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Transient versus steady state NOE in paramagnetic molecules Cu_2Co_2SOD as an example

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Truncated, steady state and transient NOE experiments have been performed on bovine Cu_2Co_2 superoxide dismutase. The effectiveness of the different NOE experiments in the general case of paramagnetic macromolecules is discussed. It is concluded that steady state NOEs give superior results. The validity of the two spins approximation is discussed, and NOE values for a fully coupled set of nuclei have been calculated. Transient NOE experiments, when properly performed, confirm the previous assignment of the hyperfine shifted signals in Cu_2Co_2SOD based on steady state NOE measurements [(1989) Inorg. Chem. 28, 4650] and eliminate any further reason of controversy on an important issue as the assignment of the ^1H NMR signals of protons of metal-coordinated imidazoles.

1. INTRODUCTION

The investigation of paramagnetic macromolecules through the detection of the nuclear Overhauser effect (NOE) in ^1H NMR spectroscopy has been pioneered by Gerd La Mar since 1983 [1–7]. Owing to the fast relaxation rates of isotropically shifted signals, the NOEs which can be detected in paramagnetic macromolecules are quantitatively very small, thus creating severe limitations to the application of this sophisticated technique. On the other hand, the information obtained from NOE studies are so precious that the research in the area is quite active [8–10].

An extensive study on $Cu(II)_2Co(II)_2$Superoxide Dismutase (SOD hereafter) based on the detection and interpretation of steady state nuclear Overhauser effects has been recently published [9]. It has been possible to perform a complete assignment of the signals of the histidines [9] and the aspartate [10] bound to the two metal ions, and complete agreement between spectroscopy and the X-ray structure [11,12] was found. It was also possible to state the absence of changes in the structure of the active site when the native zinc(II) ion is substituted with cobalt(II) ion. Subsequently, a paper based on transient NOE has been published [13] reporting different spectral connectivities with respect to those reported in [9,10] and stating that the structure in solution of Cu_2Co_2SOD is not consistent with the X-ray data on the native enzyme.

We compare here steady state NOE, truncated NOE and transient NOE techniques as far as paramagnetic molecules are concerned from both experimental and theoretical points of view, and we are going to state that: (i) steady state NOE provides better results from the qualitative and quantitative points of view; (ii) the active cavity of SOD in solution is the same as in the solid state.

2. MATERIALS AND METHODS

Native bovine Cu_2Zn_2SOD was purchased from Diagnostic Data Inc., Mountain View, CA, and used without further purification. Cu_2Co_2SOD derivative was prepared according to previously reported methodology [14,15]. Cu_2Co_2SOD solution in H_2O were prepared in 50 mM Hepes buffer at pH 7.0. All the reported experiments have been performed on a Bruker AMX 600 operating at the magnetic field of 14 Tesla. All the spectra have been recorded over 100000 Hz bandwidth, by using a superWEFT [16] or a MoDEFT [17] pulse sequence. Experiments have been performed in block-averaging mode. All the reported data consist of 7 blocks, 32768 scans each. Difference spectra have been collected using the previously reported methodology [9].

3. RESULTS AND DISCUSSION

The NOE η_J is defined as the fractional variation in intensity of a signal, let's say *J*, when another signal *I* is selectively saturated [18]. The application of decoupler power over peak *I* causes the saturation of the peak and, as a consequence, the occurrence of NOE to all the protons which are dipolarly coupled with *I*.

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Abbreviations: SOD, superoxide dismutase; NOE, nuclear Overhauser effect; NMR, nuclear magnetic resonance. *Enzymes:* Cu, Zn superoxide dismutase

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The extent of NOE is given by Eqn 1, which holds for two isolated dipolarly coupled homonuclear spins

$$\eta_J = \frac{\sigma_{IJ}}{\varrho_J}(1-e^{-\varrho_J t}) \qquad (1)$$

Here σ_{IJ} is the cross-relaxation rate which is operative between the dipolarly coupled protons I and J, ϱ_J is the selective relaxation rate of signal J which undergoes NOE, and t is the time for which signal I is kept saturated. NOE builds up as a function of the irradiation time up to a maximum value which is called steady state NOE. In this limit case Eqn 1 becomes

$$\eta_J = \sigma_{IJ}/\varrho_J \qquad (2)$$

In the case of transient NOE, a 180° pulse must be selectively applied to a signal, let's say J, in order to invert its magnetization to $-z$ [19]. During the recovery of magnetization along the z-axis a magnetization transfer occurs to all the resonances which are dipolarly coupled with J. The NOE extent in the case of two isolated dipolarly coupled homonuclear spins, is dependent on the relaxation rate of both inverted and observed signals

$$\eta_J = -\frac{\sigma}{D}(e^{-(\varrho'+D)t} - e^{-(\varrho'-D)t}) \qquad (3)$$

where

$$\varrho' = 1/2\,(\varrho_I + \varrho_J) \qquad (4)$$

and

$$D = (1/4(\varrho_I - \varrho_J)^2 + \varrho_{IJ}^2)^{1/2} \qquad (5)$$

$\eta_I = \eta_J$ even if protons I and J have different relaxation rates [20,21]. In this case t is the delay time between the application of the 180° selective pulse and the observation pulse. Fig. 1 reports the expected NOE values of truncated and transient NOEs for different ϱ values. This is the less favorable case for steady state versus transient experiment. In fact, if the ϱ values are different and the steady state experiment is performed by

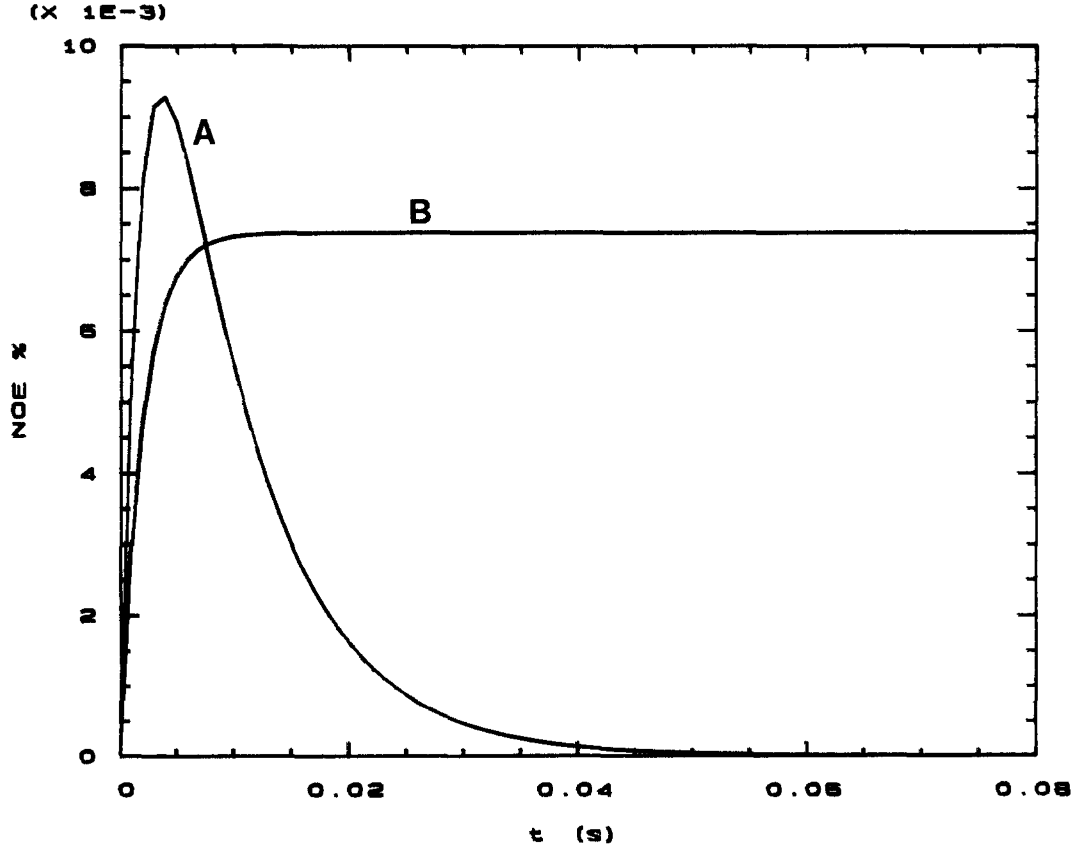

Fig. 1. Calculated time dependence of transient (A) and truncated (B) NOEs. Values have been calculated according to Eqns 1 and 3 at 600 MHz, $\tau_c = 1.4 \times 10^{-8}$ s, for two hydrogen nuclei 2.4 Å apart. Relaxation rates values are: $\varrho_I = 125\ s^{-1}$, $\varrho_J = 500\ s^{-1}$. This system corresponds to the case of peak B and H in Cu_2Co_2SOD, which have been assigned to imidazole ring vicinal protons. The time in abscissa has a different meaning in the two cases: in the case of steady state NOE t is the time during which peak I is kept saturated by continuous wave irradiation; in the case of transient NOE t is the delay time between the 180° selective pulse and the observation pulse.

observing the proton with larger ϱ values, it might happen that the transient NOE is more effective with respect to the steady state. Nevertheless, this is true only for very short times; when delays slightly longer with respect to ϱ values (of both observed and inverted signals) are applied, fast decay of transient NOE occurs. The usefulness of transient NOE mainly resides in the reduction of spin diffusion effects [19]. In fact, during the time necessary to reach the steady state condition, a magnetization transfer from the observed signal could, in turn, occur to a third dipolarly coupled spin (spin diffusion).

Let us now consider the application of NOE to paramagnetic macromolecules in the practical case of Cu_2Co_2SOD. In the native enzyme, the active site of SOD contains a copper ion and a zinc ion linked through an imidazolate bridge of a histidine residue. Three other histidines are coordinated to copper together with a semi-coordinated water molecule; two histidines and an aspartate residue are coordinated to the zinc ion in a distorded tetrahedron [11,12] (see Fig. 2). Substitution of the native Zn(II) ion with Co(II) ion yields a derivative in which 19 resonances of the ^1H NMR spectrum are spread up to +70 and down to −10 ppm [22] (Fig. 3). Our interest is now to use Cu_2Co_2SOD as an example for application of the available NOE techniques.

Table I reports the T_1 values and the observed steady state NOE values for protons of the coordinated residues, as well as the interproton distances and the calculated steady state NOE values. The NOEs are calculated on the basis of Eqn 2, where σ is given by

$$\sigma = -\frac{\hbar^2\gamma^4\tau_c}{10r_{ij}^6} \qquad (6)$$

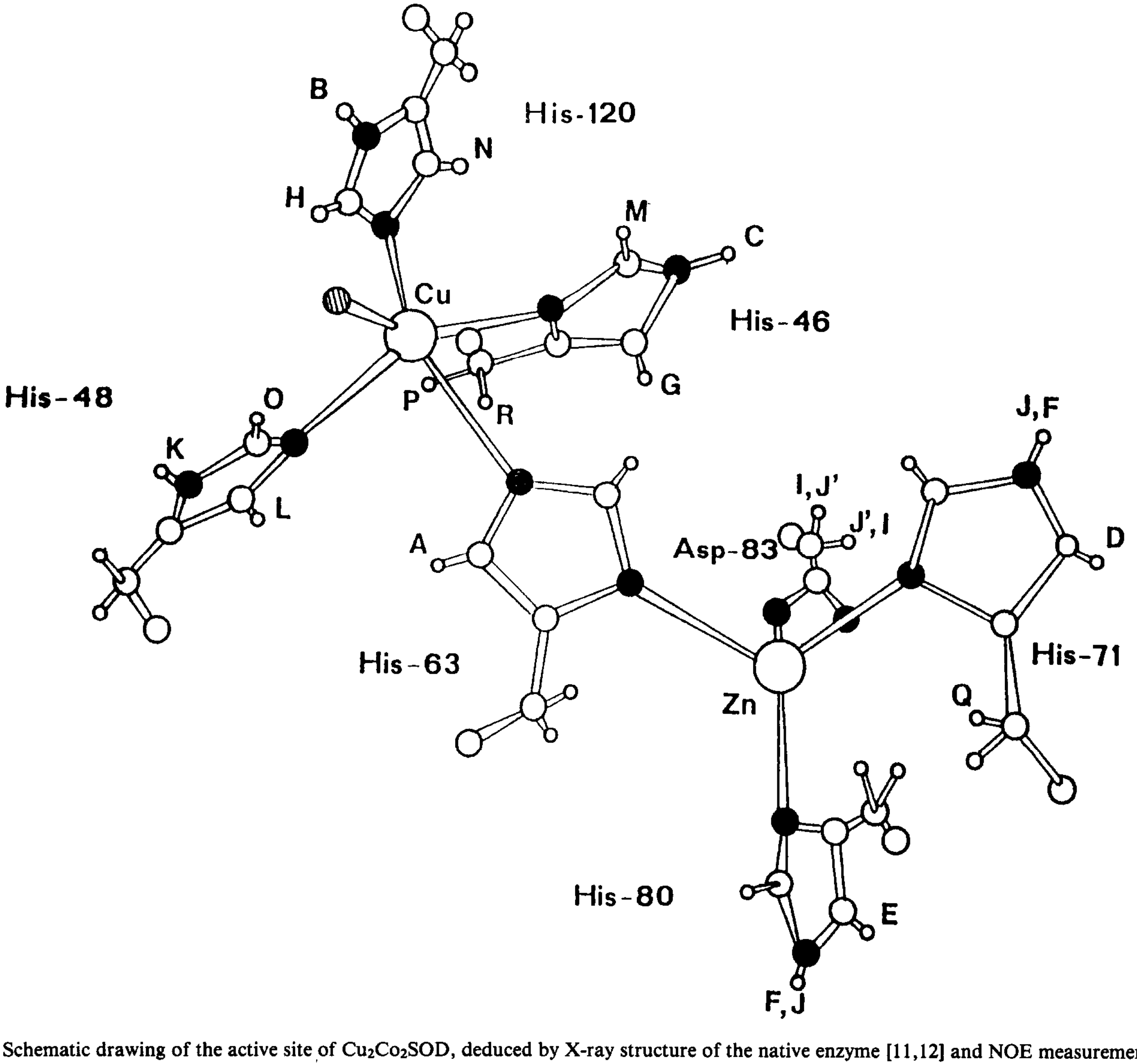

Fig. 2. Schematic drawing of the active site of Cu_2Co_2SOD, deduced by X-ray structure of the native enzyme [11,12] and NOE measurements on Cu_2Co_2SOD derivative [9,10,23]. Labelling of the protons follows the proposed assignment of the ^1H NMR spectrum [9].

Here ℏ and γ have the usual meanings, r_{ij} is the proton-proton distance and τ_c is the rotational correlation time of the molecule. In the case of Table I, a τ_c of 1.4×10^{-8} s has been used, i.e. the correlation time of Cu_2Co_2SOD of diluted solutions [23]. By considering a limit of detection of 0.2% for the NOEs, it appears that only one connectivity (Hδ1-Hε1) within each imidazole ring can be detected for histidines coordinated through Nε2, and two connectivities (Hε2-Hε1, Hε2-Hδ2) can be detected for histidines coordinated through Nδ1.

The NOEs calculated by taking into account simultaneous cross-relaxation to nearby protons are also reported (Table I, values in brackets). In this case proton *I* is allowed to receive magnetization from the saturated signal *J* and simultaneously to transfer it to other protons in its close proximity. It is shown that the largest variations in calculated NOEs for imidazole ring protons is less than 20%. So, the so-called spin diffusion is essentially lacking. Only when protons with particular geometric disposition (and with long T_1s) are considered, can spin diffusion be important. This holds for signals *L, Q* and *R* where up to 60% changes in calculated NOEs with or without spin-diffusion effects are present. It should be noted that a factor of two in NOE gives 10% difference in distances. Therefore, in the case of fast relaxing systems, steady state NOEs can be used for assignments by using Eqn 2 or, safer, the cross relaxation of *I* with all the other protons can be included [20].

In the transient NOE experiment, in order to obtain

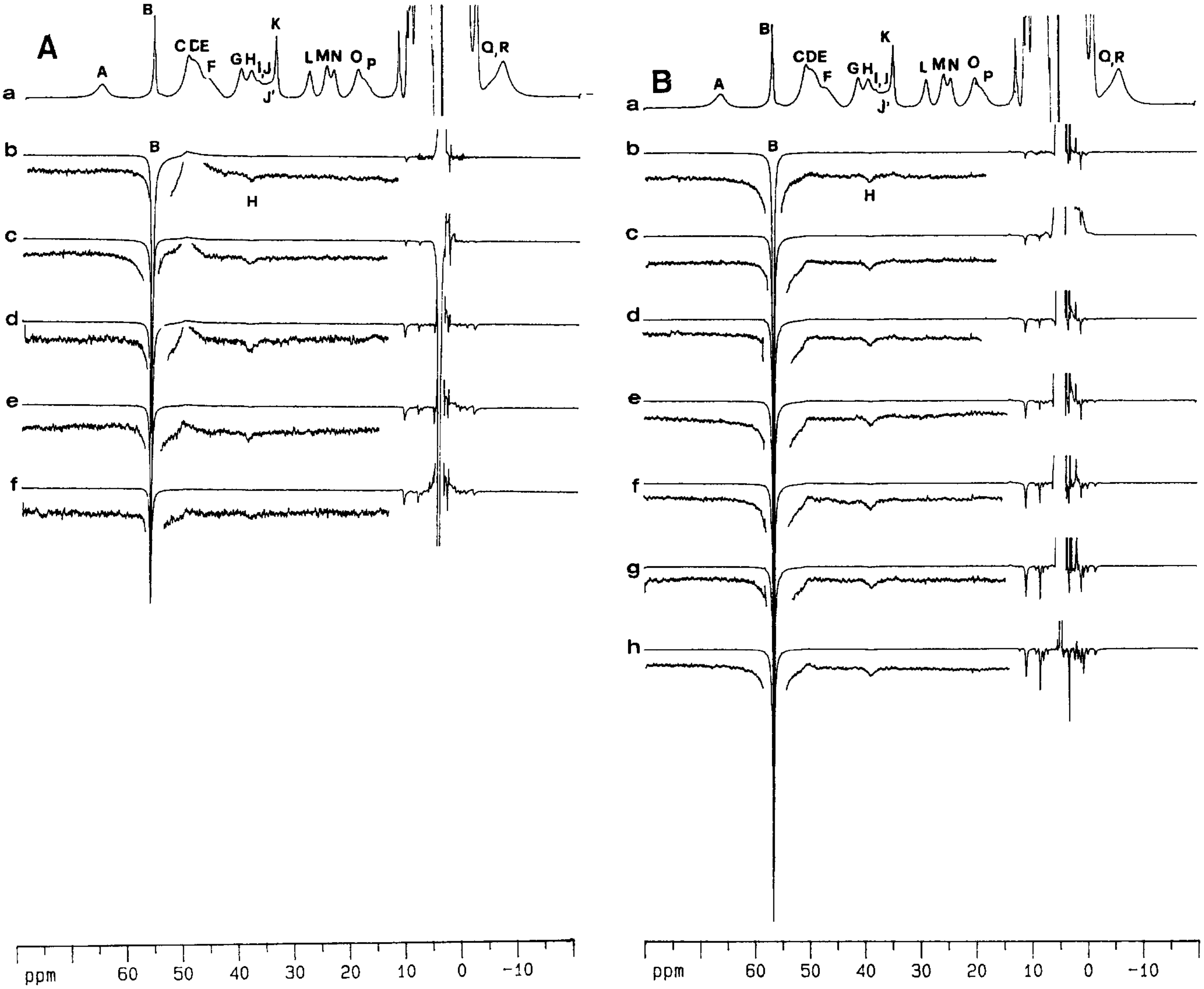

Fig. 3. (A) Transient NOE difference spectra of Cu_2Co_2SOD, obtained upon irradiation of peak B. (a) Reference spectrum of Cu_2Co_2SOD. Traces from (b) to (e) have been recorded by using delay times of 2, 4, 7, 10, 15 ms, respectively. (B) Truncated driven NOE difference spectra of Cu_2Co_2SOD, obtained upon saturation of peak B. Traces from (b) to (h) have been recorded by using a saturation time of 4, 7, 10, 15, 30, 60, 120 ms, respectively. In all cases insets are expanded a factor of ten, in order to appreciate the NOE to signal H. For comparison purposes the same number of scans have been performed in all cases. From truncated NOE experiments it unambiguously appears that, while NOE values in the region +12/−2 ppm increase up to 60 ms irradiation time, NOE to peak *H* reaches a steady state value after about 10 ms. Absolutely, no other signals appear in the region of isotropically shifted signals for long irradiation times, thus indicating that spin diffusion effects are negligible.

Table I

T_1 values, interproton distances inside the active site, observed and calculated steady state NOE values of Cu_2Co_2SOD

Saturated signal	Observed signal	T_1^a (ms)	X-ray distances	Experimental and calculated NOEs^b		
				A	B	C
A (Hδ2 His-63)	K (Hδ1 His-48)	8.0	3.81	0.6 ± 0.2	0.2	(0.2)
A (Hδ His-63)	O (Hεl His-48)	1.9	3.20	0.3 ± 0.1	0.1	(0.1)
A (Hδ2 His-63)	R (Hβ2 His-46)	2.4	3.42	0.1 ± 0.1	0.1	(0.1)
B (Hδ1 His-120)	H (Hε1 His-120)	1.8	2.38	0.9 ± 0.2	0.8	(0.8)
C (Hε2 His-46)	G (Hδ2 His-46)	3.5	2.47	1.3 ± 0.2	1.2	(1.2)
C (Hε2 His-46)	M (Hε1 His-46)	2.7	2.44	0.8 ± 0.2	1.0	(1.0)
G (Hδ2 His-46)	C (Hε2 His-46)	4.2	2.47	1.5 ± 0.4	1.4	(1.4)
K (Hδ1 His-48)	O (Hε1 His-48)	1.9	2.40	0.6 ± 0.2	0.8	(0.8)
L (Hδ2 His-48)	A (Hδ2 His-63)	1.5	2.73	0.3 ± 0.1	0.3	(0.3)
L (Hδ2 His-48)	P (Hβ1 His-46)	1.6	2.27	1.2 ± 0.3	0.9	(0.9)
L (Hδ2 His-48)	R (Hβ2 His-46)	2.4	3.19	0.5 ± 0.1	0.3	(0.3)
M (Hε1 His-46)	C (Hε2 His-46)	4.2	2.44	2.0 ± 0.4	1.5	(1.6)
M (Hεl His-46)	N (Hδ2 His-120)	2.9	2.81	-	0.5	(0.5)
P (Hβ1 His-46)	L (Hδ2 His-48)	4.3	2.27	4.9 ± 1	2.4	(2.4)
P (Hβ1 His-46)	R (Hβ2 His-46)	2.4	1.61	7.7 ± 1.0	9.8	(9.9)
Q (Hβ1 His-71)	D (Hδ2 His-71)	3.8	2.60	1.3 ± 0.2	1.0	-
R (Hβ2 His-46)	G (Hδ2 His-46)	3.5	3.27	0.6 ± 0.2	0.2	(0.4)
R (Hβ2 His-46)	L (Hδ2 His-48)	4.3	3.19	2.0 ± 0.2	0.3	(0.5)
R (Hβ2 His-46)	P (Hβ1 His-46)	1.6	1.61	4.9 ± 0.8	6.8	(6.8)

A: measured at 200 MHz; B, calculated through the two spin system approximation; C: calculated by taking into account all the imidazole protons of the histidines in the active site. Also βCH_2 protons of the histidines coordinated to copper have been considered, together with αCH of His-46 and His-63, βCH2 of Asp-83, γl methyl group of Val-118, γCH2 of Arg-143 together with the exchangeable protons of His-143 ϵNH, η_1NH2 and η_2NH2. The latter are all the protons that have been observed, through a computer graphics analysis, to be at less than 3 Å distances from at least one proton of the imidazole rings.

[a] T_1 for the signal on which NOE is detected, measured at 200 MHz [9]

[b] Experimental data have been collected at 200 MHz [9]. Eqn 2 and 6 have been used, together with the reported T_1 values and $\tau_c = 1.4 \times 10^{-8}$ s

a complete inversion of a signal, the selective 180° pulse must be sizeably shorter than $2/(T_1^{-1} + T_2^{-1})$ [20]. If this condition is not satisfied, coherence is lost during the application of the selective pulse and the effectiveness of the transient experiment decreases. The histidine ring proton signals with largest T_1 and T_2 values in Cu_2Co_2SOD are *B* and *K*. In particular, signal *B* has at 600 MHz a T_1 of 8 ms and T_2 of 1.5 ms. The origin of the large difference between T_1 and T_2 is the well-known phenomenon of Curie spin relaxation [24]. So, for signal *B*, a pulse length sizeably shorter than 2 ms should be used. Of course, the shorter the pulse, the worse the selectivity. The use of shaped pulses generated by a selective excitation unit helps to some extent to improve the selectivity of the pulse.

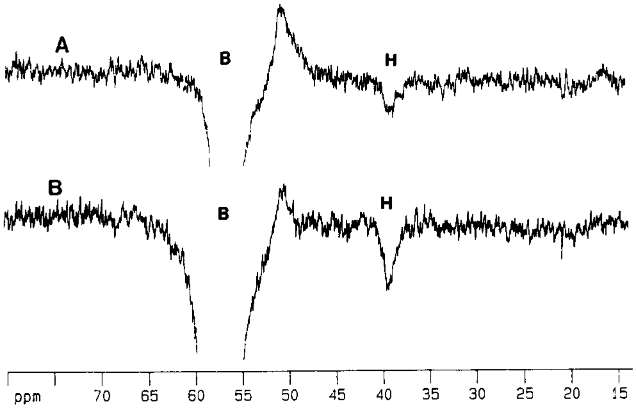

Fig. 4. Comparison of the best transient NOE (A) with the steady state (B) NOE. Spectrum (A) was recorded after 7 ms of delay time. Transient NOE on peak *H* reaches its maximum value at this delay. The two spectra have the same S/N level and are expanded 100 times with respect to the same spectra reported in Fig. 3. The NOE extent is commented in the text.

Fig. 3A reports the transient NOE at 600 MHz which has been performed by applying on signal *B* a selective 180° gaussian pulse of 0.65 ms length obtained through a selective excitation unit; Figure 3B reports the truncated NOEs upon saturation of peak *B* [9]. It can be easily observed that only a small NOE of 1.0% appears on peak *H* which, on the basis of the T_1 value of signal *H* calculated at 600 MHz, accounts for a distance of 2.4 Å. Such distance is indicative of two vicinal protons and agrees with previous assignment based on experiments performed at 200 MHz [9]. No other NOEs have been detected in the region of the isotropically shifted signals up to a 0.1% of sensitivity.

Comparison of steady state data versus transient NOE, reported in Fig. 4, indicates that the steady state NOE is actually larger also in the less favorable conditions, thus leading to greater sensitivity and smaller errors in the calculated distances. This is due to the fact that it is not possible to obtain a complete inversion of signal *B* unless the selectivity of the pulse is lost. The baseline of the difference spectra is already significantly worse in the transient NOE experiment, performed with a 0.65 ms pulse. This is an obvious consequence of the limited selectivity of the pulses which causes spillover effects difficult to eliminate in the difference spectra. Of course, no new nor different NOE is observed in the transient experiment, contrarily to what is recently reported [13].

4. CONCLUSIONS

From the reported data and considerations we state that steady state NOE represents the correct approach in the case of paramagnetic macromolecules in which the NOEs under consideration are small. The application of a selective pulse to fast relaxing signals creates several technical problems. Despite the obvious disadvantages with respect to steady state NOEs, transient experiments, if carefully performed, provide the same results as steady state experiments. Furthermore, it is possible, and easy to do, to take into account spin diffusion and to predict NOE from a fully coupled set of nuclei.

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