^2H and ^{17}O nuclei the nuclear quadrupole moment is a cause of large relaxation rates enhancements due to the coupling between the quadrupole moments and electric field gradients at the observed nucleus when bound to the protein [8]. Generic water–protein interactions may be capable of inducing large relaxation rate enhancements and to mask the effects due to paramagnetic ions [9].

Copper(II) ions are known to cause large relaxation rate enhancements owing to their favourable electronic relaxation rates and to the concomitant operativity of contact contributions [10–12]. The latter contributions may be the factor determining the linewidth and therefore the T_2^{-1} enhancements. With this in mind we have investigated the copper substituted carbonic anhydrase through ^{17}O NMR spectroscopy.

Experimental

The human B isoenzyme was purchased from Sigma (U.S.A.) and used without further purification. The bovine B isoenzyme was obtained through chromatography from a commercial mixture of isoenzymes again provided by Sigma. The apoenzymes and the copper derivatives were prepared and checked as previously reported [13]. 20% enriched $H_2^{17}O$ was added to protein solutions to final enrichments of 2–5%. The inhibitors were commercial products of analytical grade. NMR measurements were performed with a CXP Bruker spectrometer operating at 8.13 MHz; T_1 values were calculated from a best fitting treatment of the peak heights obtained

TABLE I. Water ^{17}O Transverse Relaxation Rate Enhancements (T_{2p}^{-1}, s^{-1}) in Copper(II) Carbonic Anhydrase Solutions[a] with Respect to the Native Enzyme[b], and Calculated[c] Full Paramagnetic Effect on the ^{17}O Nucleus (T_{2M}^{-1}, s^{-1}).

	T_{2p}^{-1}	T_{2M}^{-1}
CuBCAB, pH 7.4	5.2×10^2	9.6×10^6
CuBCAB + I^-, pH 6.3	9.4×10^2	1.7×10^7
CuBCAB + N_3^-, pH 7.2	8.8×10^2	1.6×10^7
CuBCAB + *p*-toluenesulfonamide, pH 6.1	~0	~0
CuBCAB + acetazolamide, pH 6.4	~0	~0
CuBCAB + oxalate, pH 7.1	~0	~0
CuHCAB, pH 5.9	5.9×10^2	1.1×10^7
CuHCAB, pH 9.4	4.6×10^2	8.5×10^6
CuHCAB + *p*-toluenesulfonamide, pH 5.5	~0	~0

[a]All the measurements are referred to 3×10^{-3} *M* solutions, at 295 K. [b]The reconstituted zinc enzyme solutions (3×10^{-3} *M* at pH between 6.2 and 9.4) give rise to T_2^{-1} values of 4.5×10^2 s^{-1}. [c]In the assumption of a single water molecule coordinated to the copper(II) ion.

through the inversion recovery method. T_2 values were obtained from linewidth measurements through the relationship $T_2 = (\pi\Delta\nu)^{-1}$.

Results and Discussion

The ^{17}O NMR line of water solutions containing the copper derivatives of both human (CuHCAB) and bovine (CuBCAB) carbonic anhydrases is sensibly broader than that of solutions containing the diamagnetic zinc enzyme. The linewidth difference is therefore attributable to the coupling between the ^{17}O nucleus and the unpaired electron, and can be directly related to the paramagnetic contribution to the ^{17}O transverse relaxation rate, T_{2p}^{-1}. The T_{2p}^{-1} values obtained for the copper enzymes and for some of their inhibitor derivatives are reported in Table I. The experimental linewidths decrease with increasing temperature in the range 275–300 K for both the diamagnetic and paramagnetic samples. The same behaviour is shown by the linewidth difference, related to the paramagnetic contribution, T_{2p}^{-1}, indicating that the exchange between free and bound water is fast on the NMR time scale. This check was performed for all the measurements reported in this communication. In no case appreciable isotropic shift from the zinc enzyme was detected, although differences of less than 10 Hz may be easily overlooked owing to the broadness of the lines. From the full paramagnetic contributions reported in Table I and the observed temperature dependence, a lower limit for the water exchange rate constant of $2–3 \times 10^7$ s^{-1} can be calculated. While the above value falls in the range of $10^6–10^9$ s^{-1} [14–16] expected for simple copper complexes, it is higher than those ($10^5–10^6$ s^{-1}) measured through kinetic techniques for the exchange of water in the native human C isoenzyme [1].

The T_1 measurements of the CuCA solutions provided values equal within the standard deviation to those of solutions of the apoenzyme and of the native enzyme (typically about 3 ms). In this case the effect of the paramagnetic center is hardly detected. Indeed, the paramagnetic effect on T_1^{-1} is mostly governed by the dipolar coupling contribution [7]. The difference between T_1^{-1} and T_2^{-1} is ascribed to the different weight of the contact term in the longitudinal and transverse nucleus–electron couplings [7]. It can be concluded that the paramagnetic effect observed on the ^{17}O linewidths is entirely due to contact contributions and hence related only to nuclei directly bound to the paramagnetic center. At variance with the results obtainable from ^1H relaxation measurements, ^{17}O relaxation is selectively related to water in the first coordination sphere.

Once established that the ^{17}O NMR linewidth is sensible to the binding of water to the paramagnetic center, the pH dependence of the linewidth reported in Fig. 1 deserves some comments. The linewidth is not markedly pH dependent in the pH range 5.5–9, with a tendency to increase at pH > 9. While it is not difficult to propose a fast exchange mechanism of the coordinated water, in the native enzyme the acidic $Zn–OH_2$ moiety has been proposed to have a pK_a around 7 [17]. The CuBCAB has been reported to have an acidic group with pK_a around 8 [18]. If such a group were the bound water the problem arises of which would be the effect on the ^{17}O NMR linewidth upon water ionization. On a system containing high spin cobalt(II) and a water molecule the ^{17}O linewidth drops when the coordinated water ionizes to OH^-, indicating that the exchange of the coordinated OH group is

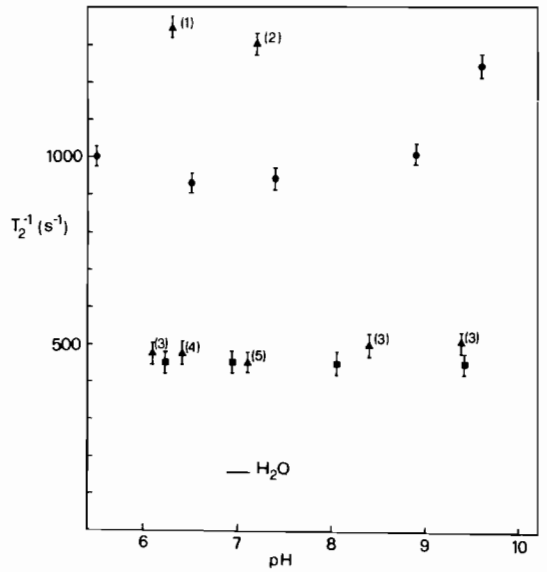

Fig. 1. pH Dependence of T_2^{-1} values for the ^{17}O signal of unbuffered water solutions containing 3.0×10^{-3} *M* copper(II) Bovine Carbonic Anhydrase B (•). The values (▲) obtained for the adducts with iodide (1); azide (2); *p*-toluenesulfonamide (3); acetazolamide (4); and oxalate (5) are also shown, together with those for 3.0×10^{-3} *M* solutions of the native bovine B isoenzyme (▪).

slow [19]; this would be a consequence of the small concentration of free OH^- ions in solution. In the present case, either water does not deprotonate or all the following equilibria are fast on the NMR

$$M-{}^*OH \overset{+H^+}{\rightleftharpoons} M-{}^*OH_2 \overset{+H_2O,\,-H_2{}^*O}{\rightleftharpoons} M-OH_2 \overset{-H^+}{\rightleftharpoons} M-OH$$

time scale at every pH. The latter possibility has been previously suggested [1]. Unfortunately ^{17}O NMR studies do not allow to definitely prove or disprove the deprotonation of the coordinated water.

Most of the monoanionic inhibitors of the native enzyme are known to bind the copper ion in CuBCAB but not to remove the coordinated water molecule [13]. The latter results come from ^1H NMR relaxation studies. ^{17}O linewidth measurements confirm that water is not substituted by this kind of ligands, the paramagnetic effect being even higher in the adducts (Table I). The bidentate oxalate ion is capable of quenching both the ^1H T_1^{-1} and the ^{17}O T_2^{-1} enhancements.

The investigated sulfonamides with both copper isoenzyme derivatives do not show any measurable T_2^{-1} enhancement as compared to the native zinc enzymes (Table I and Fig. 1). With respect to the ^1H T_1^{-1}, some sulfonamides (*p*-toluenesulfonamide and sulfanilamide) reduce the T_1^{-1} enhancement of the copper bovine derivative, giving rise to a peculiar ESR spectrum which was interpreted on the basis of a pseudotetrahedral geometry [13, 20]. Such coordination polyhedron does not contain any coordinated water. The above sulfonamides in the presence of the copper human derivative, and acetazolamide with both isoenzymes, do not appreciably reduce the ^1H T_1^{-1} enhancement; consistently, their ESR spectra have been assigned as due to five coordinated adducts, with a water molecule still present in the coordination sphere [20]. It remains therefore to explain why the two different kinds of sulfonamide adducts display the same behaviour with respect to ^{17}O NMR. In principle, a small contact term could account for the lack of T_2^{-1} enhancement for water bound to the paramagnetic center. Indeed, it has been shown that equatorial coordination of water in tetragonal complexes gives rise to $A/\hbar \cong 5 \times 10^7$ s^{-1} [14, 15] which compares with the value of 1.4×10^8 s^{-1} calculated for the pure CuCA derivatives using a value of 2×10^{-9} s for the copper electronic relaxation time [13]. Axial coordination gives rise to $A/\hbar \cong 2 \times 10^5$ s^{-1} [15], *i.e.* three orders of magnitude smaller than in the case of equatorial coordination. Therefore a more apical position of water in the square pyramidal chromophore could account for the drop of ^{17}O NMR linewidth. An alternative explanation is that the presence of the aromatic moiety of the ligand in the cavity slows down the ^{17}O exchange rate but not enough the ^1H exchange rate on the NMR time scale.

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