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The Investigation of Coordinated Water in Paramagnetic Metalloproteins through Nuclear Magnetic Resonance Spectroscopy

Ivano Bertini[a]
[a] Istituto di Chimica Generale e Inorganica, della Facoltà di Formacia, Firenze, Italy

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The Investigation of Coordinated Water in Paramagnetic Metalloproteins through Nuclear Magnetic Resonance Spectroscopy

Water molecules bound to metal ions in metalloproteins are rather common. If the metal ion is paramagnetic or if the native diamagnetic metal ion can be substituted by a paramagnetic ion, then the detection of water molecules and the investigation of their properties may be conveniently carried out through ^{1}H, ^{2}H, and ^{17}O NMR spectroscopy. The information which can be drawn from such an analysis is here critically discussed with reference to some typical metalloproteins. The importance of the electronic correlation times, as related to the geometry of the chromophore in high-spin cobalt(II) derivatives, is stressed. The acid-base properties of the coordinated water are also considered.

INTRODUCTION

There are several metalloproteins which contain at least one water molecule coordinated to the metal ion. Some representative examples are reported in Table I. The water molecule may simply contribute as a coordinated ligand to the overall properties of the metalloprotein or it may play a quite distinctive role as in the case of enzymes with hydrolytic or hydration activity. In general the coordinated water molecule exchanges with the bulk water, the exchange rate depending on the metal ion and its geometry, as occurs with simple aquo complexes.[1] The estimated lowest limit in the case of carbonic anhydrase and its derivatives in which the native zinc ion is substituted by copper(II), cobalt(II), manganese(II), nickel(II) and oxovanadium(IV) is 10^{5}–10^{7} s^{-1}.[2-4] In other systems, such as iron(III) transferrin and iron(III) methemoglobin, the exchange rate is lower, i.e., $<10^{4}$–10^{5} s^{-1}.[5-7] The detection of coordinated water may be difficult since the maximum protein concentration may be $(2–3) \times 10^{-3}$ mol dm^{-3} whereas the free water concentration is about 50 mol dm^{-3}. Furthermore, water also interacts rather firmly with the protein part of the metalloprotein, in such a way that the solvent may be classified as: (i) free for the most part; (ii) solvating the protein; (iii) bound to the metal.

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TABLE I
Some proteins containing water in the metal coordination sphere

Protein	Metal ion	Donor set	Coordinated groups	Water pK_a	Function	Ref.
Alkaline phosphatase	Zn(II)	N_4O	4 His, 1 H_2O		Phosphoenolpyruvate + H_2O ⇌ Pyruvic acid + HPO_4^{2-}	9
Benzylamine oxidase	Cu(II)	Unknown	2 H_2O + ?	8–9	$R\text{-}CH_2\text{-}NH_2 + H_2O + O_2 \rightleftharpoons RCHO + NH_3 + H_2O_2$	40
Carbonic anhydrase	Zn(II)	N_3O	3 His, 1 H_2O	7	$CO_2 + H_2O \rightleftharpoons HCO_3^- + H^+$	11,12
Catalase	Fe(III)	N_5O	1 Heme, 1 His, 1 H_2O		$H_2O_2 \rightleftharpoons H_2O + \frac{1}{2}O_2$	22,23
Concanavalin A	Mn(II)	NO_5	1 Glu, 2 Asp, 1 His, 2 H_2O		Saccharide-binding protein	54
Galactose oxidase	Cu(II)	N_2O_2X	2 His, 1 H_2O (?)		D-galactose + O_2 ⇌ D-galactosodialdose + H_2O_2	55
Liver alcohol dehydrogenase	Zn(II)	NS_2O	1 His, 2 Cys, 1 H_2O	11.2	Alcohol + NAD^+ ⇌ Aldehyde(or Ketone) + NADH	13
Methemoglobin	Fe(III)	N_5O	1 Heme, 1 His, 1 H_2O	8.1		56
Superoxide dismutase	Cu(II)	N_4O	4 His, 1 H_2O		$2\ O_2^- + 2\ H^+ \rightleftharpoons H_2O_2 + O_2$	57
Transferrin	Fe(III)	N_2O_4	2 His, 2 Tyr, HCO_3^-, H_2O	11.5	Iron transport protein	58

^1H, ^2H and ^{17}O NMR studies on diamagnetic proteins have indicated the complexity of the interaction between water and protein.[8] However, if the metal ion in the metalloprotein has unpaired electrons, then the NMR parameters of the coordinated water may be largely affected. The extent of the coupling between unpaired electrons and resonating nuclei may be large enough to be revealed through NMR experiments even when averaged over the bulk solvent molecules (Figure 1). Sometimes the metal ion to which water is bound is paramagnetic, as copper(II) in superoxide dismutase and galactose oxidase, or manganese(II) in concanavalin A, etc. Quite often water is coordinated to a diamagnetic ion as the zinc(II) ion. In such cases the native diamagnetic ion can be removed and replaced by other paramagnetic ions. The information obtained on these chemically modified species may be transferred with some caution to the native species. For several zinc enzymes it has been possible to replace the native zinc by cobalt with retention of the catalytic activity.[9] In such cases the information obtained on the high-spin cobalt(II) systems may be transferred to the native enzyme with some confidence.

Before analyzing the nature of the coupling between unpaired electrons and resonating ^1H, ^2H and ^{17}O nuclei, the acid–base properties of coordinated water will be discussed.

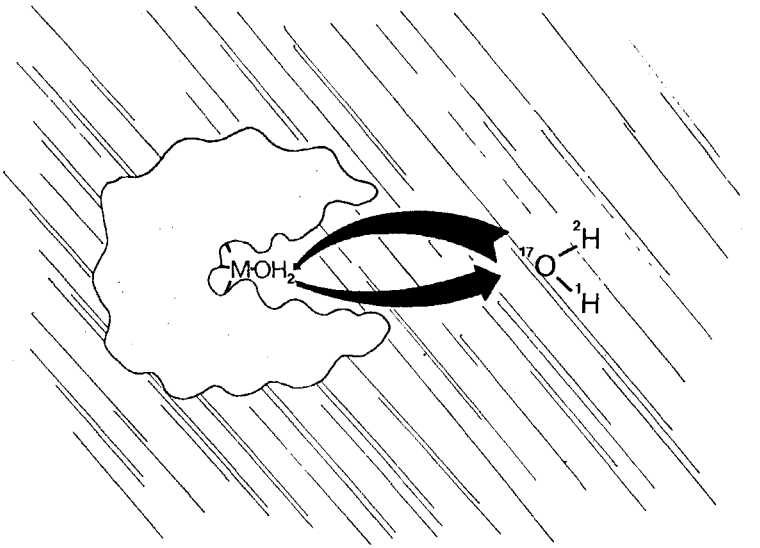


FIGURE 1 Under exchange conditions the nuclei of bulk water may sense the paramagnetic center.

THE pH-DEPENDENT PROPERTIES OF THE COORDINATED WATER

It is well known that the pK_a of water is lowered from its value of 14 upon coordination to metal ions. In the case of a single coordinated water molecule, its pK_a depends on the oxidation state of the metal ion and on the nature of the donor groups. Simple inorganic compounds illustrating such a property are rather scarce. In Table II the models investigated up to now are reported. In every case the metal is dipositive and the overall charge of the complex is 2+. The pK_a of the coordinated water ranges between 8 and 11.

A safe way of determining the pK_a of coordinated water is a potentiometric acid–base titration; however, for metalloproteins the interpretation of the titration curve is complicated by the many histidine residues with $pK_a \sim 7$, by $R\text{–}NH_3^+$ groups, etc. As it will be shown, the NMR may not reveal the hydrolysis

$$MOH_2 \rightleftharpoons MOH^- + H^+$$

equilibrium. In general, the pK_a is estimated from the pH dependence of kinetic parameters or from the pH dependence of the affinity constant of inhibitors of coenzymes. For example, several anions are capable of binding the MOH_2 species, but not the MOH species. In the case of the model complex $[Co(L)OH_2]^{2+}$ (Figure 2),[10] the pH dependence of the affinity constant for the reaction

$$[Co(L)OH_2]^{2+} + N_3^- \rightleftharpoons [Co(L)N_3]^+ + H_2O$$

displays a pK_a of 9.0, equal to that found from acid–base titration. Similar methods based on the pH dependence of the affinity constants of inhibitors

TABLE II
pK_a values of coordinated water in some $MLOH_2^{2+}$ complexes

	Donor set	pK_a	Ref.
$Co(TPyMA)H_2O^{2+}$ [a]	N_3O	9	10
$Zn(CR)H_2O^{2+}$ [b]	N_4O	8.7	59
$Co(CR)H_2O^{2+}$ [b]	N_4O	~8	59
$Co(TMC)H_2O^{2+}$ [c]	N_4O	8.4	41
$Co(TPT)H_2O^{2+}$ [d]	N_4O	10.8	60
$Cu(TPT)H_2O^{2+}$ [d]	N_4O	9.8	60

[a] TPyMA = tris(3,5-dimethyl-1-pyrazolylmethyl)amine (Figure 2).
[b] CR = condensation product of 2,6-diacetylpyridine and dipropylenetriamine.
[c] TMC = 1,4,8,11-tetramethyl-1,4,8,11-tetraazacyclotetradecane.
[d] TPT = *N,N,N*-tris(3-aminopropyl)amine.

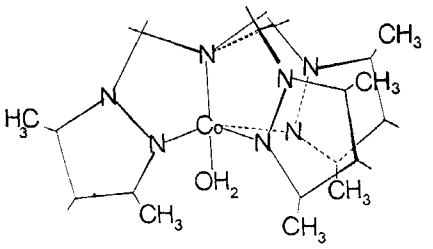


FIGURE 2 Scheme of the probable structure of the complex [Co tris(3,5-dimethyl-1-pyrazolylmethyl)amine OH_2]$^{2+}$.

for carbonic anhydrase[9,11,12] or of NADH for liver alcohol dehydrogenase[13] have permitted an estimate of the pK_a of a possible dissociation equilibrium. In the former case the pK_a is rather low, probably as a result of hydrophobic interaction within the enzymatic cavity coupled with the dipositive charge of the zinc-donor moiety. Other metalloproteins, in which negative residues like Glu are coordinated to the metal, display higher pK_a values, as in the case of liver alcohol dehydrogenase.[13]

Together with the dissociation of the coordinated water, in the case of iron(III) heme residues a change in the spin multiplicity of the metal center may occur. A typical example is that of methemoglobin whose low pH form has $S = 5/2$ and the high pH form has $S = 1/2$.[14,15]

THE NATURE OF THE INTERACTION BETWEEN UNPAIRED ELECTRONS AND RESONATING NUCLEI

Unpaired electrons behave like magnetic bars whose moments are much larger than those associated with the nuclei ^1H, ^2H and ^{17}O. Furthermore, electron spins relax several orders of magnitude faster than nuclear spins. As a consequence, the nuclei which are coupled with unpaired electrons have a relaxation pathway which may be very efficient. The relaxation mechanisms are essentially twofold: one due to the dipolar coupling, i.e., due to through-space coupling between magnetic bars, and the other due to the Fermi contact term arising from the delocalization of unpaired spin through the chemical bond. In the case of a metal ion following the spin-only theory, i.e., no zero-field splitting and no magnetic anisotropy, the dipolar contribution to the nuclear relaxation rate enhancement, T_{iM}^{-1}, is given by the following expression[16]

$$T_{1M}^{-1} = \frac{1}{15}\left(\frac{S(S+1)\gamma_I^2 g^2 \mu_B^2}{r^6}\right)\left(\frac{6\tau_{c1}}{1+\omega_I^2\tau_{c1}^2} + \frac{14\tau_{c2}}{1+\omega_s^2\tau_{c2}^2}\right), \qquad (1)$$

$$T_{2M}^{-1} = \frac{1}{15}\left(\frac{S(S+1)\gamma_I^2 g^2 \mu_B^2}{r^6}\right)\left(4\tau_{c1} + \frac{3\tau_{c1}}{1+\omega_I^2\tau_{c1}^2} + \frac{13\tau_{c2}}{1+\omega_s^2\tau_{c2}^2}\right), \quad (2)$$

where S is the total electron spin of the ion, γ_I is the nuclear gyromagnetic ratio, μ_B is the Bohr magneton, ω_I is the nuclear resonance frequency, ω_s is the electron resonance frequency, and r is the electron–nuclear distance. The correlation times τ_{ci} are related to the electronic relaxation times T_{1e} and T_{2e} in a simplified and pragmatic way through the following equations:

$$\tau_{c1}^{-1} = \tau_r^{-1} + T_{1e}^{-1}, \quad (3)$$

$$\tau_{c2}^{-1} = \tau_r^{-1} + T_{2e}^{-1}, \quad (4)$$

where τ_r is the rotational correlation time which can be roughly estimated through the Stokes–Einstein equation and which is about 10^{-8} s [17] for molecules with molecular weight of 30 000. In regard to the coordinated water, it has been suggested that the rotational correlation time is affected by the possible rotation of the molecule about the metal–oxygen axis.[18] Here it is assumed that the correlation times τ_{c1} and τ_{c2} are equal. Both T_{1M} and T_{2M} are field dependent, since ω_I and ω_s are field dependent. Therefore it can be stated that the dipolar coupling between unpaired electrons and resonating nuclei depends on the correlation times and on the applied magnetic field in a way similar to that shown in Figure 3.

The contact contribution to the nuclear relaxation rate enhancement is given by[19]

$$(T_{1M}^{-1})_{\text{contact}} = \frac{1}{3}S(S+1)\left(\frac{A}{\hbar}\right)^2\left(\frac{2\tau_{e2}}{1+\omega_s^2\tau_{e2}^2}\right), \quad (5)$$

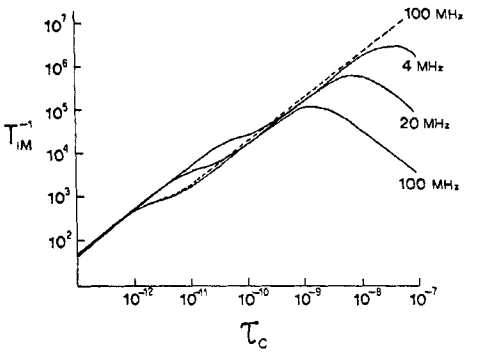


FIGURE 3 τ_c dependence of the paramagnetic relaxation rates T_{1M}^{-1}(——) and T_{2M}^{-1}(----) for a single water proton bound to an $S = 1/2$ ion. T_{1M}^{-1} is reported at three different proton Larmor precession frequencies.

$$(T_{2M}^{-1})_{contact} = \frac{1}{3} S(S+1) \left(\frac{A}{\hbar}\right)^2 \left(\tau_{e1} + \frac{\tau_{e2}}{1 + \omega_s^2 \tau_{e2}^2}\right), \quad (6)$$

where A is the Fermi hyperfine coupling constant, τ_{ei} can be assumed to be T_{ie}, and the other symbols have their usual meaning. By comparing Eqs. (1), (2) and (5), (6), it is apparent that $(T_{2M}^{-1})_{contact}$ is larger than $(T_{1M}^{-1})_{contact}$ and that the difference increases with increasing τ_e. In general it is true that $(T_{1M}^{-1})_{dip} > (T_{1M}^{-1})_{contact}$ whereas $(T_{2M}^{-1})_{contact}$ may be as large or larger than $(T_{2M}^{-1})_{dip}$. Therefore the T_1 investigations allow the use of Eq. (1) to extract structural information on the sample. The analysis of the T_2 data is, in general, less straightforward.

Equations (1), (2), (5), (6) refer to the full paramagnetic effect on a non-exchanging nucleus. When the nuclear species under consideration is in fast exchange with a bulk diamagnetic environment, the experimental relaxation rate enhancements T_{1p}^{-1} measured on the averaged signal are related to (T_{1M}^{-1}) through the relation $T_{1p}^{-1} = fT_{1M}^{-1}$, where f is the molar fraction of nuclei bound to the paramagnetic center. Under these circumstances the exchange rate τ_M^{-1} is an additive term to the τ_{ci}^{-1} and τ_{ei}^{-1} correlation times and is, in general, negligible.

When τ_M is of the order of magnitude of T_{1M} or larger, then $fT_{1p} = (T_{1M} + \tau_M)$ or $fT_{1p} = \tau_M$, respectively.[20,21] The proton exchange in iron(III) proteins has been found to fall in both fast[22,23] and slow[5-7] exchange ranges. Temperature-dependent investigations can yield information on the kinetic parameters involved in the water detachment process.

The dependence of fT_{2p} on τ_M is more complicated since exchange broadening effects may also be operative. Although the analysis is still possible,[24] it is not treated further here since there are no meaningful applications in the investigation of metal-coordinated water in metalloproteins.

COMMENTS ON THE NUCLEAR PROPERTIES OF ^1H, ^2H AND ^{17}O

^1H has a large magnetic moment and the coupling with unpaired electrons is quite efficient. Therefore, the ^1H T_1^{-1} measurements usually provide the extent of such coupling. By performing the measurements at various magnetic fields it is possible, through Eq. (1), to determine τ_c and a geometrical factor which includes the number of protons and their distances.[25] This can be illustrated by rewriting Eq. (1) as follows:

$$T_{1p}^{-1} = \frac{[E]}{111} GKf(\tau_c), \quad (7)$$

where [E] is the enzyme derivative concentration, and G is given by

$$G = \sum_i \frac{n_i}{r_i^6},$$

where n_i is the number of protons interacting with the paramagnetic center at a distance r_i. Sometimes it happens that τ_c is not field independent in the field range investigated, especially because of the presence of zero-field splitting; in these cases information on such a dependence may be obtained.

The τ_c values estimated by this procedure for the various metal ions for fields of $\simeq 1$ T under the condition that $\tau_c = T_{1e}$ are reported in Table III. The values depend on the particular metal ion and on the geometry of the chromophore. Indeed, the presence of low lying excited states provides efficient "two phonon" electronic relaxation mechanisms.[26]

Analysis of the ^1H T_2^{-1} values start by factorizing dipolar and contact contributions and may allow an estimate of other parameters like the hyperfine coupling constant.

^2H and ^{17}O nuclei have relatively low nuclear magnetic moments and have a quadrupole moment which provides independent relaxation mechanisms. The coupling with unpaired electrons therefore does not cause large relaxation rate enhancements. In order to detect such contributions concentrated solutions are needed so as to increase the mole fraction of coordinated water. Furthermore, the correlation times should be particularly favorable, i.e., as large as possible. For example, ^2H T_1^{-1} measurements have been reported for a 10^{-3} M solution of a metalloprotein containing a water molecule in the coordination sphere, the metal ion being Gd^{3+}, with a τ_c of $\simeq 10^{-8}$ s.[27]

^{17}O T_1^{-1} measurements have never permitted a determination of any paramagnetic effect in metalloprotein solutions. However, when $\tau_c > 10^{-11}$ s, the large hyperfine coupling constant due to direct binding of oxy-

TABLE III
Electronic relaxation times (s) for some metal ions

		Ref.			Ref.
Ti^{3+}	$\sim 10^{-9}$	a	Fe^{3+}(l.s.)	$<10^{-12}$	64
VO^{2+}	$\sim 10^{-8}$	62	Fe^{2+}	$\sim 10^{-12}$	65
V^{3+}	$<3 \times 10^{-12}$	a	Co^{2+}(tetr.)	10^{-10}–10^{-11}	25,66
V^{2+}	10^{-9}–10^{-10}	37	Co^{2+}(five-coord. h.s.)	10^{-11}–10^{-12}	25
Cr^{3+}	10^{-9}–10^{-10}	37	Co^{2+}(oct. h.s.)	10^{-12}–10^{-13}	35
Mn^{3+}	$<4 \times 10^{-12}$	a	Ni^{2+}	10^{-10}–10^{-12}	63,67
Mn^{2+}(h.s.)	10^{-8}–10^{-9}	37	Cu^{2+}	10^{-8}–10^{-9}	63,68
Fe^{3+}(h.s.)	10^{-10}–10^{-11}	63			

[a] Estimated from Ref. 61.

gen to the metal ion gives rise to a sizeable $(T_2^{-1})_{contact}$ contribution which may easily be determined. Reports are available on systems containing copper(II)[3] and manganese(II)[28,29] proteins. In concentrated solutions of the simple copper aquo complex, the isotropic shift could also be measured and therefore the correlation time could be evaluated.[30]

AN EXAMPLE: WATER ^1H T_1^{-1} INVESTIGATION OF METAL SUBSTITUTED CARBONIC ANHYDRASE

Carbonic anhydrase is a zinc-containing metalloenzyme with molecular weight 30 000.[11,12] The coordinated water molecule is necessary for the catalytic hydration of CO_2. The zinc ion can be removed and replaced by cobalt without loss of the catalytic activity. Other metal-substituted derivatives show a quite reduced activity. The metal ions are bound to the protein part through three histidine residues. The ^1H T_1^{-1} NMR values of water solutions containing paramagnetic metal ions are sensibly enhanced (Figure 4) with respect to the native enzyme.[4,31] Many anions and sulfonamides are capable of inhibiting the catalytic activity of the enzyme by binding to the metal ion. A water proton T_1^{-1} investigation at variable magnetic fields on solutions containing the cobalt(II) and copper(II) derivatives has allowed a determination of τ_c and the G factor [Eq. (7)] which includes the number of protons and their distances.[25] In the case of the copper derivatives[32] (Figure 5) τ_c is always the same and in the usual range of 10^{-8}–10^{-9} s. The G factors fall into two ranges, $\simeq 5 \times 10^{-15}$ pm^{-6} and $(0.5\text{–}0.9) \times 10^{-15}$ pm^{-6}, respectively. In the former case the geometric factor can be interpreted on the

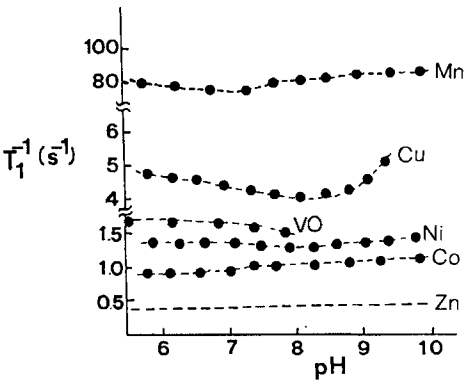


FIGURE 4 Water proton T_1^{-1} values of water solutions containing several metal-substituted bovine carbonic anhydrase B. The enzyme concentrations were 10^{-3} M.

	G (pm^{-6})	τ_c (s)
Cu BCAB pH 5.5	5.6×10^{-15} (± 11%)	2.7×10^{-9} (± 10%)
Cu BCAB·N_3^-	5.3×10^{-15} (± 9%)	3.1×10^{-9} (± 7%)
Cu BCAB·$C_2O_4^{2-}$	0.93×10^{-15} (± 5%)	3.1×10^{-9} (± 5%)

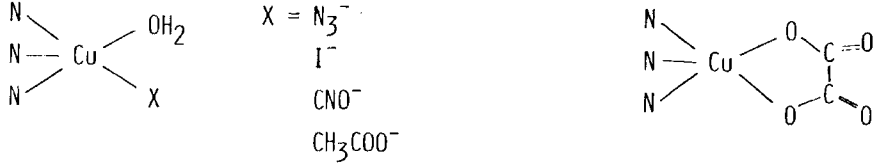


FIGURE 5 *G* and τ_c values and chromophores of copper(II) carbonic anhydrase (bovine isoenzyme B) and of some of its inhibitor derivatives.

basis of a coordinated water molecule, whereas in the latter case the water is removed from coordination, the small G values indicating a second-sphere interaction. A water molecule with a Cu–O distance of 200 pm is expected to provide a G value of 4×10^{-15} pm^{-6}.

Quite more instructive is the investigation of the cobalt derivative, since there are a variety of structures with different relaxation properties.[33,34] In Figure 6 are reported some typical chromophores whose G and τ_c values are reported in Table IV. It is evident that when a water molecule is in the coordination sphere G is $(3–5) \times 10^{-15}$ pm^{-6}, independently of the geometry. As in the case of copper, G values of 1×10^{-15} pm^{-6} indicate second-sphere interactions. The variation of τ_c with stereochemistry is most interesting. It is $\simeq 10^{-11}$ s for tetracoordinated tetrahedral chromophores, whereas it is $\simeq 10^{-12}$ s for pentacoordinated chromophores and $\simeq 10^{-13}$ s for hexacoordinated hexamethanol complexes.[35] The theoretical justification for such values is based on the number of low lying electronic excited states, which increases with the coordination number. Indeed, octahedral complexes have a T_{2g} ground state, tetrahedral complexes an A_{2g} ground state and a square pyramidal chromophore has E_g and A_g levels lowest and very close in energy.[36] The value of τ_c as determined from NMR experiments can therefore be taken as a criterion for assigning the stereochemistry in high-spin cobalt(II) chromophores.

In the case of the manganese(II) system, T_{1e} and hence τ_c is not field independent. Probably the zero-field splitting is small enough that its modulation deriving from donor displacements within the chromophore is capable of affecting the electronic relaxation times. Within this framework T_{1e} is required to depend on another correlation time, τ_v (the zero-field splitting

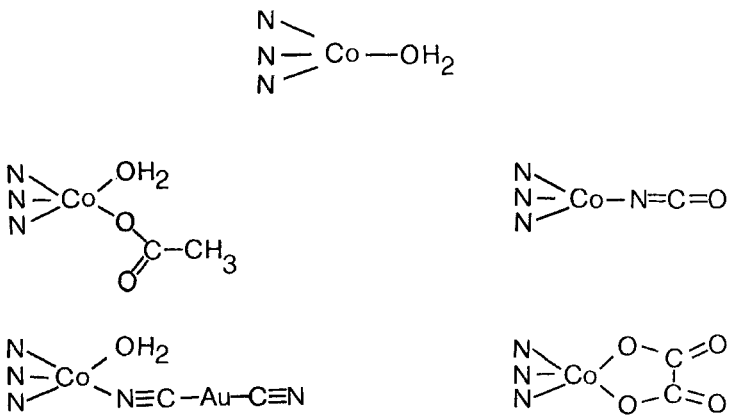


FIGURE 6 Chromophores of cobalt(II) carbonic anhydrase (bovine isoenzyme B) and of some of its inhibitor derivatives.

TABLE IV

Best fit values of the geometrical factor (G) and of the correlation time τ_c for some cobalt(II) substituted carbonic anhydrase (bovine isoenzyme B) derivatives[a]

	G (pm^{-6})	τ_c (s)
Co(BCAB) pH 6.0	3.3×10^{-15}(±17%)[b]	3.3×10^{-11}(±26%)
Co(BCAB) NCO^-	1.4×10^{-15}(± 7%)	3.1×10^{-11}(± 4%)
Co(BCAB) $Au(CN)_2^-$	3.6×10^{-15}(± 4%)	4.2×10^{-12}(± 3%)
Co(BCAB) CH_3COO^-	5.5×10^{-15}(± 4%)	4.2×10^{-12}(± 3%)
Co(BCAB) $C_2O_4^{2-}$	0.97×10^{-15}(± 1%)	5.3×10^{-12}(± 1%)
$CoL(H_2O)^{2+}$ (Figure 2)	5.7×10^{-15}(±50%)	5.3×10^{-12}(±45%)
$Co(MeOH)_6^{2+}$ [35]		5.0×10^{-13}

[a] From Ref. 25 except when specified.
[b] Standard deviations in brackets.

modulation correlation time), through the following expression derived for the distortion undergone by the aquo complex in collisions with other water molecules[37]:

$$\frac{1}{T_{1e}} = B\left(\frac{\tau_v}{1 + \omega_s^2\tau_v^2} + \frac{4\tau_v}{1 + 4\omega_s^2\tau_v^2}\right). \tag{8}$$

The parameter B is related to the magnitude of the zero-field splitting. Now it has been possible to extract, through Eqs. (7) and (8), the values of G, τ_v and B from the experimental ^1H T_1^{-1} values (Table V). Again the G values can provide direct information concerning the presence of coordinated water.[25,38]

In principle there is no reason why τ_c should be constant with the applied magnetic field or that it should follow a simple relationship; this can only be stated *a posteriori* in the given range of magnetic fields investigated. Indeed, in the case of the nickel derivative, it was not possible to extract any information about τ_c or G. The nickel derivative, if hexacoordinated, should have a $^3A_{2g}$ ground state. However, the experimental data could not be fitted through Eq. (7), nor through both Eqs. (7) and (8). Presumably the magnetic field dependence of τ_c is rather complex. Attempts were made to

TABLE V

Best fit parameters governing the water proton relaxation rate for manganese(II) substituted carbonic anhydrase(bovine isoenzyme B) derivatives[a]

	G (pm^{-6})	B (rad s^{-1})^2	τ_v (s)
Mn (BCAB) pH 8.9	3.3×10^{-15}(±7%)[b]	4.7×10^{19}(±12%)	3.2×10^{-12}(±13%)
Mn (BCAB) N_3^-	3.6×10^{-15}(±4%)	6.9×10^{19}(± 5%)	4.7×10^{-12}(± 5%)

[a] From Ref. 25.
[b] Standard deviations in brackets.

investigate the hexaaquonickel(II) complex.[39] However, in this case the experimental $^1H\ T_1^{-1}$ data were almost the same between 4 and 60 MHz, allowing no possibility to analyze experimental data.

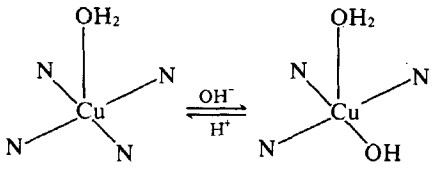
THE DISSOCIATION OF WATER AND THE NMR PARAMETERS

The dissociation of the coordinated water and formation of the hydroxo derivative has two major consequences: (*i*) the number of protons which "feel" the paramagnetic center becomes halved; (*ii*) the direct exchange of coordinated OH groups with free OH^- anions cannot be fast enough on the NMR time scale at low pH since the concentration of OH^- is small and its supply to the metal site is diffusion limited. According to the former consequence, the $^1H\ T_1^{-1}$ values of aqueous solutions of benzylamine oxidase (Table I) are reported to decrease by 50% with increasing pH.[40] Again, according to the latter consequence the $^{17}O\ T_2^{-1}$ enhancements of ^{17}O aqueous solutions of the model complex $[Co(TMC)H_2O]^{2+}$ (Table II) decrease to zero upon increasing the pH.[41]

However, the water dissociation is not in general easily followed by NMR spectroscopy. In fact, the metal–oxygen distance is expected to decrease upon water dissociation with a consequent increase in the geometrical factor G of the electron–proton coupling [Eq. (7)] which may compensate for the loss of a proton. More dramatic is the change of the electronic structure often accompanied by a conformational change of the magnetophore, which relaxes the τ_c parameter. Finally, the second sphere of hydration may contribute quite differently to the overall nuclear relaxation rates. Therefore the water and corresponding hydroxo derivatives are just two different, unrelated systems whose $^1H\ T_1^{-1}$ values cannot be significantly compared. For example, the $^1H\ T_1^{-1}$ values of cobalt bovine carbonic anhydrase B are essentially pH independent[42] whereas the human isoenzyme displays smaller values at low pH[43,44] when the OH_2 species is presumably present (!). In the case of methemoglobin, although the solvent proton relaxation rate is largely due to outer-sphere relaxation, the $^1H\ T_1^{-1}$ values are insensitive to the acid–base equilibrium despite a magnetic susceptibility transition. Probably the appearance of an exchangeable water molecule in the heme pocket propitiously balances the expected decrease.[7]

The $^{17}O\ T_2^{-1}$ values of water solutions of copper carbonic anhydrase reveal the presence of oxygen bound to the copper ion under rapid exchange conditions.[3] Such values are again pH independent in the pH range 5.5–11. Therefore if the $CuOH_2 \rightleftharpoons CuOH$ equilibrium occurs, it should also provide a pathway for the oxygen of the hydroxo group to exchange with the solvent.

In the case of superoxide dismutase, the water proton relaxation rate increases dramatically with increasing pH, corresponding to a pK_a of 11.5.[45] This has been tentatively accounted for on the basis of a large decrease of the copper–oxygen distance upon water dissociation,[45] or alternatively, on the basis of a OH ligand replacing a histidine nitrogen present in the coordination sphere together with the water molecule[46]:



Parallel ^{17}O T_2^{-1} investigations have shown that the coordinated water (below pH 10) displays little hyperfine coupling owing to the long copper–oxygen distance and to its presumably axial position. Large pH dependent T_2^{-1} enhancements are measured above pH 10 with a pK_a of ~11.4.[47] Such data appear consistent with the latter interpretation.

GENERAL REMARKS

When water is directly coordinated to a paramagnetic ion in a metalloprotein, it may be selectively revealed by NMR spectroscopy. Under fast exchange conditions, water proton T_1^{-1} values are related to the number of exchangeable protons which are coupled with the paramagnetic center through a dipolar mechanism. The extent of such a coupling depends on the magnitude of the applied external magnetic field and on a correlation time which, in turn, may be related to other structural properties of the metal donor moiety. Owing to limitations in metal concentrations, the paramagnetic contribution to the resulting T_1^{-1} values may be easily appreciated for τ_c values larger than 10^{-12} s (see Table III).

The use of ^2H and ^{17}O nuclei is limited to those cases in which significant Fermi contact contributions occur and in which the τ_c values are larger than 10^{-11} s. Therefore, T_2^{-1} or linewidth measurements are more suitable although structural information is more difficult to obtain.

The paramagnetic contribution to T_1^{-1} values can be analyzed in terms of the simple Solomon equations [Eqs. (1) and (7)]. It should be kept in mind, however, that such equations only hold in the absence of zero-field splitting and magnetic anisotropy. Furthermore, they ignore relaxation effects of unpaired spin density delocalized onto the ligands,[48] in this case on the oxygen atom. More sophisticated equations are available, although the

author's advice is to keep the number of parameters as low as possible and to interpret them *cum grano salis.* Variable magnetic field investigations provide independent information and should be performed whenever possible. They are particularly useful when the correlation time turns out to be magnetic field independent. The T_1^{-1} values on different isotopes would also be quite meaningful and useful. For example, both ^1H and ^2H T_1^{-1} measurements could be performed in such a way as to determine τ_c at a given magnetic field. However, except in exceptional cases, this technique is rarely applied to metalloproteins.[49]

The analysis in terms of the above equations may provide a parameter which contains the number of protons and their distances (G factor) as well as the correlation time. When the relaxation rates of different compounds are compared, it should be verified either that τ_c remains constant or its effects should be evaluated. In the case of cobalt carbonic anhydrase all the inhibitors cause a decrease of water ^1H T_1^{-1} values,[33] but for two opposing reasons[25]: (*i*) water is removed and τ_c has remained substantially unchanged; (*ii*) water is *not* removed but τ_c has decreased by an order of magnitude. For the same reasons the NMR parameters may not be useful for detecting the acidic dissociation of the coordinated water.

An important consequence of the knowledge of the electronic relaxation time is that the magnitude of the linewidth can be *a priori* evaluated through relationships like Eqs. (2) and (6); if there are sizeable dipolar and contact shifts and not dramatic line broadening ($\tau_e \neq 10^{-11}$) the signals of protein protons close to the paramagnetic center may be recorded and easily identified.[50-52]

When the exchange rate is slow on the NMR time scale, the measured effects are those of outer-sphere solvation[7]; these effects are present even when there is no water coordinated to the paramagnetic metal ion. Attempts at the theoretical interpretation of such data are also available.[7,49]

IVANO BERTINI
Istituto di Chimica Generale e Inorganica, della Facoltà di Farmacia, Via G. Capponi, 7, Firenze, Italy

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