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# Advances in the NMR investigation of paramagnetic molecules in solution

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CONTENTS



## A. INTRODUCTION

The proton magnetic resonance of paramagnetic complexes is a technique used since its beginning in the laboratory of Prof. Luigi Sacconi. The first publications in this field, dealing with nickel(II) complexes with Schiff bases between salicylaldehyde and *N*,*N*-substituted ethylendiamines, were submitted in 1965 and appeared in 1966 [1,2] at a time when the number of papers on the subject of NMR of paramagnetic complexes published in the international literature was of the order of a few tens [3–7].

The potential for this technique to provide unique information on the structure and electronic states of metal complexes was immediately realized in a laboratory which had the coordination properties of the first transition elements as its main field of study [8–22].

From those first papers on simple paramagnetic metal complexes, our research group later evolved toward the study of more complex systems, such as metalloproteins, using this technique as the major spectroscopic tool for obtaining detailed structural information on such very large chemical systems [23–25].

The potential of this technique grew with the years due to the development of instrumental facilities and to a better theoretical comprehension of several factors which can affect the detectability of the NMR signals in paramagnetic systems. To

this expansion of the frontiers of NMR of paramagnetic molecules, we have fruitfully contributed [24–26], especially with respect to magnetically coupled paramagnetic systems [27–29].

The aim of this review is to summarize the improvements brought about by our group in the comprehension of the NMR parameters of paramagnetic species, in particular metalloproteins, and to review the state of the art in detecting 2D NMR spectra of paramagnetic systems.

## B. NMR PARAMETERS IN PARAMAGNETIC SPECIES

The presence of unpaired electrons strongly affects the properties of magnetic nuclei interacting with them. The interaction can occur through space, i.e. the magnetic moment associated with the spin moment of the electrons interacts with the magnetic moment originated by the nuclear spin moment, or can occur through chemical bonds, i.e. some fraction of the unpaired electron spin can delocalize, through different mechanisms, directly on the resonating nucleus.

The extent and the type of interaction are determined by the nature of the paramagnetic centre, by its coordination geometry and by the nature of its ligands. The presence of hyperfine interaction determines a sizeable contribution both to the chemical shift and to the relaxation rates of the nucleus under investigation, which in most cases is the dominant contribution to the NMR parameters.

### *(i) The hyperfine shift: contact and pseudocontact contributions*

The contribution to the chemical shift of a nucleus due to coupling with unpaired electrons is called the hyperfine shift. If the anisotropy of the hyperfine interaction, in frequency units, is smaller than the rotational frequency, as generally happens in solution, then the observed hyperfine shift is an average and is called the isotropic shift [23,24].

Furthermore, the magnetic nucleus senses the electron magnetic moment averaged over all its possible orientations owing to fast electron relaxation rates.

The contribution to the shift arising from the interaction with the spin density present on the nucleus itself is called the contact shift or the Fermi contact shift, because Fermi theoretically justified the presence of finite electron density at the nucleus [30]. It is provided by the relation [31]

$$\frac{\Delta\nu}{\nu_0} = -\frac{A_c}{\hbar\gamma_N B_0}\langle\hat{S}_z\rangle = \frac{A_c}{\hbar}\frac{g_e\mu_B S(S+1)}{3\gamma_N kT} \tag{1}$$

The second equality holds for an orbitally non-degenerate ground state under isotropic conditions. In eqn. (1), $A_c$ is the contact hyperfine coupling constant, $\langle\hat{S}_z\rangle$ is the expectation value of $\hat{S}_z$, $S$ is the spin quantum number of the paramagnetic centre, $\gamma_N$ is the magnetogyric ratio of the nucleus, and the other symbols have their

usual meanings. $A_c$ is proportional to the amount of unpaired spin density at the resonating nucleus. Due to its nature, $A_c$ is independent of the orientation of the molecule with respect to the magnetic field.

Only a small fraction of the unpaired spin density is present at the resonating nucleus. This fraction of unpaired spin density can be the result of a direct delocalization via chemical bonds [32] or of spin polarization effects between singly occupied and doubly occupied orbitals [24,33,34].

The dipolar contribution originates from the dipolar coupling of the nucleus with the electron spin density of the metal ion or spread over the ligands. The dipolar coupling depends on the relative location of the two magnetic moments which are oriented along the magnetic field. Upon rapid rotation, as occurs in solution, this contribution is averaged and is called pseudocontact. If the electron magnetic moment is isotropic ($g = 2$), the average upon rotation is zero. If the electron magnetic moment is not isotropic, i.e. it changes its magnitude as the orientation of the molecules changes with respect to the external magnetic field, then the average upon rotation is different from zero. The anisotropic nature of the electron spin moment, and then the presence of magnetic anisotropy, is due to the existence of an orbital contribution to the electron magnetic moment. The name pseudocontact contribution derives from the fact that this term becomes isotropic due to fast rotation of the molecule. In the point–dipole approximation, i.e. when the unpaired electrons are assumed to be delocalized on the metal ions, the pseudocontact contribution is given by (in the case in which the magnetic susceptibility tensor has an axial symmetry) [35,36]

$$\frac{\Delta\nu}{\nu_0} = \frac{1}{4\pi 3r^3}(3\cos^2\theta - 1)(\chi_{\parallel} - \chi_{\perp}) \tag{2}$$

where $r$ is the metal–nucleus distance, $\theta$ is the angle formed by $r$ with the molecular $z$ axis, $\chi_{\parallel}$ and $\chi_{\perp}$ are the equatorial and axial components of the magnetic susceptibility tensor. Since this contribution depends on $r^{-3}$, the analysis of the pseudocontact shift values can provide structural information.

*(ii) Relaxation rates enhancement*

The dipolar interaction is often the dominant contribution for the nuclear relaxation rates, even for nuclei separated by a few bonds from the metal ion. This contribution depends on $S$, on the inverse of the sixth power of the unpaired electron–nucleus distance, on a function of the magnetic field and on the correlation time $\tau_c$ [26,37–39].

$$T_{1M}^{-1} = \frac{2}{15}\left(\frac{\mu_0}{4\pi}\right)^2 \frac{\gamma_N^2 g_e^2 \mu_B^2 S(S+1)}{r^6}\left[\frac{7\tau_c}{1+\omega_S^2\tau_c^2} + \frac{3\tau_c}{1+\omega_I^2\tau_c^2}\right] \tag{3}$$

$$T_{2M}^{-1} = \frac{1}{15}\left(\frac{\mu_0}{4\pi}\right)^2 \frac{\gamma_N^2 g_e^2 \mu_B^2 S(S+1)}{r^6}\left[4\tau_c + \frac{13\tau_c}{1+\omega_S^2\tau_c^2} + \frac{3\tau_c}{1+\omega_I^2\tau_c^2}\right] \tag{4}$$

where $\omega_S$ and $\omega_I$ are the electronic and the nuclear Larmor frequencies, respectively. They depend on the magnetic fields; the ratio $\omega_S/\omega_I$ is 658 if the nucleus is a proton and is higher for other nuclei.

The dependence upon $r^{-6}$ directly provides structural information on the distance between the nucleus and the metal ion. This is true in the simplified point–dipole approximation where the unpaired electrons are localized on the metal ion. However, through the bond between the metal and the donor atom of the ligand, some spin density can delocalize over the ligands, thus making the analysis of $T_{1,2}^{-1}$ more complicated [26].

The correlation time for the dipolar interaction is determined by the fastest rate among the mechanisms which produce reorientation of the electron and nuclear spins, i.e. rotation, electron relaxation and chemical exchange

$$\tau_c^{-1} = \tau_r^{-1} + \tau_s^{-1} + \tau_m^{-1} \tag{5}$$

In the absence of chemical exchange, in small complexes $\tau_c$ can be dominated by $\tau_r$ or $\tau_s$, depending on the nature of the metal ions; in macromolecules, where rotation is slowed down, $\tau_c$ is always determined by $\tau_s$.

The contact coupling also determines a contribution to the relaxation rates. These contributions are

$$T_{1M}^{-1} = \frac{2}{3}S(S+1)\left(\frac{A_c}{\hbar}\right)^2 \frac{\tau_c}{1+\omega_S^2\tau_c^2} \tag{6}$$

$$T_{2M}^{-1} = \frac{1}{3}S(S+1)\left(\frac{A_c}{\hbar}\right)^2\left[\tau_c + \frac{\tau_c}{1+\omega_S^2\tau_c^2}\right] \tag{7}$$

where all the symbols have been previously defined.

The correlation time $\tau_c$ in eqns. (6) and (7) is given by [39]

$$\tau_c^{-1} = \tau_s^{-1} + \tau_m^{-1} \tag{8}$$

i.e. it is not modulated by molecular tumbling. From eqns. (3) and (6), it can be observed that the dipolar term is often the dominant contribution to $T_{1M}^{-1}$, whereas in the case of $T_{2M}^{-1}$, the contact term can be dominant in the case of high $\tau_S$ values and large values of the hyperfine coupling constant [40].

Finally, a third contribution, particularly relevant for $T_2^{-1}$, is the so-called Curie relaxation, which originates from the rotation of the static magnetic moment resulting from the different population of the electronic spin levels [41,42]. It depends on the square of the magnetic field and is particularly large in the case of nuclei with large $\gamma_N$ ($^1$H) in large molecules (high $\tau_r$) with high $S$ value. This contribution to the linewidth could prevent the detectability of signals of protons close to the paramag-

netic centres in macromolecules at high magnetic fields; in such cases, low magnetic fields (if still available!) are needed.

From eqns. (3), (4), (6) and (7), it can be observed that $T_{1,2}^{-1}$ contain two parameters ($S$ and $\tau_c$) which are directly related to the nature, oxidation state, and coordination number of the metal ion. $S$ is directly related to the oxidation number and to the spin state of the metal ion present in the molecule investigated [43]; $\tau_s$ depends on the nature of the metal ion, on its $S$ spin number and its coordination geometry.

When the electronic excited levels are high in energy compared with the ground state, high $\tau_s$ values are expected. On the other hand, in the case of low-lying energy levels which are populated at room temperature, low $\tau_s$ values are observed. The availability of excited states depends on the nature of the metal ion and on its coordination geometry. Let us take high-spin Co(II) as an example: in the case of octahedral symmetry, it has an orbitally triply degenerate ground state ($^4T_{2g}$). Low-symmetry components and spin–orbit coupling split the level into six Kramers doublets, all thermally populated. In the case of tetrahedral symmetry, the ground state is orbitally non-degenerate ($^4A_2$) and the zero-field splitting is small. Therefore excited levels are high in energy and electron relaxation is more than one order of magnitude slower than in the octahedral coordination.

In the case of protons, for which experimental results will be discussed, the value of $\tau_c$ determines the detectability of signals, especially in macromolecules where $\tau_c = \tau_s$. In the latter case, if $\tau_s > 10^{-10}$ s and $r < 5$ Å, a proton would have too short a $T_2$ and therefore too large a linewidth to be detected. In addition, the Curie contribution to $T_2$ could also be sizeable. Examples of this situation are copper-containing proteins [44]. Copper(II) has a long electron relaxation time ($\tau_s$ around $2–3 \times 10^{-9}$ s). For small complexes, $\tau_c$ is dominated by $\tau_r$ ($10^{-11}$ s) and the $^1$H NMR signals can be resolved; as the molecular weight increases and $\tau_r > 10^{-9}$ s, the lines become too broad and protons closer than 6 Å to the copper centre cannot be detected. The only way to overcome this problem is to shorten the electron relaxation time of copper through magnetic interaction with a fast relaxing paramagnetic centre. This will be discussed in the next section.

### *(iii) NMR parameters in dimers*

If more than one paramagnetic metal ion is present, the metal ions can be magnetically coupled. This coupling can dramatically affect the NMR parameters [27(a),27(c),27(d)–29]. Let us consider a system constituted by two metal ions. The more complex case of a larger number of paramagnetic centres will be treated in Sect. D.

The presence of magnetic coupling between the electron spin moments of the two paramagnetic centres determines the occurrence of new electron spin energy levels which are relatively (depending on the magnitude of the magnetic coupling

constant) low in energy with respect to the ground state and which can be populated at room temperature.

If a slow-relaxing metal ion is coupled with a fast-relaxing one, the former can relax through the mechanisms of the latter, which are more efficient [27(a),27(c),27(d)–29]. As a consequence of magnetic coupling, one spin moment can cause fluctuating magnetic fields, and then induce relaxation, on the other spin moment, both through scalar and dipolar interactions. If a spin moment has a fast electron relaxation rate, the fluctuations due to its own relaxation also generate new relaxation pathways for the slow-relaxing ion.

Magnetic coupling is effective for shortening the electron relaxation time of the slow-relaxing metal ion, even for very small values of the coupling constant $J$. Variations of more than one order of magnitude have been reported to occur in the case of a slow-relaxing ion ($\tau_s$ ca. $10^{-9}$ s) coupled with a fast-relaxing ion ($\tau_s$ ca. $10^{-12}$ s), also for very small $J$ values [27(b)].

As far as the nuclear spins are concerned, let us consider the case of a nucleus which senses only one metal ion of the pair.

We start considering the effect of coupling on the shifts. The contact shift depends on $\langle \hat{S}_z \rangle$, i.e. on the magnetic susceptibility [24]. If the coupling constant is smaller than $kT$ and the new electron spin levels originated by the coupling are all populated, the magnetic susceptibility, and therefore the shift, is not affected by the presence of another metal ion. On the other hand, if the magnetic coupling is large, then a variation of the magnetic susceptibility, and therefore of the contact shift, occurs. The contact shift for a nucleus coupled with a metal ion in a dimer is given by [28]

$$\frac{\Delta\nu}{\nu_0} = \left(\frac{A_c}{\hbar}\right)\frac{g_e\mu_B}{\gamma_N 3kT}\frac{\Sigma_i c_i S_i(S_i+1)(2S_i+1)e^{-(E_i/kT)}}{\Sigma_i(2S_i+1)e^{-(E_i/kT)}} \tag{9}$$

where $c_i$ is a coefficient which takes into account the contribution of the spin function of the isolated metal ion to the spin function of the dimer, $S_i$ are the spin levels of the coupled system, and the other symbols have their usual meanings.

The effect of magnetic coupling on the contact contribution changes with temperature as the populations of the various spin levels change with temperature. The experimental temperature dependence of the shifts can be analyzed with eqn. (9). In the case of two unlike metal ions antiferromagnetically coupled, the nuclei interacting with one or the other metal ion will have opposite temperature dependence for their shifts.

By analysis with eqn. (9), discrimination between the two metal ions and assignments of the signals to the two metal ions domains can be performed [45].

Finally, magnetic coupling affects the nuclear relaxation rates due to the change in $\tau_s$ (in the case of heterodimers). The nuclear magnetic moment couples with each of the new $S_i$ levels of the pair and the contribution from each depends on their $S_i$ values and on their populations, which in turn depend on temperature. Equations

for $T_{1M}$ and $T_{2M}$ have been derived in the case of coupled dimers [27–29,45–47].

$$T_{1M_1}^{-1} = \frac{2}{15}\left(\frac{\mu_0}{4\pi}\right)^2 \frac{\gamma_N^2 g_e^2 \mu_B^2}{r_1^6} \Sigma_i c_i^2 S_i(S_i+1)(2S_i+1) \frac{e^{-(E_i/kT)}}{\Sigma_i(2S_i+1)e^{-(E_i/kT)}}$$
$$\times \left[\frac{7\tau_{c_i}}{1+\omega_S^2\tau_{c_i}^2} + \frac{3\tau_{c_i}}{1+\omega_I^2\tau_{c_i}^2}\right]$$
$$+ \frac{2}{3}\left(\frac{A_c}{\hbar}\right)^2 \Sigma_i c_i^2 S_i(S_i+1)(2S_i+1) \frac{e^{-(E_i/kT)}}{\Sigma_i(2S_i+1)e^{-(E_i/kT)}} \frac{\tau_{c_i}}{1+\omega_S^2\tau_{c_i}^2} \tag{10}$$

$$T_{2M_1}^{-1} = \frac{1}{15}\left(\frac{\mu_0}{4\pi}\right)^2 \frac{\gamma_N^2 g_e^2 \mu_B^2}{r_1^6} \Sigma_i c_i^2 S_i(S_i+1)(2S_i+1) \frac{e^{-(E_i/kT)}}{\Sigma_i(2S_i+1)e^{-(E_i/kT)}}$$
$$\times \left[4\tau_{c_i} + \frac{13\tau_{c_i}}{1+\omega_S^2\tau_{c_i}^2} + \frac{3\tau_{c_i}}{1+\omega_I^2\tau_{c_i}^2}\right]$$
$$+ \left(\frac{A_c}{\hbar}\right)^2 \Sigma_i c_i^2 S_i(S_i+1)(2S_i+1) \frac{e^{-(E_i/kT)}}{\Sigma_i(2S_i+1)e^{-(E_i/kT)}} \left[\tau_{c_i} + \frac{\tau_{c_i}}{1+\omega_S^2\tau_{c_i}^2}\right]$$
$$+ \frac{\omega_I^2 g_e^2 \mu_B^4}{(3kT)^2 r_i^6} \Sigma_i c_i^2 S_i^2(S_i+1)^2(2S_i+1) \frac{e^{-(E_i/kT)}}{\Sigma_i(2S_i+1)e^{-(E_i/kT)}} \left[4\tau_r + \frac{3\tau_r}{1+\omega_I^2\tau_r^2}\right] \tag{11}$$

where $c_i$ are coefficients which take into account the contribution of the spin moment of the isolated metal ion (1) to each new spin level $S_i$ of the pair and $r_1$ is the distance between metal 1 and the observed nucleus.

In general, $\tau_c$ is different for each $S_i$ level ($\tau_{c_i}$). If $\tau_c$ is dominated by $\tau_r$ or $\tau_m$, $f(\tau_c)$ is the same for all levels. When $\tau_s$ dominates $\tau_c$, in the absence of specific information, a single $\tau_s$ can tentatively be used.

It is important to note that, even in the case of a low $J$ value, and in the case in which all the spin levels are populated, the $T_1$ value of a nucleus coupled with a metal ion belonging to a pair is not the same as in the isolated case. This is due to the fact that relaxation rates depend on the square of the interaction energy.

### C. ADVANCEMENT IN THE NMR TECHNIQUES ON PARAMAGNETIC MOLECULES

The assignment of the NMR spectra of paramagnetic species has always been a difficult task as the chemical shift values cannot be predicted simply on the basis of the proton type (aliphatic, aromatic, etc.) [23,24].

The isotropic shift is the sum of two contributions which can be opposite in sign and which are determined by many factors. However, since the early times of NMR of paramagnetic molecules, theoretical calculations on the isotropic shift were performed which allowed a semi-quantitative interpretation of the spectroscopic data [2,3(a),11,48–52]. For a long time, the assignment of NMR spectra of paramagnetic

molecules was made by comparison with similar systems with assigned spectra, by chemical substitution of groups in the molecule and by intuition!

Then, the possibility of isotopic substitution, essentially in the case of $^1H$ and $^2H$, provided a precious tool for the assignment of spectra [53–56]. In such a way, it was possible to investigate an extensive series of iron porphyrin complexes as well as iron-containing proteins. Studies of the $^{13}C$ and $^{15}N$ paramagnetic shift of $^{13}CO$ and $C^{15}N^-$ bound to low-spin ferric complexes of porphyrin derivatives and hemoproteins have appeared since the 1970s [57,58]. Non-haeme iron-containing proteins and their model complexes have also been investigated [59–61].

Only in the last decade has the successful application of advanced experimental techniques, such as nuclear Overhauser effect (NOE) measurements [62,63], allowed researchers in the field to assign NMR spectra on a firmer basis. This methodological advancement is particularly important in the case of metalloproteins, where the signals of the protons of residues present in the active site and sensing the paramagnetic centre, could be covered by the intense diamagnetic envelope of the signals of the rest of the protein [64].

NOE experiments measure the fractional change in the intensity of a signal when another signal is saturated [62–64]. The change is different from zero when dipolar coupling is operative between the two nuclei. This effect is proportional to $r_{ij}^{-6}$, where $r_{ij}$ is the distance between nuclei $i$ and $j$. Therefore, these measurements provide unique structural information. The effect is also proportional to the selective $T_1$ of the observed signals. In the case of paramagnetic molecules, characterized by short relaxation times, the effect can be very small [64–69].

Application of the NOE technique to such systems was first exploited by La Mar and coworkers and used in the assignment of the spectra of some haeme-containing proteins and in the elucidation of the structure–function relationship in these systems [65–67]. This technique was later used by other groups to perform the assignment of paramagnetically shifted signals [68–70].

Our group in Florence had successfully applied these measurements to a large variety of metalloproteins, such as superoxide dismutase [71–74], ferredoxins [75,76], high potential iron–sulphur proteins (HiPIPs) [77–79], and metallothioneins [80]. As an example, we present here the assignment of the $^1H$ NMR spectra of cobalt(II)-substituted superoxide dismutase ($Cu_2Co_2SOD$) and of the $Fe_2S_2$ ferredoxins obtained through NOE experiments.

Copper, zinc superoxide dismutase ($Cu_2Zn_2SOD$) is a dimeric metalloenzyme, MW 32 000, each subunit containing a copper(II) and a zinc(II) ion bridged by an histidinato ligand [81–83]. Zinc(II) has a pseudotetrahedral geometry and its coordination is completed by an aspartate ligand and by two histidines. The copper(II) has a distorted square pyramidal geometry with three more histidines and a water molecule [84,85]. The $^1H$ NMR spectrum of this enzyme does not show any isotropically shifted proton resonance outside the diamagnetic envelope [86]. Indeed,

the protons near the copper(II) ions are expected to be broadened beyond detectability owing to the large effect of the slow-relaxing copper(II) ion on the $T_{2M}^{-1}$ [26,40].

Replacement of the zinc(II) ion with cobalt(II) or nickel(II) ions [82,87,88], gives rise to derivatives ($Cu_2Co_2SOD$ or $Cu_2Ni_2SOD$) which have the same structural properties [71,89] and the enzymatic activity of the native enzyme. Through the histidinato bridge, copper is magnetically coupled with cobalt or nickel, which have much faster electron relaxation rates than the copper(II) ion. As a consequence, the magnetic coupling between the two metal centres produces a shortening in $\tau_s$ of copper and therefore a decrease in $T_{2M}^{-1}$ for the protons sensing the copper ion. The electronic relaxation time of the copper(II) ion in the native enzyme is estimated to be $3 \times 10^{-9}\ s^{-1}$ [90] and that of the cobalt(II) ion in the $E_2Co_2SOD$ derivative (where E means empty) to be $3 \times 10^{-11}\ s^{-1}$ [91], whereas in the $Cu_2Co_2SOD$ derivative, a $\tau_s$ for the Cu(II) of $1.7 \times 10^{-11}\ s^{-1}$ has been estimated [91]. A similar effect of a decrease of the linewidth of the proton signals of residues coordinated to copper, which is magnetically coupled with fast-relaxing metal ions, was previously observed in some small complexes [92].

Figure 1 reports the spectra of the $Cu_2Co_2SOD$ and $Cu_2Ni_2SOD$ derivatives. In both cases, several relatively sharp and well-resolved signals are observed in the range 90 to −20 ppm which are attributed to protons of the residues coordinated to both metal ions. An $^1H$ NOE study on $Cu_2Co_2SOD$ [71] and a 2D NMR investigation on $Cu_2Ni_2SOD$ [93] have allowed us to assign all the proton resonances of the ligands of copper(II) and cobalt(II)/nickel(II) ions. In Fig. 2(A), some sample NOE spectra obtained on $Cu_2Co_2SOD$ are reported [91]. Such an investiga-

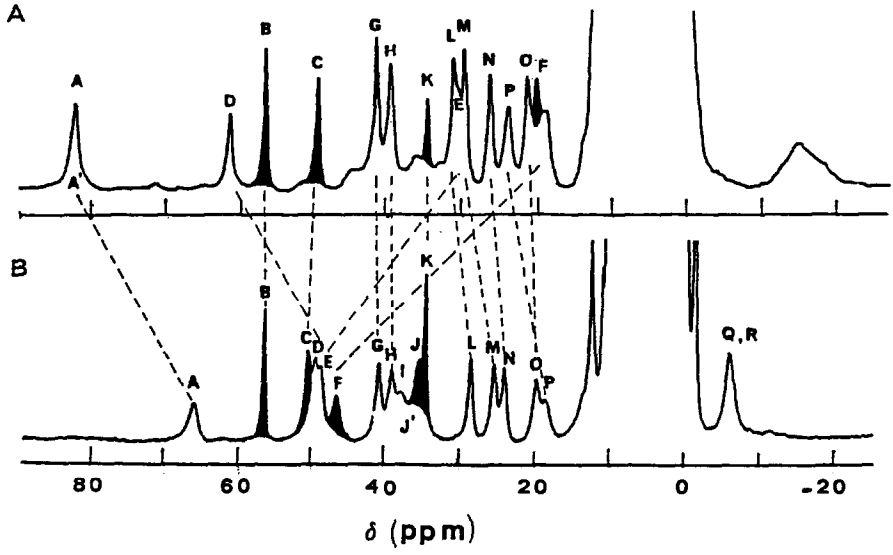


Fig. 1. 200 MHz $^1H$ NMR spectra of $Cu_2Ni_2SOD$ (A) and $Cu_2Co_2SOD$ (B). Signals are labelled according to assignments reported in ref. 71.

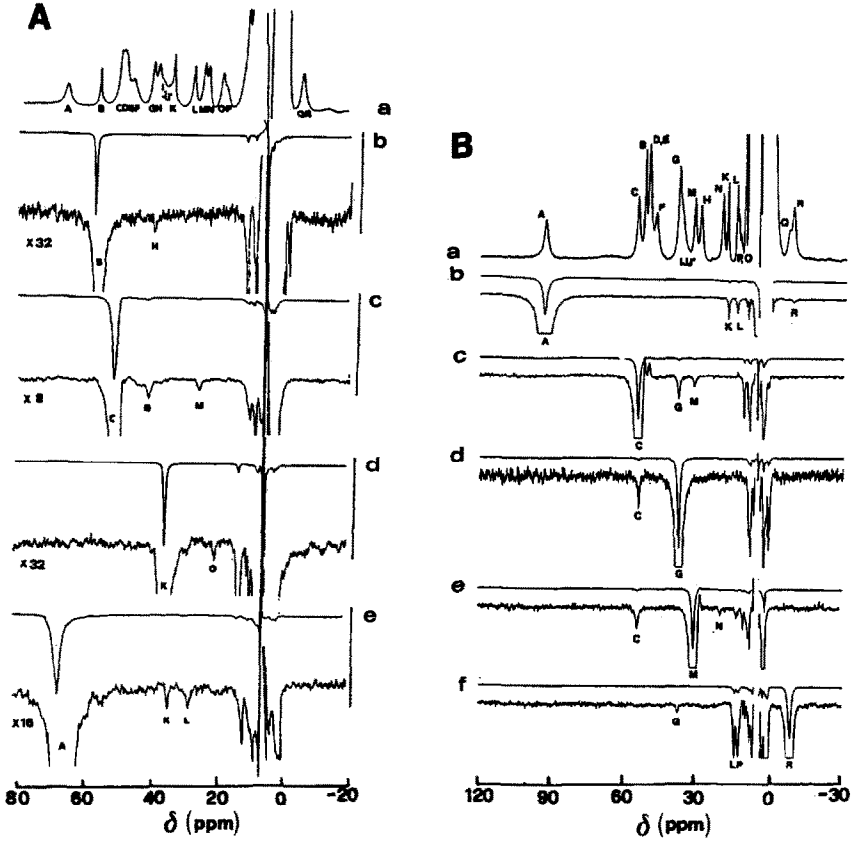


Fig. 2. $^1$H NOE difference spectra recorded at 200 MHz on $Cu_2Co_2SOD$ (A) and on $Cu_2Co_2SOD + N_3^-$ (B) in $H_2O$ solutions. A, (a) Reference spectrum; (b)–(e) show NOE difference spectra obtained by saturating signals B (b), C (c), K (d) and A (e), respectively. (Adapted from ref. 71.) B, (a) Reference spectrum; (b)–(f) NOE difference spectra obtained by saturating signals A (b), C (c), G (d), M (e) and R (f), respectively. (Adapted from ref. 72.) The smallest observed NOE effect (0.3%) is observed on signal R upon saturation of signal A (B, trace b). The other labelled NOE signals range from 0.5% to 9%.

tion has also been performed in the presence of inorganic anions such as azide [72] which have been reported to inhibit enzymatic activity [82,83,94]. Data obtained in the presence of azide (Fig. 2(B)) have been compared with those obtained in the absence of inhibitors. As can be seen, the methodology had been developed to detect NOE as small as 0.2% [72]. Such studies allowed us to observe that: (i) the structure of the active site in solution when zinc is substituted by Co(II) is fully retained [71]; (ii) the binding of azide to the enzyme causes lengthening of the His-48 to copper(II) bond, accomplished by a movement of the copper(II) ion inside the cavity. No geometrical rearrangements of copper ligands have been detected within experimental error ($\leqslant$10%) [72]. The use of fast-relaxing paramagnetic metal ions magnetically

coupled with paramagnetic metal ions with unfavourable electronic relaxation rate is therefore, a useful tool for investigating the coordination site of the latter ion.

In Fig. 3, the $^1$H NMR spectrum of a $Fe_2S_2$ ferredoxin is reported [95]. This class of proteins [96,97], involved in the respiratory chain as an electron-transfer protein, contains an $Fe_2S_2$ cluster in which the two iron ions are bridged by two inorganic sulphide bridges and whose coordination is completed by two cysteines to each iron atom [98]. In the resting state, the protein contains two iron(III) ions, which are characterized by long $\tau_s$. As a consequence, the signals of the $\beta$-$CH_2$ of the metal-bound cysteines are very broad in the $^1$H NMR spectrum [99]. Upon reduction of the protein, one iron(III) becomes iron(II), which has a short electron relaxation time. The two iron ions are magnetically coupled with a $J$ constant of about 200 $cm^{-1}$. Consequently, $\tau_s$ of Fe(III) is shortened by the presence of the fast-relaxing magnetically coupled Fe(II) and the signals of protons of cysteines coordinated to iron(III) become sharp enough to be easily detected. Assignment of the signals has been performed through NOE experiments, despite the fast relaxation of the paramagnetically shifted signals ($T_1$ from 0.4 to 11 ms) [95]. Such assignment allowed us to identify the cysteines coordinated to the iron(III), i.e. to discriminate which iron accepts the electron upon reduction.

In the early 1980s, the introduction of 2D NMR provided a formidable step ahead, particularly fruitful in the investigation of solution structure and dynamics of protein, DNA fragments and other biological macromolecules [100–103]. With these experiments, both scalar interactions, i.e. $J$ coupling or through bond interactions, and dipolar interactions can be easily determined by the detection of cross peaks between signals. COSY and TOCSY are the basic experiments for detecting scalar interactions [104–106] whereas NOESY experiments provide information on such dipolar connectivities [107,108]. These techniques allow the assignment of even very crowded and overlapped spectra, thus providing the resolution of the structure of macromolecules in solution and understanding of their dynamics. The potential of this methodology is now well settled, with tens of structures of biological molecules solved by NMR. An enormous number of pulse sequences have been designed for

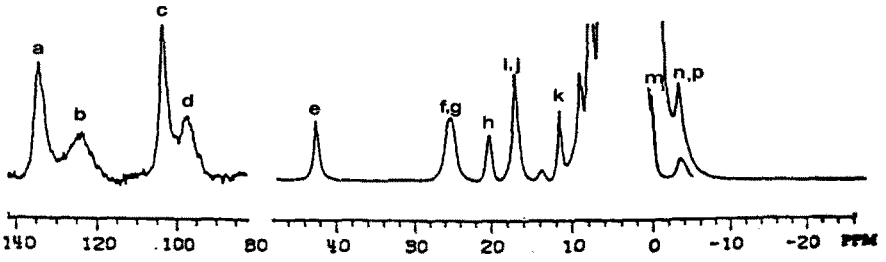


Fig. 3. 360 MHz, 303 K $^1$H NMR spectrum of reduced $Fe_2S_2$ ferrodoxin from *Spirulina Platensis* in $D_2O$ solution. The 140–80 ppm region is expanded 5 times vertically to show clearly the broad peaks a–d. (Adapted from ref. 95.)

the investigation of structure and dynamic of diamagnetic molecules in solution [109]. Recently, the introduction of multidimensional (3D, 4D and even 5D) NMR has also provided a further powerful investigation tool [110–112].

Application of 2D techniques to paramagnetic systems was delayed due to the intrinsic difficulties of these systems: large spectral width and short relaxation times. Therefore fast relaxation of NMR signals requires that the 2D experiments in paramagnetic systems be "tuned" to the properties of the signals investigated [113–116]. The first 2D experiments performed on paramagnetic systems were applied to the detection of chemical exchange between different species (EXSY experiments) [113,117]. Using this technique, Xavier and coworkers have investigated the electron transfer in cytochrome $c_3$ protein from *Desulphonbrio gigas* [119].

Since both NOESY and COSY information decays as a function of the relaxation rates, very short recycle delays must be used when information on fast-relaxing signals is collected. Partial suppression of the diamagnetic signals provided by fast recycling enhances the detectability of the isotropically shifted resonances. Moreover, fast recycling allows one to acquire a large number of scans for each experiment. In the case of COSY experiments, using short acquisition times we have been able to detect cross peaks between signals ca. 900 Hz broad [118]. To obtain these results, data must be collected in the "ancient" magnitude mode instead of the widely used phase-sensitive mode [119]. Since the large linewidth of the paramagnetic signals prevents the resolution of the multiplets when the spectra are collected in the phase-sensitive mode, the antiphase components of the cross peaks, if unresolved, tend to cancel each other, thus significantly reducing the intensity of the cross peaks [116,120].

The detection of NOESY cross peaks in paramagnetic systems can be optimized by using appropriately short mixing times. Mixing times from 2 to 15 ms are commonly used, with relaxation delays from 30 to 200 ms. In such conditions we have been able to detect cross peaks between protons 3 Å apart, with signals having $T_1$ up to 5 ms [93].

An elegant and significant application of 2D spectroscopy to paramagnetic molecules is given by the study of peroxidases.

Peroxidases are a class of proteins containing an haeme moiety, which react with $H_2O_2$ or alkyl peroxides to perform a variety of oxidation reactions [121–123]. The fifth position of the haeme iron is occupied by the nitrogen of an histidine (proximal histidine) residue [124]. The $^1H$ NMR spectra of the high-spin form show well-resolved signals spread over 100 ppm of spectral width, characterized by short $T_1$ and large linewidth, due to the long electron relaxation of high spin iron(III) [125–127]. The advancement of the 2D NMR techniques allowed the detection of cross peaks in the 2D spectra on these systems, thus providing the assignment of isotropically shifted signals [117,119].

Only signals of the iron ligands outside the diamagnetic envelope are observed due to the negligible magnetic anisotropy of iron(III) which therefore induces very

small pseudocontact shifts on the protons of residues not directly coordinated to the metal.

On the other hand, the low spin form obtained by $CN^-$ binding, in which iron has a short $\tau_s$, also provides sharp signals with a large pseudocontact shift contribution for non-ligand protons [65(c),69,128–130].

The 1D and 2D NMR characterization has been performed, after the elegant characterization of horseradish peroxidase (HRP) by La Mar and coworkers [129], on cytochrome *c* peroxidase (Ccp) [69,130,131], some of its mutants [132] and on ligninase (LiP) [125–133]. Cytochrome *c* peroxidase, present in yeast, catalyzes the oxidation of cytochrome *c*. Ligninase catalyzes the oxidation of aromatic rings in lignine, a high molecular weight polymer which is hardly biodegradable, present in wood. The 2D NOESY map of Ccp-CN is shown in Fig. 4 [131].

COSY and NOESY experiments, performed on both CcP-CN and LiP-CN, allowed us to assign most of the haeme signals and several signals arising from residues close to the active site [125–131]. The NMR data showed that the structure of the active site of the two enzymes and of the Ccp mutants are very similar but not identical. The major difference among the investigated proteins is in the geometrical arrangement of the proximal histidine. The shifts of the protons of the proximal histidine signals, which account for such differences, correlate with the redox potential of the $Fe^{3+}/Fe^{2+}$ pair [123].

In reviewing advancements and perspectives of the NMR of paramagnetic sytems, we must mention some recent results on the analysis of the dipolar part of the isotropic shift to predict the magnetic susceptibility tensor originated by a metal ion inside the active cavity of an enzyme. We have already reported that the dipolar isotropic shift contains a geometrical factor which depends on the orientation of the magnetic susceptibility tensor in the molecular frame. If the dipolar shifts can be factored out, then the extent and the orientation of the magnetic susceptibility tensor of the system are obtained.

This technique has been used for many years in the case of metalloproteins in which the native metal ion is substituted with a trivalent ion of the lanthanide series [134–138]. Since all Ln(III) ions except Gd(III) induce an isotropic shift almost completely dipolar in origin, all the isotropically shifted resonances contain geometrical information [139–143].

More recently, this methodology has been used when isotropically shifted resonances of non-metal bound residues are assigned. Protons of residues not coordinated to the metal may sense an isotropic shift which is only dipolar in origin; this contribution can simply be calculated if the shift of the signal in the absence of an isotropic contribution is known. Using this methodology, Emerson and La Mar have predicted the orientation of the $\chi$ tensor of met-cyanomyoglobin [144] by taking into account a large number of protons, which sense small dipolar shifts, detected through 2D experiments [145]. In our laboratory, we have successfully used this

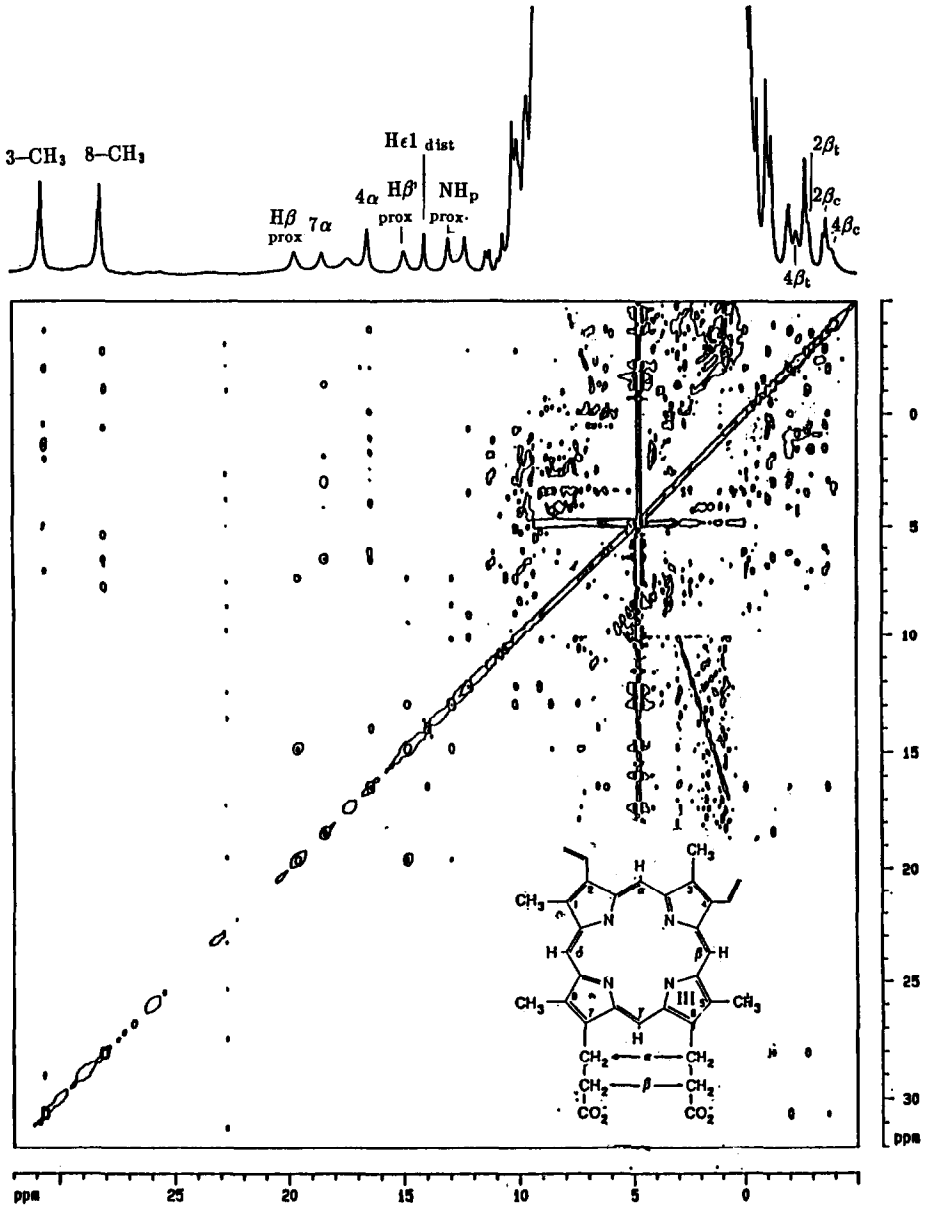


Fig. 4. 600 MHz, 298 K NOESY spectrum of a water solution of CcP–$CN^-$ [131] obtained with 15 ms of mixing time, together with 1D reference spectrum. Assignment of signals in the 1D spectrum refers to the reported scheme of the haeme. (Adapted from ref. 131.)

methodology to estimate the orientation of the magnetic $\chi$ tensor of Co(II) ion inside the Zn(II) site of superoxide dismutase [74] and of the Co(II) substituted carbonic anhydrase in the presence of perchlorate [146].

Knowledge of the $\chi$ tensor allows assignment of further signals and the prediction of the shift values when some perturbations (mutants, adducts, etc.) are introduced into the system.

### D. NMR AS A UNIQUE TOOL IN THE INVESTIGATION OF METAL CLUSTERS IN PROTEINS

The electronic structure of metal clusters is a fascinating problem which has attracted the interest of inorganic and bioinorganic chemists for many years [147,148]. Understanding of the effect of magnetic coupling among metal ions on the electronic spin levels is the basis for the interpretation of a large series of experimental data, such as Mössbauer measurements [149–151], ENDOR [152], EPR [153,154], MCD [155], resonance Raman [156] and NMR [75–79,157–166].

Our laboratory, following development of the equations describing the NMR parameters for dinuclear species, has proceeded to study trinuclear [167] and tetranuclear metal clusters [168].

The most general form of the spin Hamiltonian term, which describes the coupling between four metal ions according to the coupling scheme of Fig. 5 is

$$\hat{H} = J_{12}\hat{S}_1\hat{S}_2 + J_{13}\hat{S}_1\hat{S}_3 + J_{14}\hat{S}_1\hat{S}_4 + J_{23}\hat{S}_2\hat{S}_3 + J_{24}\hat{S}_2\hat{S}_4 + J_{34}\hat{S}_3\hat{S}_4 \quad (12)$$

where $\hat{S}_1$, $\hat{S}_2$, $\hat{S}_3$ and $\hat{S}_4$ are the spin angular momentum operators of the four metal centres and $J_{mn}$ are the two-centre isotropic exchange coupling constants.

By applying Hamiltonian (12) to the spin functions containing the isolated metal ion spin functions, the spin function of the new $S_i'$ states of the cluster can be obtained. Only for three or less different $J$ values, can analytical equations for the spin level energies be obtained. From the energy separations of the levels, their

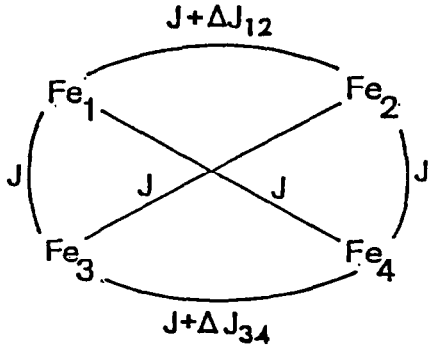


Fig. 5. Magnetic exchange coupling scheme in $Fe_4S_4$ clusters.

populations can be calculated through their Boltzmann distribution and finally the contact shifts can be computed as a function of temperature.

We applied the above treatment to a cluster of three high spin iron(III) and one high spin iron(II). This type of cluster is contained in the oxidized form of high-potential iron-sulphur proteins (HiPIP), which in nature perform one-electron transfer [169–171]. The iron ions are bridged by sulphide bridges in a cubane structure. Four coordination of each iron ion is completed by a cysteine residue [172,173]. The electron delocalization in such systems can be quite relevant; indeed, Mössbauer spectroscopy shows that, in the cluster, there are two iron 3+ and two iron 2.5+, i.e. one iron(III) and the iron(II) which gives rise to a so-called mixed valence pair [150,151].

Several different HiPIPs can be extracted by different bacteria. So far, we have characterized HiPIPs from *Chromatium Vinosum* [78], *Ectothiorodospira Halophila* [77] and *Rhodococcus Gelatinosus* [168]. The NMR spectra of these proteins, reported in Fig. 6, show eight isotropically shifted signals which are both downfield and upfield. These signals have been assigned through NOE, NOESY and COSY experiments to the $\beta$-$CH_2$ protons of the four cysteine ligands to the iron ions [76–80,168]. The spectra of *C. Vinosum* and *R. Gelatinosus* HiPIPs show six downfield and two upfield $\beta$-$CH_2$ cysteine signals. In the case of *E. Halophila*, there are four downfield and four upfield $\beta$-$CH_2$ cysteine protons. One more isotropically downfield shifted signal, labelled E, is detected for *C. Vinosum* and *R. Gelatinosus* and it has been assigned to one $\alpha$-CH of a cysteine residue.

The temperature dependence of the shifts is reported in Fig. 7. Signals A–D experience a Curie behaviour in all the derivatives investigated, signals F and G for *R. Gelatinosus* experience an anti-Curie behaviour, the signals Y and Z and the signals W and X for *E. Halophila* have a pseudo-Curie behaviour. In order to account for their temperature dependence, we solved the Hamiltonian (9) under different assumptions in the coupling scheme of Fig. 5. Assuming that $J_{13} = J_{14} = J_{23} = J_{24} = J$, $J_{12} = J + \Delta J_{12}$ and $J_{34} = J + \Delta J_{34}$, i.e. in the case in which the coupling between the two iron(III) ions ($Fe_1$ and $Fe_2$) and the iron(III)–iron(II) mixed valence pair ($Fe_3$ and $Fe_4$) is different from the coupling constant between all the other ions, the energies for the electron spin levels in the cluster are given by

$$E(S'_{12}S'_{34}S') = \frac{J}{2}[S'(S'+1)] + \frac{\Delta J_{12}}{2}[S'_{12}(S'_{12}+1)] + \frac{\Delta J_{34}}{2}[S'_{34}(S'_{34}+1)] \tag{13}$$

where $S'$ ranges between $(S_1 + S_2) + (S_3 + S_4)$ and $|(S_1 + S_2) - (S_3 + S_4)|$, $S'_{12}$ ranges between $S_1 + S_2$ and $|S_1 - S_2|$, $S'_{34}$ ranges between $S_3 + S_4$ and $|S_3 - S_4|$.

The contact shift can now be calculated by taking into account the contribution of the population of each $S'$ level of the cluster.

$$\frac{\Delta\nu}{\nu_0} = \frac{g_e\mu_B}{h\gamma_N 3kT} A_m \frac{\Sigma_i C_{im} S'_i(S'_i+1)(2S'_i+1)e^{-(E_i/kT)}}{\Sigma_i(2S'_i+1)e^{-(E_i/kT)}} \tag{14}$$

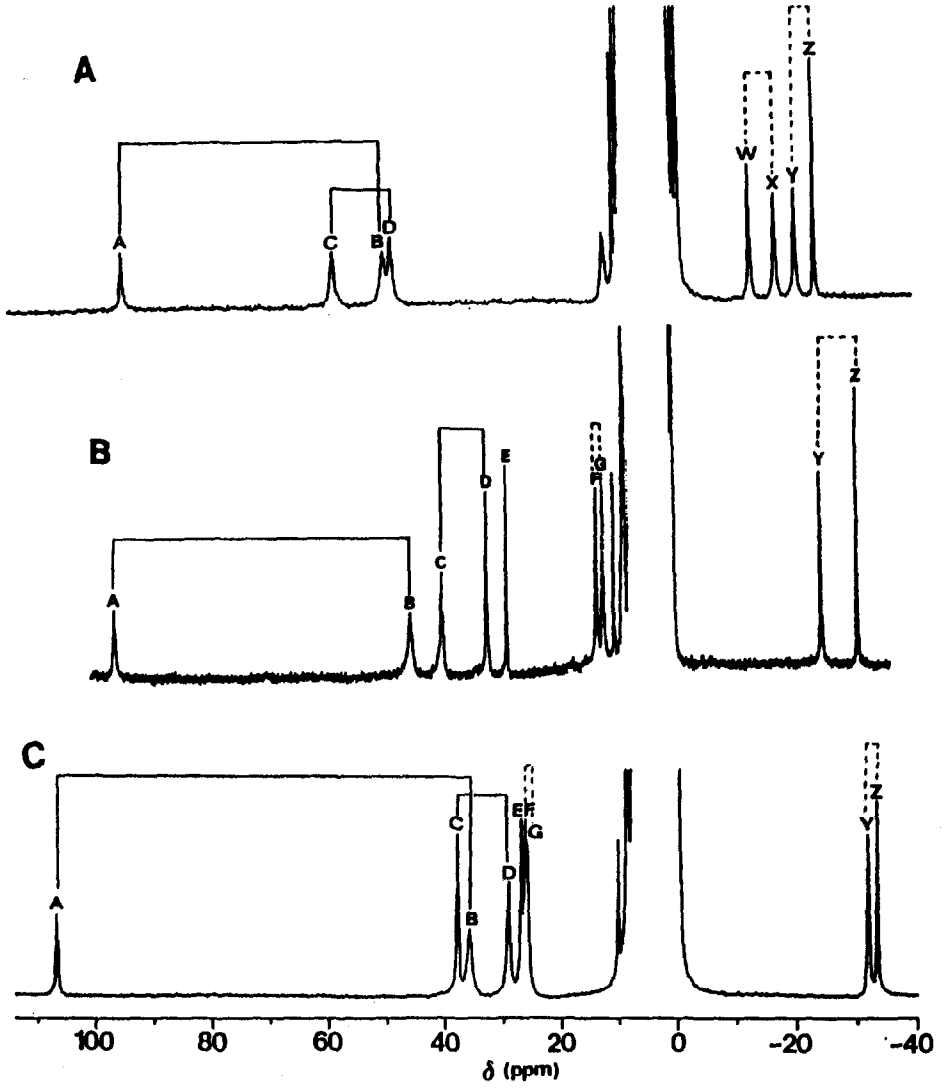


Fig. 6. 600 MHz, 303 K $^1$H NMR spectra of oxidized HiPIPs from *E. Halophila* (A, HiPIP II), *R. Gelatinosus* (B), and *C. Vinosum* (C). Spectra were recorded in $D_2O$ solutions, at pH = 5.1. (Taken from ref. 168.)

where the sum is performed over all the $S_i'$ levels of the cluster, as in the case of dimers and $C_{im}$, similar to the case of dimers, is a coefficient which takes into account the contribution of the spin function of the isolated metal ion to the spin function of the cluster [78,168]. $A_m$, i.e. the contact hyperfine coupling constant between the resonating nucleus and the isolated metal centre, is assumed to be equal for all the eight protons. We choose as a starting set of $J$ parameters a larger coupling between the metal ions of higher oxidation states, as observed in a large series of dinuclear

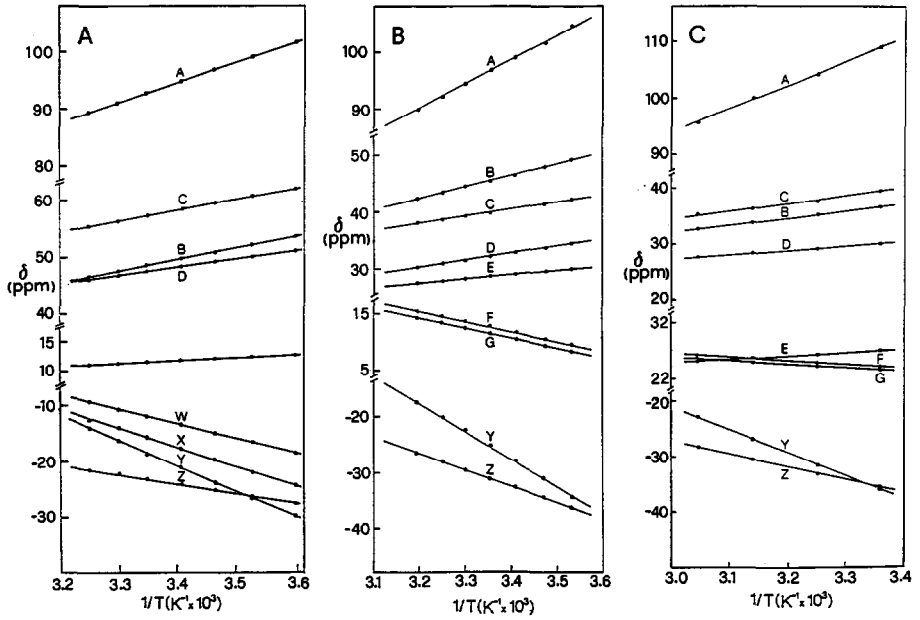


Fig. 7. Temperature dependence of the chemical shifts of the hyperfine shifted resonances of oxidized HiPIPs from *E. Halophila* (A, HiPIP II), *R. Gelatinosus* (B), and *C. Vinosum* (C). (Adapted from ref. 168.)

systems from magnetic measurements [174]. In the scheme with 3 different $J$ values, the calculated temperature dependence of the chemical shifts is reported in Fig. 8(A) (see figure legend for details). These $J$ values also reproduce the ground state $S = 1/2$, found from the fitting of the Mössbauer data. In this situation, $Fe_1$ and $Fe_2$, the two ferric ions, are equivalent and induce on the protons sensing them an upfield shift with Curie behaviour.

The calculated pattern is similar to that experimentally observed in the case of *E. Halophila*, thus assigning the downfield signals to protons interacting with the Fe(III)–Fe(II) pair, while those upfield are due to protons interacting with the two iron(III) ions [78,79]. In order to rationalize the shift pattern observed in *C. Vinosum* and *R. Gelatinosus*, we further decrease the symmetry of the system by considering $J_{14} < J_{13} = J_{14} = J_{24}$, i.e. by decreasing the coupling between one of the pure ferric ions and the Fe(II) ion of the mixed valence pair. This causes a removal of the degeneracy of the two pure ferric ions and in Fig. 8(B) and (C), the results of the calculations are reported. Under the conditions of Fig. 8(C), the shift induced by $Fe_1$ is downfield and the temperature dependence goes from a pseudo-Curie to an anti-Curie behaviour. This is the pattern of signals F and G in *C. Vinosum* and *R. Gelatinosus*, which thus correspond to the signals W and X in the *E. Halophila*.

Recently, through NMR spectroscopy, it has been possible to specify which are the cysteines bound to the Fe(III)–Fe(III) pair and which are the cysteines bound to the iron ions of the mixed valence pair [79,166]. This has been possible in the case

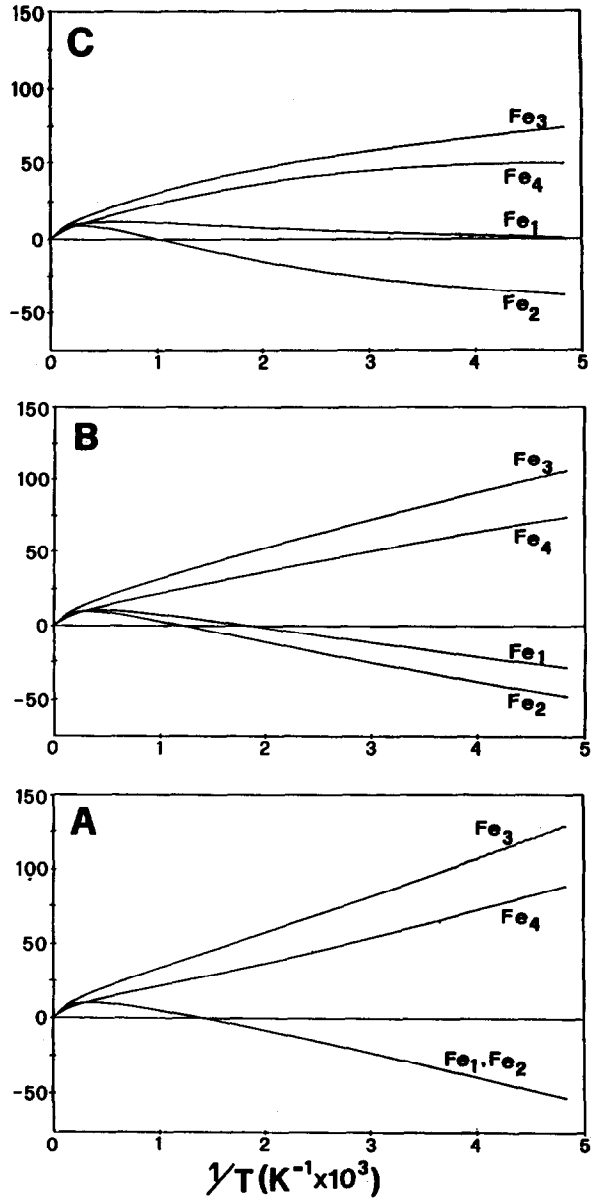


Fig. 8. Temperature dependence of the $^1H$ NMR hyperfine shifts of HiPIPs calculated with $J_{12} = 400\ cm^{-1}$, $J_{34} = 200\ cm^{-1}$, $J_{13} = J_{14} = J_{23} = 300\ cm^{-1}$ and $J_{14} = 300\ cm^{-1}$ (A), $270\ cm^{-1}$ (B), $230\ cm^{-1}$ (C). (Adapted from ref. 168.)

of HiPIP from *Chromatium Vinosum* from which the X-ray structure has been solved in both the oxidized and reduced states [172,173]. In this system, despite the line broadening induced by the presence of paramagnetic metal ions onto the protons of the aminoacid residues in the proximity of the cluster, a series of 2D experiments, devoted to the detection of connectivity between the $\beta$-$CH_2$ and the $\alpha$-CH of the four iron coordinated cysteines and the rest of the protein has been performed. Figure 9 shows COSY and NOESY experiments on the reduced state which shows signals spread from +17 to −0.3 ppm. The four $\beta$-$CH_2$ cysteines resonances a–y, b–z, c–w, d–e are detected together with three of the four $\alpha$-CH resonances. The presence of two triptophanes and one phenylalanine residues near three of the four cysteines has allowed the unequivocal assignment of each $\beta$-$CH_2$ pair to a specific cysteine residue. As an example, we report in Fig. 10(A) and (B) the observed NOESY correlations which the NH exchangeable protons of triptophanes (labelled w1 and w2) and one $\alpha$-CH cysteine proton at 8.52 ppm give with signals belonging to a tryptophane, whose typical spin system pattern is observed in the TOCSY reported in Fig. 10(C). Another example of the connectivities between the $\beta$ and $\alpha$ protons of the iron-coordinated cysteines and the aromatic residues near the cluster is the cross peak in the NOESY spectrum (Fig. 10(D)) between the $\beta$-$CH_2$ proton at 16.1 ppm

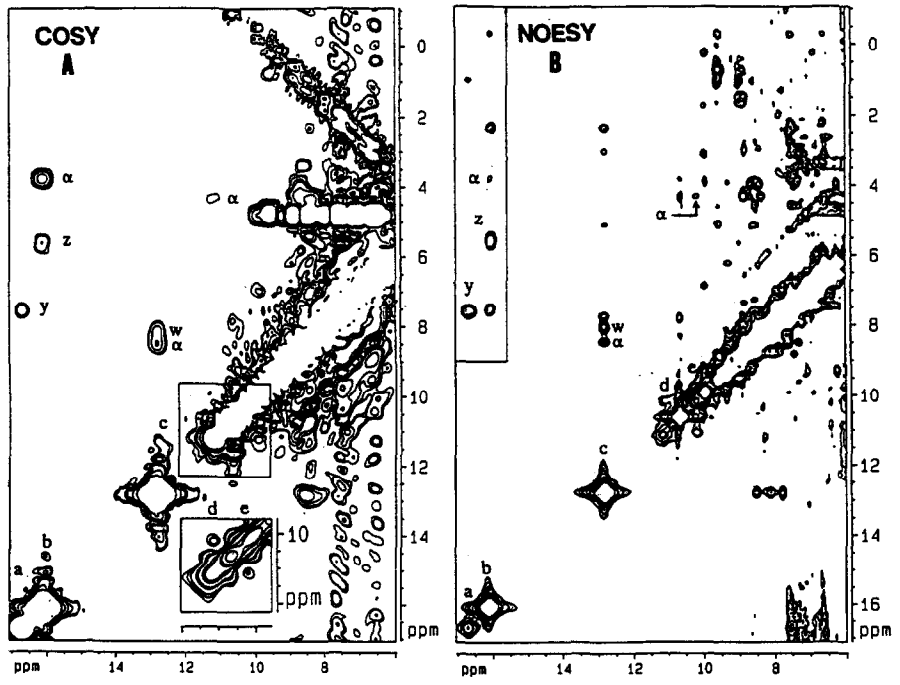


Fig. 9. 600 MHz, 300 K $^1$H NMR COSY (A) and NOESY (B) spectra of reduced *C. Vinosum* HiPIP. COSY were recorded in magnitude mode, NOESY in phase-sensitive mode (TPPI). Mixing times of 10 ms were used for NOESY spectrum. (See ref. 79 for experimental details.)

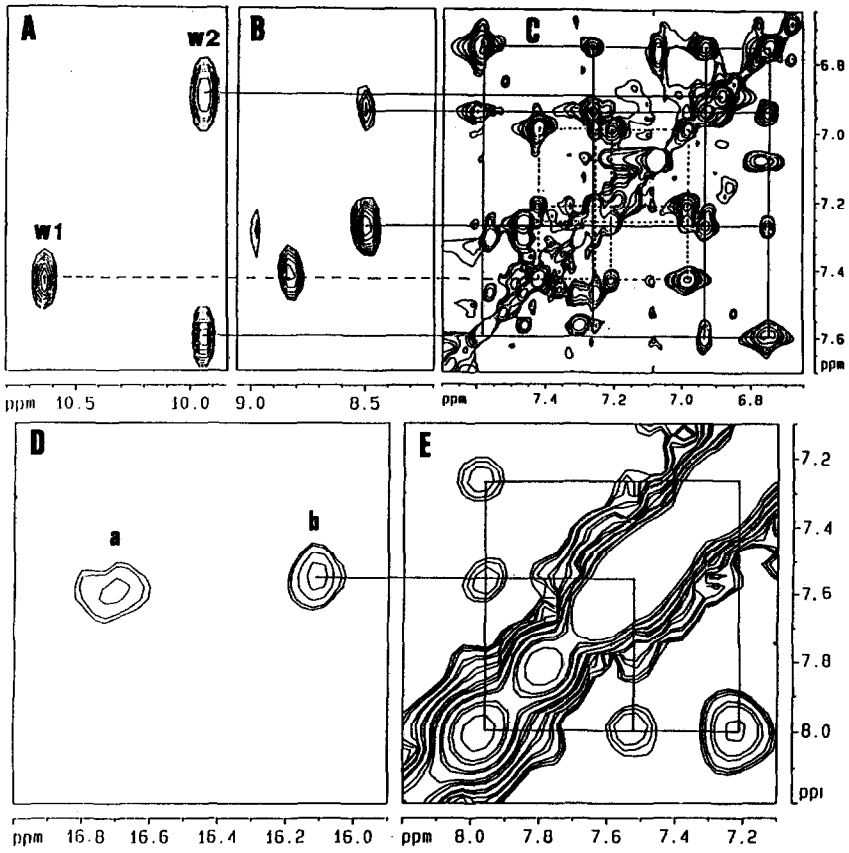


Fig. 10. 600 MHz, 300 K NOESY (A, B, and D), TOCSY (C) and COSY (E) spectra on the aromatic region of reduced *C. Vinosum* HiPIP. The region of the TOCSY spectrum in which the spin system pattern of Trp residues occur is shown. The patterns of two different tryptophanes are drawn. The COSY spectrum permits detection of the scalar spin pattern of a phenylalanine. The NOESY spectra allow detection of through space connectivities from the ring protons of the tryptophanes to the respective NH protons (A), from a phenylalanine to a signal of a cysteine $\beta$-$CH_2$ (C), from a cysteine $\alpha$-CH proton to the H$\varepsilon$3 and H$\zeta$3 protons of one tryptophane residue (B). All these residues have been assigned on the basis of the observed connectivities [79].

with a signal at 7.52 ppm, which from COSY experiments (E), can be assigned to a phenylalanine spin system. Since, as previously reported in this section, the theoretical model indicates which are the $\beta$-$CH_2$ protons coordinated to the pure ferric iron ions and which to the mixed valence pair, the assignment of the iron ions which accept the electron upon reduction is straightforward [79,166].

Both the oxidized and the reduced forms of the protein were investigated. Since the exchange rate between the two oxidation states is slow on the NMR time scale, EXSY experiments provide correlations between $\beta$-$CH_2$ and $\alpha$-CH cysteine signals

of the oxidized and reduced states; as a consequence, information obtained over the protein in the two oxidation states is complementary.

An extensive spectroscopic characterization has also been performed on the ferredoxin from *Clostridium Pasterianum*, which contains two non-equivalent $Fe_4S_4$ clusters. EXSY experiments between the oxidized, intermediate and reduced forms allowed us to attribute the signals to each cluster [75(b)]. The temperature dependence of the $\beta$-$CH_2$ signals of the reduced $Fe_4S_4^+$ form was determined and the shift pattern rationalized with an appropriate choice of $J$ values for Hamiltonian (12) [75,76].

## E. CONCLUSIONS

The importance of NMR in the investigation of paramagnetic molecules has been established for many years. The paramagnetic ion, with its peculiar magnetic properties "highlights" its neighbours, conferring distinctive properties in terms of chemical shift and/or relaxation properties to the nuclei influenced by the hyperfine interaction.

This is particularly useful in the study of paramagnetic metalloenzymes, because the metal site represents the catalytic core of the molecule, therefore information about the nature of the ligands, coordination number, binding mode of substrates and inhibitors, as well as dynamic information about electron or ligand exchange can easily be obtained.

While the availability of higher magnetic fields is not expected to provide further advancements in this area in the future, owing to the line broadening induced by the field on the paramagnetic signals, the continuous evolution of instrumental performance, as well as the development of more potent algorithms for treating experimental data, will allow one to overcome the present limitations of 1D and 2D experiments in detecting very broad signals or with very short longitudinal relaxation times, contributing to a more generalized approach to the study of paramagnetic systems using NMR.

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