Eur Biophys J (1991) 20:269–279

European Biophysics Journal

^{1}H NMR investigation of reduced copper-cobalt superoxide dismutase

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Received May 8, 1991 / Accepted in revised form September 24, 1991

Abstract. Human copper-cobalt superoxide dismutase in the reduced form has been investigated through ^{1}H NMR techniques. The aim is to monitor the structural properties of this derivative and to compare them with those of reduced and oxidized native superoxide dismutases. The observed signals of the cobalt ligands have been assigned as well as the signals of the histidines bound to copper(I). The latter signals experience little pseudocontact shifts which allow a rough orientation of the magnetic susceptibility tensor in the molecular frame. The connectivities indicate that, although the histidine bridge is broken in the reduced form, the interproton distances between ligands of both ions are essentially the same.

Key words: ^{1}H NMR – Metal substitution – Superoxide dismutase

Introduction

Copper, zinc superoxide dismutase (SOD, hereafter) is a well known dimeric enzyme of MW 32 000, each subunit containing one zinc and one copper ion (Fridovich 1983, 1987; Bertini et al. 1990). As far as the copper (II) containing oxidized form is concerned, the investigations range from the X-ray structure (Tainer et al. 1982, 1983), to EPR (Fee and Gaber 1972; Brigg and Fee 1978; Bertini et al. 1988), CD (Pantoliano et al. 1982; Banci et al. 1990b) and EXAFS (Blackburn et al. 1983). The structure revealed the existence of a histidinato bridge between copper and zinc. ^{1}H NMR investigations of the cobalt-copper derivative have been quite helpful in understanding the binding of inhibitors (Bertini et al. 1985a; Banci et al. 1990a, 1988, 1989b). ^{1}H NOE measurements have allowed the monitoring of inter histidine distances which have been found to be consistent with the X-ray structure (Banci et al. 1990a, 1989a, 1991).

Relatively little is known about the reduced enzyme because it contains two d^{10} ions and cannot be investigated through the usual spectroscopic techniques (Mota de Freitas et al. 1990). An EXAFS study has proposed three coordination around copper (I) (Blackburn et al. 1984). Investigation of the $Cu(I)_2Co(II)_2$ derivative revealed three NH's, one for each histidine coordinated to cobalt (II) (Bertini et al. 1985b). This was an evidence that the histidinato bridge between His-63 and copper ion was broken (see Fig. 1). In a recent NMR study on the reduced native enzyme all but one of the histidine protons of both copper and zinc domains were identified and assigned (Bertini et al. 1991). The interproton connectivities pro-

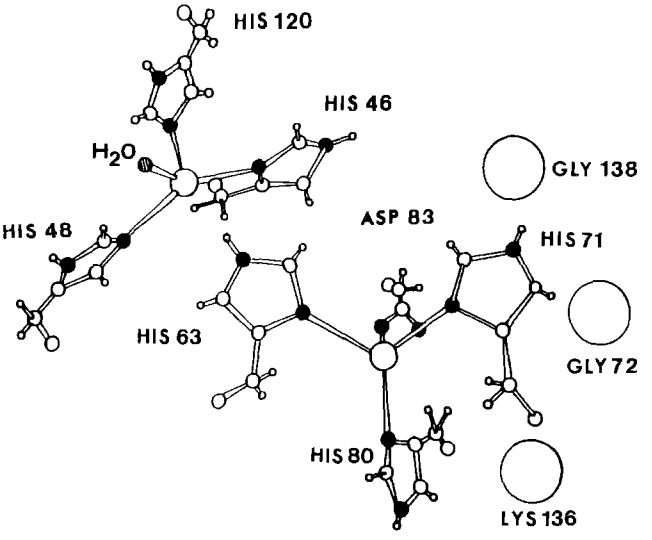

Fig. 1. Schematic view of the active site of $Cu(I)_2Zn(II)_2SOD$ adapted from the X-ray structure of the oxidized form of the bovine enyzme. Cu(I) is on the left hand side and Zn(II) on the right hand side

Abbreviations: WEFT, water eliminated Fourier transform; NOE, nuclear Overhauser effect; NOESY, NOE spectroscopy; COSY, correlation spectroscopy; TOCSY, total correlation spectroscopy; SOD, superoxide dismutase; $E_2Co(II)SOD$, SOD with empty copper site (E = empty) and with cobalt(II) in the Zinc(II) site

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vided a structure very similar to that of the oxidized form, although the imidazole moiety which is bridging the metal ion in the oxidized form is now protonated and possibly tilted away from the original position.

We have now extended the investigation to the $Cu(I)_2Co(II)_2$ derivative with the aim of *1*) assigning the hyperfine shifted signals of the cobalt (II) ligands; *2*) measuring pseudocontact shifted signals of the protons of copper (I) ligands in order to use cobalt (II) as a probe to obtain structural information on the copper domain; *3*) revealing connectivities through 1D and 2D NMR between protons which sense the paramagnetic center to different extents. From these measurements further independent information on the structure of the cobalt substituted reduced enzyme are obtained.

Together with the $Cu(I)_2Co(II)_2$ derivative we have investigated the $Ag(I)_2Co(II)_2$ species in order to complete the picture of the structure of the metal sites when the copper site is occupied by a different metal ion.

Experimental part

Human SOD, obtained from human SOD gene expressed in yeast (Beyer et al. 1987), was a gift of Dr. R. A. Hallewell of Chiron Corporation, Emeryville, USA. Selectively deuteriated SOD at the $\varepsilon 1$ position of histidines was prepared as reported elsewhere (Banci et al. 1990c). The reduced form of the enzyme, $Cu(I)_2Zn(II)_2$SOD was obtained upon addition of solid sodium dithionite to the native enzyme in 10 mM phosphate buffer at pH 5.0 (Cass et al. 1977; Lippard et al. 1977). The metal substituted derivatives were obtained from the apoenzyme following an already reported procedure (Valentine and Pantoliano, 1982). Metal ions were removed from SOD through dialysis against 10 mM EDTA in 50 mM acetate buffer at pH 3.8 (McCord and Fridovich 1969). The excess of chelating agent was removed through dialysis against 100 mM NaCl in 50 mM acetate buffer at pH 3.8, and then against 50 mM acetate buffer (Forman et al. 1973). The $Cu(II)_2Co(II)_2$ derivative was obtained through addition of a stoichiometric amount of Co(II) and then Cu(II) to the apoenzyme at pH 5.5. Metal incorporation was followed spectrophotometrically (Fee 1973). The buffer was changed through dialysis against 50 mM phosphate, pH 5.5 and the derivative was reduced to $Cu(I)_2Co(II)_2$ SOD through addition of sodium dithionite (Bertini et al. 1985b) as reported for $Cu(I)_2Zn(II)_2$SOD (Cass et al. 1977; Lippard et al. 1977).

The $Ag(I)_2Zn(II)_2$ and $Ag(I)_2Co(II)_2$ derivatives were prepared through slow addition of stoichiometric amounts of an aqueous solution of 9 mM $AgNO_3$ to the apoprotein (0.5 mM) at pH 5.0 in 50 mM sodium phosphate (Beem et al. 1977). The buffer was then changed by concentrating the sample by ultrafiltration and diluting it with 25 mM potassium phosphate at pH 7.8, and two equivalents of Zn(II) or Co(II) were added. The pH was then decreased to 5.5 and 5.0, respectively.

^{1}H NMR experiments were performed on a Bruker MSL 200, Bruker AC 200, Bruker AC 300 and a Bruker AMX 600. The spectra reporting the isotropically shifted signals have been recorded at 200 MHz over a 50 kHz bandwidth by using a super WEFT pulse sequence (Inubushi and Becker 1983). The spectral region between +15 and −5 ppm has been investigated at 600 MHz by using a presaturation pulse sequence. Alternatively either a 1-1 echo-sequence (Sklenar and Bax 1987) or a binomial pulse sequence 1-$\bar{3}$-3-$\bar{1}$ (Hore 1983) were used in order not to excite water protons. ^{1}H NOE experiments have been performed by using either the super WEFT or the 1-$\bar{3}$-3-$\bar{1}$ pulse sequence, and they have been collected using the previously reported methodology (Banci et al. 1990a, 1989a, 1990c; Ramaprasad et al. 1984; Unger et al. 1985). T_1 values have been calculated by using the inversion recovery method (Bertini and Luchinat 1986). 2D experiments have been performed at 600 MHz over a 25 ppm spectral region. NOESY spectra have been recorded using mixing times of 100–150 ms. They have been collected in the phase sensitive mode using the time proportional phase increment method (TPPI) (Marion and Wutrich 1983). 512 FIDs were collected using 2K data points each. Zero filling in the F1 dimension was applied in order to obtain a 2K × 1K 2D data point matrix. COSY spectra have been recorded in Magnitude mode (Aue et al. 1976). 128 FIDs have been collected over 1K data points. Zero filling in the F1 dimension was such as to obtain a 1K × 1K or 512 × 512 symmetrized data points matrix. In all cases presaturation pulses have been applied during both relaxation delay and mixing time to suppress the solvent peak.

Results and discussion

Table 1 summarizes the assignment of the signals for the $Cu(I)_2Co(II)_2$ derivative, together with those of $Cu(II)_2Co(II)_2$SOD and $Cu(I)_2Zn(II)_2$. Such assignment has been proposed on the basis of the experimental data reported and discussed below.

Cobalt-coordinated residues

The ^{1}H NMR spectrum of the $Cu(I)_2Co(II)_2$ derivatives at pH 5.5 at 200 MHz and 298 K is reported in Fig. 2 together with that of $Ag(I)_2Co(II)_2$SOD. When the experiments are performed in D_2O eight resonances are detected in the region between +70 and +15 ppm. They reasonably belong to residues coordinated to Co(II). The spectra of the two derivatives show minor differences in this region. A broad resonance, signal F, is observed in the $Ag(I)_2CO(II)_2$ derivative. This resonance is unresolved in the case of $Cu(I)_2Co(II)_2$SOD, but can be detected through NOE experiments (see later). When the derivatives are dissolved in D_2O (Fig. 2b and 2d), resonances A, C and G disappear; they can be thus assigned to the three NH protons of the histidines coordinated to cobalt (II) (Bertini et al. 1985b). The pH dependence of the spectrum performed on the bovine isoenzyme (Fig. 3) indicates that peaks A and G decrease in intensity and disappear around neutral pH, while no substantial decrease in the intensity of peak C is detected up to pH 9.5.

Table 1. Chemicals shifts[a], T_1 values[b] and proposed assignment for signals of the ^{1}H NMR spectrum of $Cu(I)_2Co(II)_2SOD$, $Cu(II)_2Co(II)_2SOD$, $Cu(I)_2Zn(II)_2SOD$ at 300 K at pH 5.5

Signal	$Cu(I)_2Co(II)_2SOD$			$Cu(II)_2Co(II)_2$	$Cu(I)_2Zn(II)_2$
	δ (ppm)	T_1 (ms)	Proposed Assignment	δ (ppm)	δ (ppm)
A	63.2[c]	1.4	Hε2 *His*-63	–	12.44[h]
B	55.5[c]	1.8	Hδ2 *His*-71	49.4[g]	6.82[h]
C	49.4[c]	2.4	Hε2 *His*-71	46.7 or 35.4[g]	15.40[h]
D	48.7[c]	2.4	Hδ2 *His*-80	48.8[g]	6.86[h]
E	45.5[c]	1.5	Hδ2 *His*-63	66.2[g]	6.05[h]
F	44[c]	$\leq$1.5	Hβ *Asp*-83	37.4 or 35.6[g]	
G	38.8[c]	1.3	Hε2 *His*-80	35.4 or 46.7[g]	12.70[h]
H	33.2[c]	1.1	Hβ *Asp*-83	35.6 or 37.4[g]	
I	14.07[cd]	80	Hε2 *His*-43	14.07[d]	14.07[h]
K	12.90[cd]	220	Hδ1 *His*-43	12.90[d]	12.90[h]
L	12.24[c]	40	Hδ1 *His*-48	34.5[g]	12.54[h]
M	11.02[c]	50	Hα2 *Gly*-138	–	4.18[f]
N	10.44[c]		Hα1 *Gly*-138	–	4.08[f]
Z	– 6.4[c]	1.4	Hβ2 *His*-71	– 6.2[g]	
1	8.60[cdef]		Hε1 *His*-43		8.61[h]
2	8.58[cde]		Hε1 *His*-48		8.54[h]
3	8.55[c]		HN *Gly*-72(*Lys*-136)		
4	8.54[de]		Hε1 *His*-110		8.52[h]
5	8.35[de]		Hε1 *His*-120	39.0[g]	8.29[h]
6	7.14[cde]		Hδ2 *His*-43		7.14[h]
7	5.90[c]		Hδ2 *His*-48		
8	3.30[c]		Hα1 *Gly*-72		
9	2.84[cf]		Hβ2 *His*-48	12.52[c]	3.26[cf]
10	2.56[f]		Hβ1 *His*-48	6.35[c]	3.13[f]

[a] Chemical shift values are given with 2 or 3 or 4 figures according to their linewidth
[b] T_1 measurements of the isotropically shifted signals are performed at 200 MHz; in the other cases they are performed at 600 MHz
[c] assigned on the basis of ^{1}H NOE experiments and X-ray data
[d] assigned through comparison with the spectrum of $Cu(I)_2Zn(II)_2SOD$
[e] assigned through comparison between the spectra of $Cu(I)_2Co(II)_2SOD$ and selectively deuterated $Cu(II)_2Co(II)_2SOD$
[f] assigned from NOESY spectrum in D_2O
[g] from Banci et al. (1989a)
[h] from Bertini et al. (1991)

In the case of $Ag(I)_2Co(II)_2SOD$ signal A starts disappearing at pH around 5.0 and is competely lost at pH 5.5 (data not shown).

The remaining five resonances are expected to arise from three meta-like Hδ2 histidine protons and two βCH_2 protons from Asp-83. Indeed, previously reported data on both $Cu(II)_2Co(II)_2$ and $E_2Co(II)_2$ derivatives selectively deuterated at the ε1 position of the histidine residues indicated that the Hε1 resonances are broadened beyond detection (Banci et al. 1990c). A spectrum of a $Cu(I)_2Co(II)_2$ derivative selectively deuterated at the ε1 position of the histidine residues in D_2O solution is also reported in Fig. 2e. It appears that all the five non-exchangeable signals in the downfield region are maintained, and we conclude that also in this derivative the Hε1 histidine protons are broadened beyond detection.

Figure 4 shows the ^{1}H NOE difference spectra obtained upon saturation of several hyperfine shifted peaks in the $Cu(I)_2Co_2SOD$ derivative. Peak A gives rise to a NOE of 1.9% with peak E (Fig. 4b). The calculated interproton distance from the T_1 of signal E is consistent with A and E being ortho to one another on a histidine ring, A being the exchangeable Hε2 and E being the Hδ2 proton. A 10% NOE to peak F has been obtained upon saturation of resonance H (Fig. 4c). The same connectivity has been confirmed by saturating the pair of signals E and F (Fig. 4d). Although the T_1 of signal F could not be measured accurately, it can be estimated to be $\leq$1.5 ms. Using such an estimate the NOE observed is consistent with the assignment of F and H, both no exchangeable, as βCH_2 protons of Asp-83. An analogous conclusion had been previously reached in the E_2Co_2SOD derivative (Banci et al. 1990c). Saturation of signal G, another exchangeable Hε2 histidine proton, has been performed with the aim of finding the corresponding Hδ2 signal. Signals B and D are the only candidates. To discriminate between them we ran two different NOE experiments using the off-resonance irradiation symmetrical to either B or D. While in the first experiment no evidence was found for NOE to B (Fig. 4e), in the second experiment a small but reliable NOE to peak D was detected (Fig. 4f). From these experiments dipolar connectivity between D and G is established; the NOE extent has been estimated to be about 1.2%, again consistent with the expected interproton distance. Through the same technique signal C, the third exchangeable Hε2 histidine proton, has been irradiated in order to check that signal B belongs to the corresponding Hδ2 proton: the off-resonance irradiation has been set symmetrical to signal B (Fig. 4g) and a small NOE has been detected to peak B. As a consequence, signals B and C are confirmed to belong to the same histidine.

Other histidines

The diamagnetic region of the ^{1}H NMR spectra in D_2O of $Cu(I)_2Co(II)_2SOD$, is reported in Fig. 5; the inset shows the diamagnetic region where the Hε1 histidine protons are expected to fall for the spectra of $Cu(I)_2Co(II)_2SOD$ and $Cu(I)_2Co(II)_2SOD$ selectively deuterated at Hε1 together with the same region of the spectrum of $Cu(I)_2Zn(II)_2SOD$. Five Hε1 resonances are expected to be observed in the $Cu(I)_2Co(II)_2$ derivative of

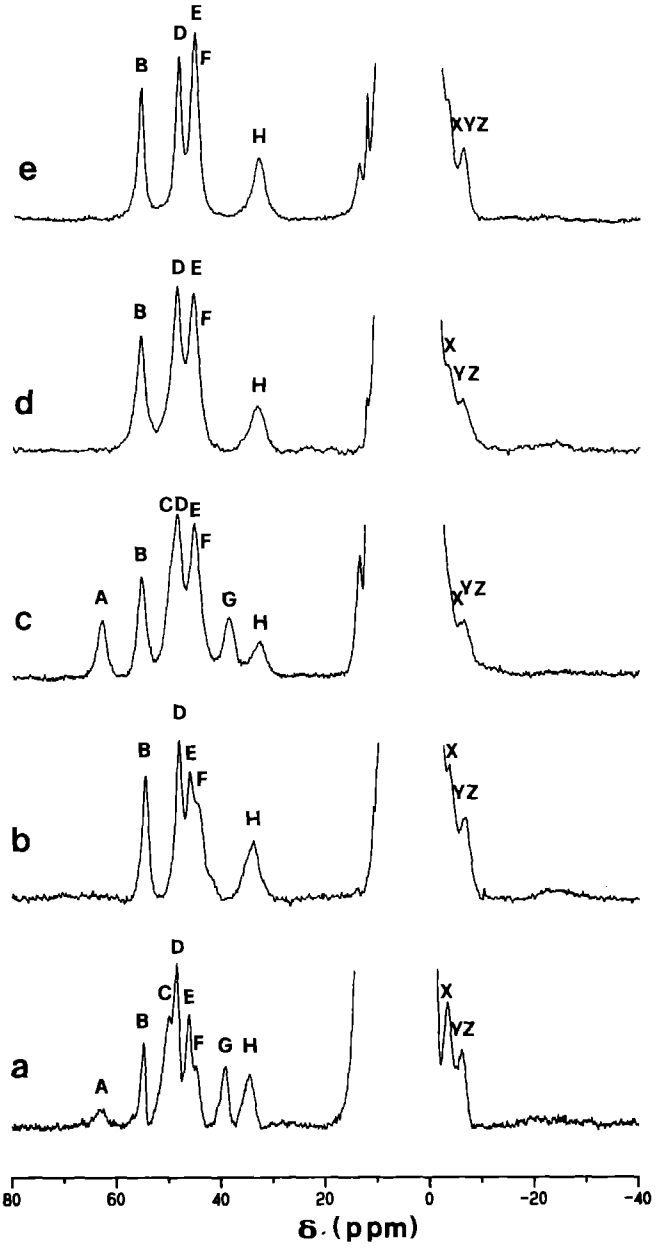


Fig. 2. 200 MHz, 300 K, ^{1}H NMR spectra of the isotropically shifted region of $Ag(I)_2Co(II)_2SOD$ in H_2O (**a**) and D_2O (**b**), together with those of $Cu(I)_2Co(II)_2SOD$ in H_2O (**c**) and D_2O (**d**). The samples are in 50 mM phosphate buffer. Samples **a** and **b** are at pH 5.0, samples **c** and **d** are at pH 5.5. The spectrum in D_2O, at pH 5.5, of $Cu(I)_2Co(II)_2SOD$ selectively deuterated at the ε1 position of the histidine residues is also reported (**e**)

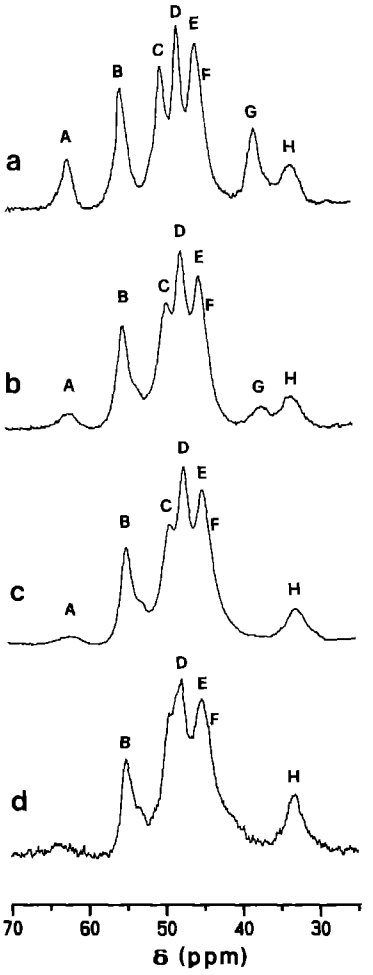


Fig. 3. 200 MHz, 300 K ^{1}H NMR spectra of the isotropically shifted region of $Cu(I)_2Co(II)_2SOD$ at pH 5.0 (**a**), at pH 6.4 (**b**), at pH 6.8 (**c**), at pH 9.5 (**d**) in 50 mM phosphate buffer

the eight observed in the reduced native enzyme, because, as already pointed out, the three Hε1 protons of the histidines coordinated to cobalt are expected to be isotropically shifted and broadened beyond detection. Four of the five signals are marked with asterisks in the diamagnetic derivative, the fifth being in a crowded region of the spectrum (Bertini et al. 1991). Signals 1, 2, 4 and 5 disappear in the selectively deuterated sample. 2D experiments have confirmed the assignment of signal 1 as an Hε1 proton. Indeed, from NOESY experiments in D_2O on $Cu(I)_2Co(II)_2SOD$ and $Cu(I)_2Co(II)_2SOD$ selectively deuterated at Hε1 we observe in the latter case the absence of all the cross peaks of signal 1 which unambiguously confirms the assignment of signal 1 as an Hε1 proton (Fig. 6). The connectivity between signal 1 and the signal at 7.14 ppm, labelled 6, can be assigned to an Hε1–Hδ2 connectivity. Therefore four of the five Hε1 protons from histidines not coordinated to cobalt(II) are identified. Detection of the fifth signal, expected at about 6.8 ppm and assigned to *His*-46 Hε1 in the $Cu(I)_2Zn(II)_2$ derivative, is possibly hampered by the many aromatic signals present in that region or by a dipolar shift contribution from the cobalt(II) ion, or both. Hε1 of *His*-46 is the closest to cobalt(II) among the non-cobalt coordinated histidine protons.

The spectra in the imidazole NH region (15–10 ppm) of $Cu(I)_2Zn(II)_2SOD$, $Ag(I)_2Co(II)_2SOD$, $E_2Co(II)_2$-SOD and $Cu(I)_2Co(II)_2SOD$ in H_2O are reported in Fig. 7 (from a to d, respectively). The spectrum of $Cu(I)_2Zn(II)_2SOD$ (Fig. 7a) has been already published (Cass et al. 1977; Lippard et al. 1977; Bertini et al. 1991) and it is reported for comparison purposes. All the signals of Fig. 7 disappear when the derivatives are dissolved in D_2O solutions except signal M in trace a, b and d. The three signals a, e and g in the spectrum of $Cu(I)_2Zn(II)_2SOD$ have been already assigned to NHε2

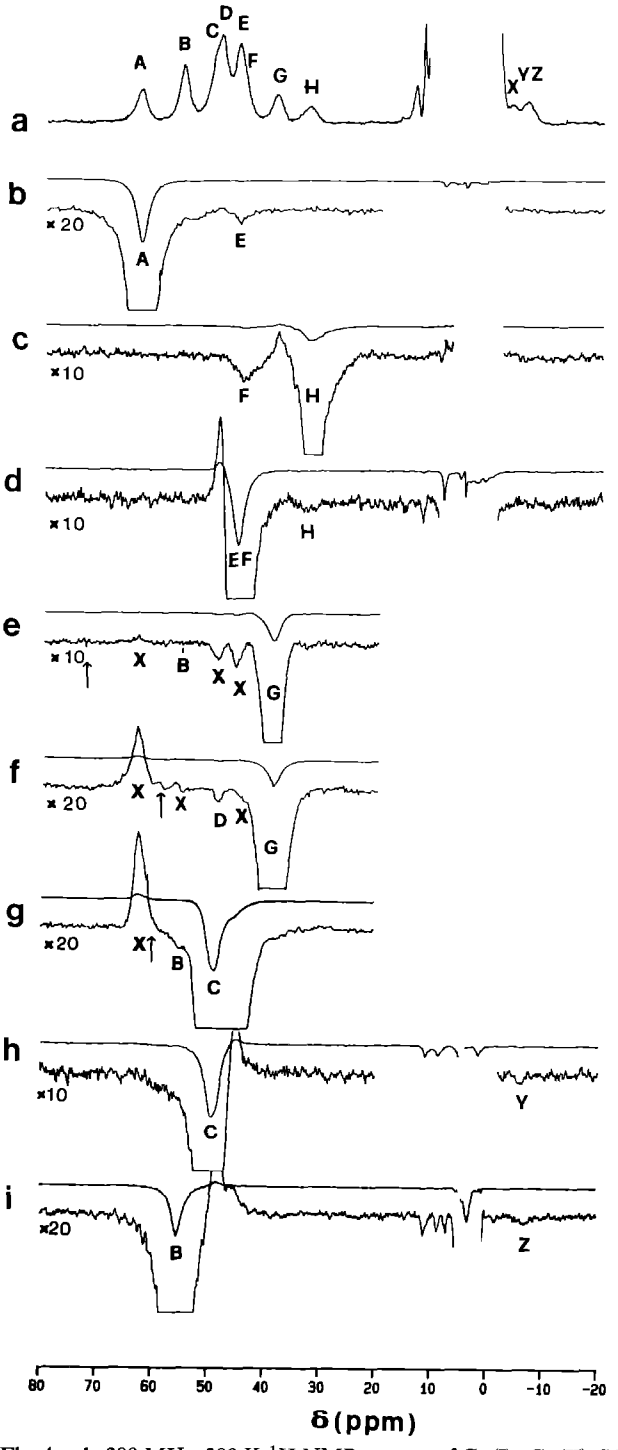


Fig. 4a–i. 200 MHz, 300 K ^{1}H NMR spectra of $Cu(I)_2$ $Co(II)_2SOD$ in H_2O: **a** reference spectrum; **b–h** steady-state NOE difference spectra obtained by saturating peaks A (**b**), H (**c**), E–F (**d**), G (**e** and **f**), C (**g** and **h**), B (**i**). Signals G and C have been saturated in two different condition (see text). The lower trace for each difference spectrum are vertically expanded as reported in the figure. Crosses indicate off resonances effects; arrows indicate the positions of the off resonance saturating pulse. The sample is in 50 mM phosphate buffer at pH 5.5

imidazole protons of *His*-71, *His*-80 and *His*-63, respectively (Stoesz et al. 1979; Bertini et al. 1991). Since these residues are coordinated to the zinc(II) ion, the corresponding signals in the cobalt(II) substituted derivatives have been already identified in the far downfield region.

On the other hand, little or no change is expected to occur upon cobalt substitution in the shift of NH signals far from the active site. The two NH protons of *His*-43 (not coordinated to any metal and far from the cobalt site), which had been assigned to peak b and d in the $Cu(I)_2Zn(II)_2SOD$ derivative, should not change their chemical shifts upon cobalt(II) substitution. Accordingly, signals I and K of the cobalt substituted derivatives are assigned to NHε2 and NHδ1 protons of *His*-43, respectively.

NOE experiments have been performed on signals I and K. Whenever NOE experiments involved signals in the diamagnetic region series of experiments at different irradiation times (build-up profiles) have been performed, in order to monitor and minimize spin-diffusion effects. Two major NOEs (8.60 and 7.14 ppm) were detected by saturating I, and one (8.60 ppm) by saturating K (Fig. 8). The NOE on the signal at 8.60 ppm is common to both experiments. This signal is already assigned as histidine Hε1 from selective deuteriation (signal 1 in Fig. 5). The relative NOE extent allows us to assign the signal at 7.14 ppm as Hδ2 of *His*-43. The data are consistent from both qualitative and quantitative point of view with experiment performed on the $Cu(I)_2Zn(II)_2SOD$ derivative under the same experimental conditions.

Saturation of peak L has been performed with irradiation times of 16 and 96 ms (Fig. 9). Two primary NOEs at 8.58 ppm and 2.84 ppm are observed. The former signal (signal 2) is an Hε1 proton as independently confirmed (Fig. 5). The absence of connectivity with another aromatic signal might indicate that the two protons corresponding to signals L and 2 belong to either *His*-120 or 48, since they are bound to the metal through Nε2 (see Fig. 1).

Another broad exchangeable proton, signal J, appear under signal I when slow relaxing protons are suppressed by fast acquisition (Fig. 7e). This signal could be assigned to Hε2 of *His*-46, which is the copper coordinated histidine closest to the cobalt ion (5.3 Å vs. 7.5 Å of Hε2 of *His*-48), or to a peptide NH proton in the vicinity of the cobalt site. Saturation of signal J cannot be performed without strongly affecting signals I, K, L. Therefore we cannot discriminate between the two possibilites.

Among the other Hε1 signals (Fig. 5), signal 5, at 8.35 ppm, can be assigned to Hε1 *His*-120 by simple comparison with the reduced native system in which Hε1 *His*-120 has been detected at 8.29 ppm. Owing to the large metal-to-proton distances Hε1 of *His*-120 is expected to be only slightly affected by cobalt substitution. Since signal 1 is already assigned to *His*-43, signals 2 and 4 most probably belong to *His*-48 and 110, respectively. The NH proton of the latter was unobserved also in the reduced native enzyme because of fast exchange with the bulk solvent (Bertini et al. 1991).

NOEs from the hyperfine shifted signals

Figure 10 shows the NOEs obtained in the diamagnetic region by irradiation of the hyperfine shifted signals. By

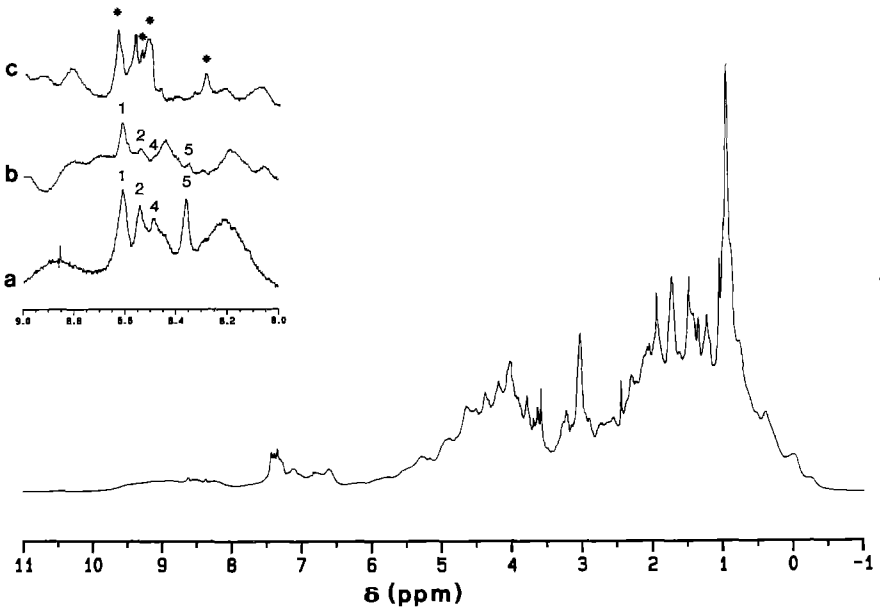
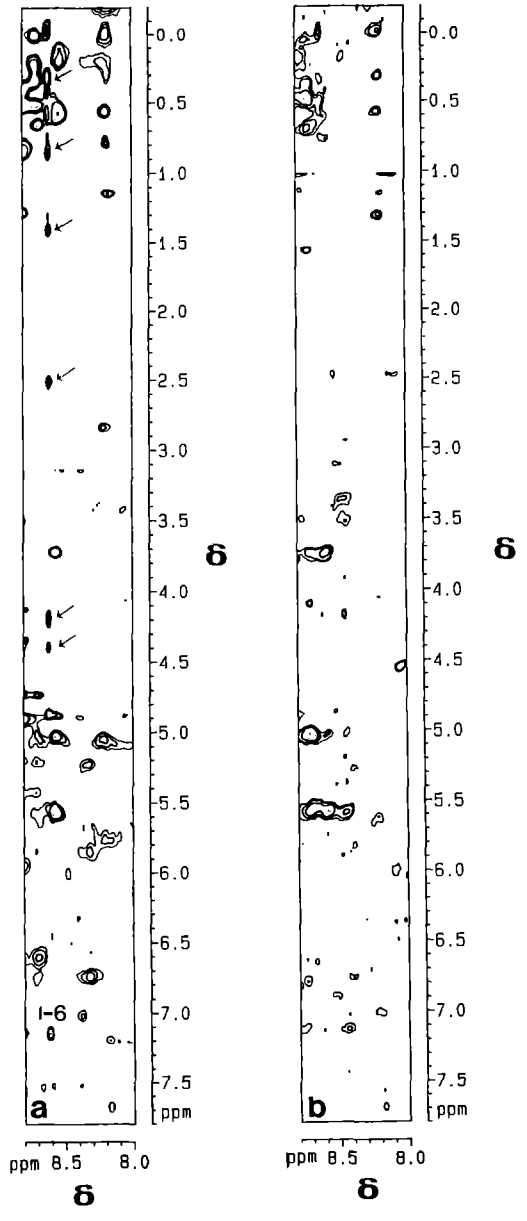


Fig. 5. Diamagnetic region of the 600 MHz ^{1}H NMR spectrum of $Cu(I)_2Co(II)_2SOD$ in 50 mM phosphate buffer, at 300 K. In the inset the expanded region between 9.0 and 8.0 ppm for $Cu(I)_2Co(II)_2SOD$ (**a**), selectively deuterated $Cu(I)_2Co(II)_2SOD$ (**b**) and $Cu(I)_2Zn(II)_2SOD$ (**c**) are reported



irradiating signal A an NOE on signal 2 is observed together with an NOE on a signal at 8.21 ppm (Fig. 10b). Irradiation of signal E gives NOE to signal 2 as well as to signal L (Fig. 10c and d). The connectivity between signals 2 and L has already been discussed. This is a clear correlation between the ring signals of a cobalt-coordinated histidine (signals A and E) and those of another histidine not coordinated to the cobalt ion (signals L and 2). The structural data available point to *His*-63 and *His*-48 as the only possible candidates. Signal A is thus assigned as *His*-63 Hε2, E as *His*-63 Hδ2, L as *His*-48 Hδ1 and 2 as *His*-48 Hε1. The signal at 2.84 ppm (signal 9) experiencing NOE upon saturation of L could be assigned as the Hβ2 proton of the same histidine, consistent with the structural data. A NOESY experiment has allowed us to detect its partner at 2.57 ppm (signal 10) (Fig. 11).

Besides the already described NOEs, signal E also gives an NOE at 5.90 ppm. The NOE is better detected when the experiment is performed in D_2O (Fig. 10d). A possible assignment for this resonance would be the Hδ2 of *His*-48

Saturation of peak D in D_2O solution yields other NOEs in the region between 1 and 2 ppm and in the region between 9 and 10 ppm (Fig. 10e) while saturation of peak G gives rise to NOEs in the diamagnetic region, at 8.76, 2.07 and 0.95 ppm (Fig. 10f).

By saturating signal B for about 60 ms several NOEs have been observed (Fig. 10g). Two large NOEs have been detected at 8.55 and 3.30 ppm of about 14% and 15%

Fig. 6 a, b. Contour plot of the region between 8.8–8.0 ppm and 7.8–−0.2 ppm, in which the connectivities of the Hε1 protons are expected to appear, of the NOESY spectrum recorded at 600 MHz and 300 K of $Cu(I)_2Co(II)_2SOD$ (**a**) and $Cu(I)_2Co(II)_2SOD$ selectively deuterated at Hε1 position (**b**). The cross peaks indicated with the arrows and the connectivity 1–6 belong to the same column of spectrum (**a**); they are missing in the selectively deuterated sample. Samples are in 50 mM phosphate buffer at pH 5.5, in D_2O

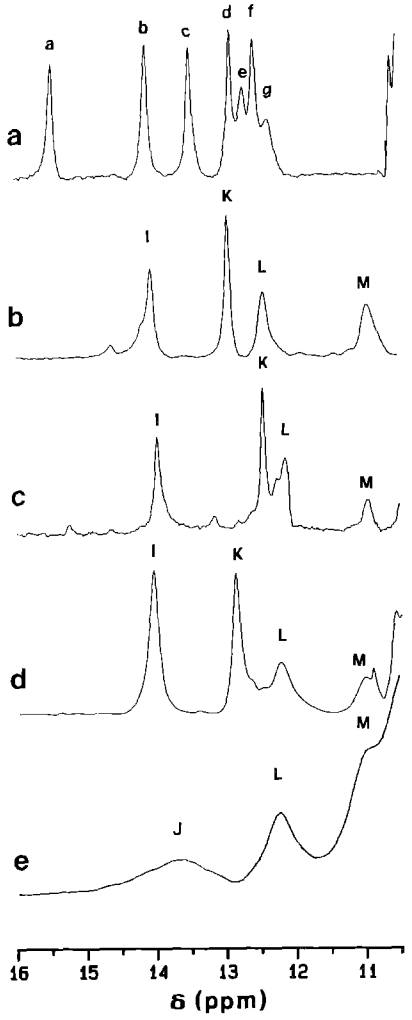


Fig. 7a–e. 300 K, ^{1}H NMR spectra in H_2O of the region between 16.0 and 10.5 ppm of $Cu(I)_2Zn(II)_2SOD$ (**a**), $Ag(I)_2Co(II)_2SOD$ (**b**), $E_2Co(II)_2SOD$ (**c**), $Cu(I)_2Co(II)_2SOD$ (**d**, **e**). The samples **a**, **c**, **d**, **e** are in phosphate buffer, at pH 5.5. Sample **b** is at pH 5.0. Spectra **a**–**d** are recorded at 600 MHz with 1 s of relaxtion delay, spectrum **e** is recorded at 200 MHz using 82 ms of relaxation delay

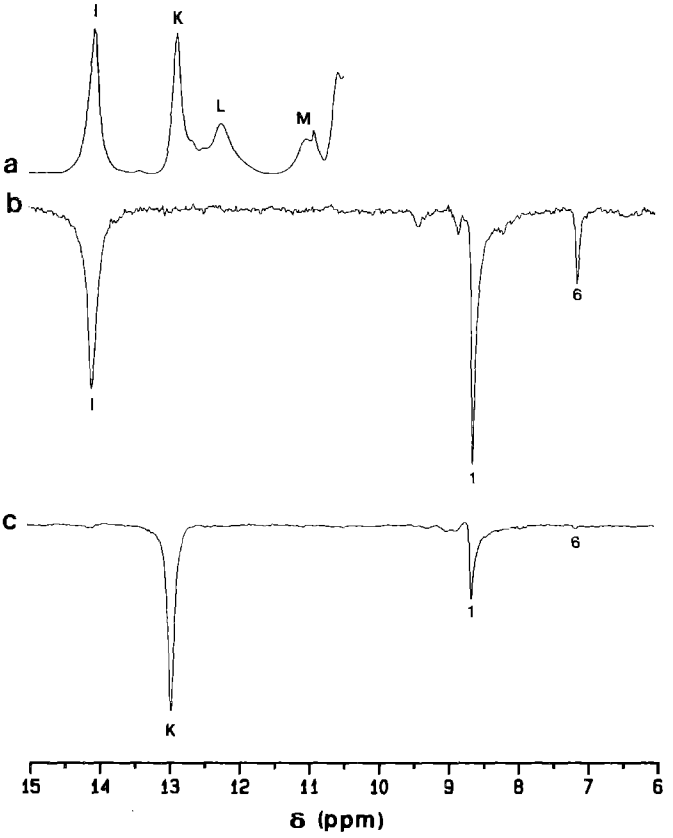


Fig. 8a–c. 600 MHz, 300 K, ^{1}H NMR spectra between 16.0 and 10.5 ppm, in H_2O at 303 K, of $Cu(I)_2Co(II)_2SOD$. Reference spectrum in the region of the irradiated signals (**a**); NOE difference spectrum obtained by saturating peak I (**b**); NOE difference spectrum obtained by saturating peak K (**c**). The sample is in 50 mM phosphate buffer at pH 5.5

respectively (signals 3 and 8), the latter possibly due to several overlapped resonances; other NOEs have been observed at 1.13 ppm and on a signal at 6.95 ppm. 1% NOEs have also been detected on signal Z at −6.4 ppm (Fig. 4i) and on signal M at 11.02 ppm. The repetition of the same experiment in D_2O solution confirm all the previous observed NOEs except that on signal 3 which drastically reduces in intensity. This accounts for the assignment of signal 3 as an exchangeable NH peptide proton. Repetition of the experiment at shorter irradiation time allows us to confirm that both 8.55 and 3.30 ppm are primary NOEs. Signal 3 can be tentatively assigned from X-ray data to the Hα1 proton of *Gly*-72.

Saturation of peak C also yields several NOEs (Fig. 10h). Two NOEs, both of 5%, have been observed to peak M, at 11.02 ppm, which also undergoes NOE of a smaller extent upon saturation of B, and to a peak at 8.76 ppm, under 60 ms of selective saturation on peak C. Furthermore NOEs are detected to a signal at 10.44 ppm (signal N), to a broad resonance at 8.10 ppm and to a peak at 1.50 ppm. Also in this case the NOEs extent at short saturation period allows one to better observe the cross relaxation which only depends on the proton-proton distance. At 10 ms of irradiation time the NOE on signal M appears to be the largest among the previously observed NOEs which can be detected in Fig. 10h. Signal N and the signal at 1.50 ppm also appear to arise from primary NOEs. Furthermore, another signal appears at 4.10 ppm, which at larger irradiation time is undetectable owing to the less satisfactory suppression of water signal. On the other hand, the repetition of the experiments on signal C performed by varying the offset between the on resonance and the off resonance irradiation, together with the comparison with Fig. 10e, allow us to distinguish the peak which, in Fig. 10h, are due to the saturation of peak D, saturation that occurs when C is saturated. Under this condition the NOE to peak M, N and signals at 8.10 and 4.10 ppm are unambigously due to the saturation of C, while the NOE at 8.76 and, at least partially, the NOE at 1.50 arise from saturation of D. Finally, a small NOE is detected at −6.1 ppm (signal Y in Fig. 4h). Such NOE is not observed when signal D is saturated in D_2O, i.e. in the absence of signal C.

From the observed NOEs from signals B and C on the one hand and D and G on the other, no clearcut evidence is gained to discriminate between *His*-71 and *His*-80.

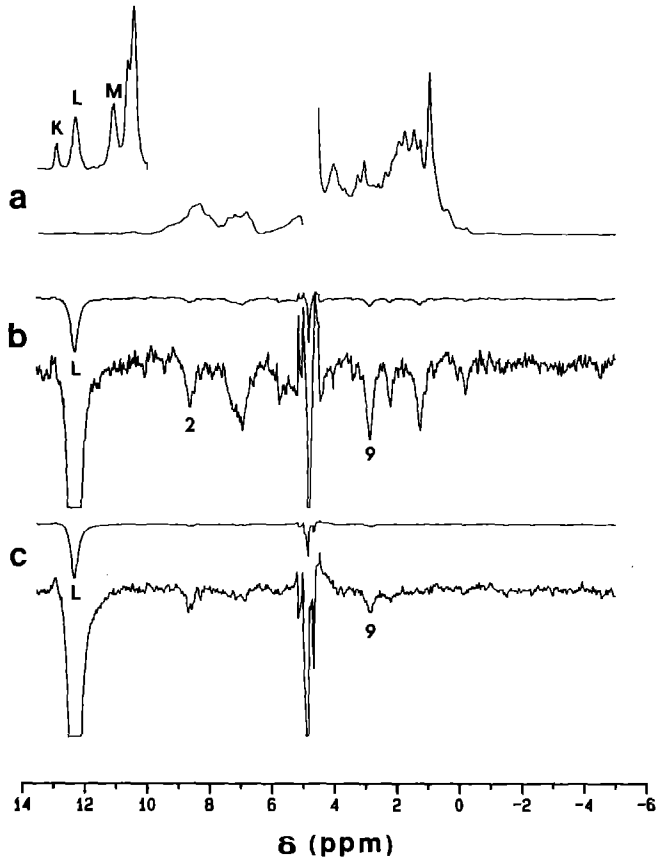


Fig. 9a–c. 300 MHz, 300 K, ^1H NMR spectrum in H_2O of $Cu(I)_2Co(II)_2SOD$ between 13.5 and −5.0 ppm: reference spectrum (**a**); NOE difference spectra obtained by saturating signal L with different irradiation times: 96 ms (**b**), 16 ms (**c**). The lower trace for each difference spectrum are expanded a factor of ten. The sample is in 50 mM phosphate buffer at pH 5.5

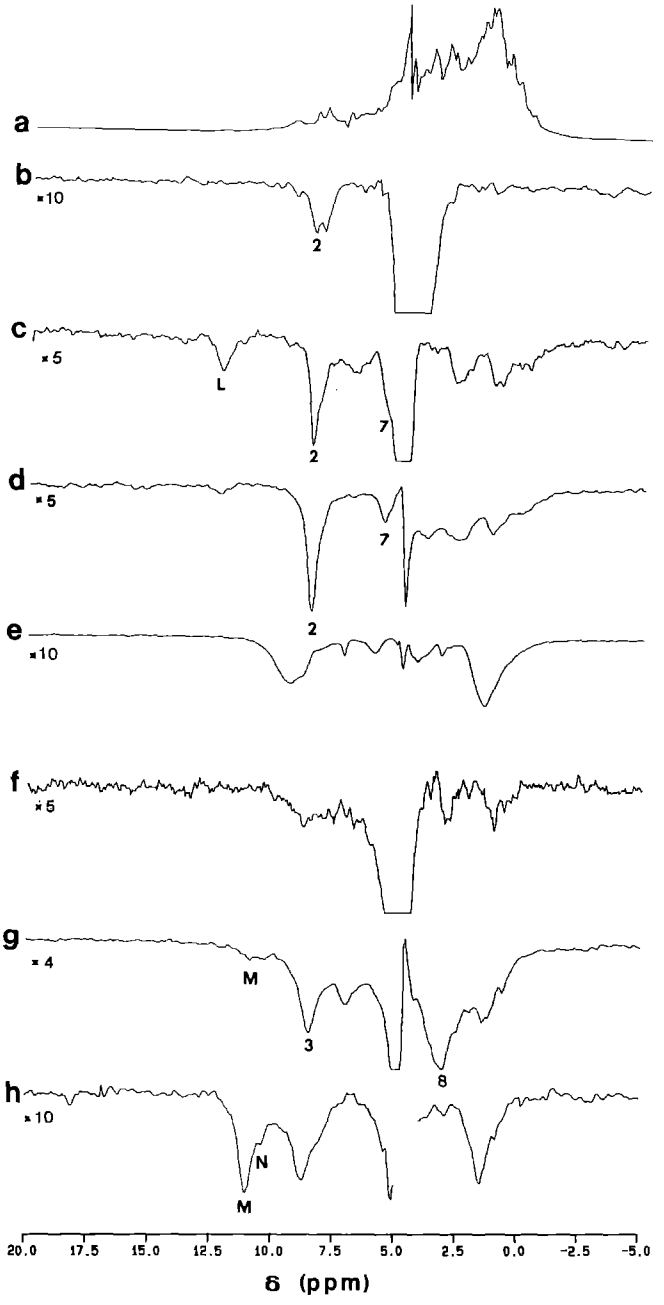


Fig. 10a–h. 200 MHz, 300 K, ^1H NMR spectrum in H_2O of $Cu(I)_2Co(II)_2SOD$ between 20 and −5 ppm. Reference spectrum (**a**); NOE difference spectrum obtained by saturating peak A (**b**), peak E in H_2O (**c**) and D_2O (**d**), peak D (**e**), peak G (**f**), peak B (**g**), peak C (**h**). The lower trace for each difference spectrum are expanded as reported in the figure

However, we can tentatively assign signals B and C to *His*-71 and signals D and G to *His*-80, on the basis of the following considerations: the X-ray structure indicates that *i*) Hδ2 of *His*-71 and Hβ2 of the same histidine are 2.8 Å apart and they are expected to give rise to an NOE. The NOE observed between signal B and signal Z accounts for this proton-proton connectivity. No other βCH_2 of cobalt coordinated histidines is so close to the ring proton. Furthermore, Hβ2 of *His*-71 is the farthest from the metal among the four βCH_2 protons of *His*-71 and *His*-80, and therefore supposedly better observable. The assignment of Hβ2 of *His*-71 as the signal at −6.4 ppm is in agreement with the analogous assignment of a signal at −6.2 ppm in $Cu(II)_2Co(II)_2SOD$ which has been performed on the basis of the NOE data (Banci et al. 1989a); *ii*) *His*-71 is the only residue of the active site that is buried, and its NH is expected to be the slowest exchanging NH proton. Therefore signal C is the best candidate for NH of *His*-71. With this working hypothesis we proceed to the examination of the NOEs from signals B, C, D, and G.

Among the NOEs from signal B, NH of *Gly*-72 and of *Lys*-136 are the best candidates for the signal at 8.55 ppm since they are at 3.8 and 3.7 Å respectively from the Hδ2 of *His*-71. Although the intensity of the NOE would suggest an even shorter distance, no exchangeable protons are observed in the X-ray structure at less than 3.7 Å.

As far as the NOEs from signal C are concerned, X-ray data indicate that the closest protons to the NHε2 of *His*-71 are the Hα1 of *Leu*-126, the Hα1 of *Gly*-138 and the Hα2 of *Gly*-138 which are at 2.3, 2.8 and 3.1 Å, respectively. Interestingly, the Hα1 of *Gly*-138 is also at 3.1 Å from Hδ2 of *His*-71 (signal B). A NOE to peak M, of smaller extent, has been detected also by saturating B. The NOE extent and the T_1 values of signal M account for a distance of 2.8 Å between proton M and NH of

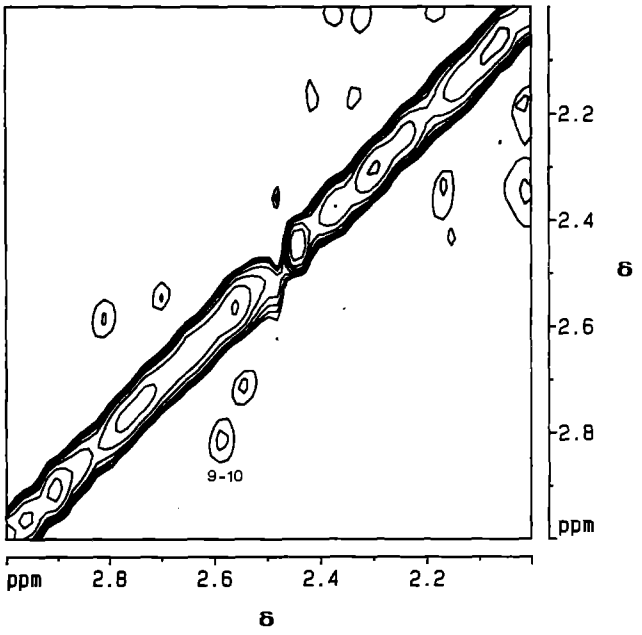


Fig. 11. Contour plot of the diamagnetic region between 3.0 and 2.0 ppm of the NOESY spectrum recorded at 600 MHz and 300 K of $Cu(I)_2Co(II)_2SOD$ in 50 mM phosphate buffer at pH 5.5, in D_2O

His-71 and a 30% larger distance between proton M and Hδ2 of *His*-71. The interproton distances calculated by the NOEs are, within the extimated error of 10%, consistent with the distances as predicted by X-ray data. Hence, we tentatively propose to assign resonance M as the Hα1 of *Gly*-138. The metal-to-proton distances are 7.1 Å for Hα1 of *Leu*-126, 5.9 Å for Hα1 of *Gly*-138 and 6.0 Å for Hα2 of *Gly*-138. These distances provide a further criterion to distinguish between the Hα of *Gly*-138 and of *Leu*-126. Since the chemical shift of the Hα protons in a diamagnetic protein is typically 4–5 ppm, signal M is affected by a sizeable dipolar isotropic contribution to the chemical shift and therefore is not likely to arise from Hα1 of *Leu*-126. Also the Hα2 of *Gly*-138 is expected to be affected by a dipolar shift analogous to the observed for its geminal proton, hence we assign the primary NOE detected at 10.44 ppm (signal N) obtained by saturating signal C to the Hα2 of *Gly*-138.

Further analysis of $Cu(I)_2Zn(II)_2SOD$ and $Cu(II)_2Co(II)_2SOD$

The analysis of the 1D epxeriments have allowed us not only to assign the isotropically shifted resonances of the $Cu(I)_2Co(II)_2SOD$ and $Ag(I)_2Co(II)_2SOD$ derivatives, but also to firmly assign some of the Hε1 and NHε2 resonances of the histidines coordinated to copper(I). Furthermore, analysis of the NOEs from the paramagnetic to the diamagnetic region have allowed us to propose an assignment for several signals of residues which are not directly coordinated to the metal ions but which may sense the contribution of the cobalt(II) ion to their chemical shift and relaxation times. A simple subtraction between the chemical shifts in the $Cu(I)_2Co(II)_2SOD$ and the $Cu(I)_2Zn(II)_2SOD$ derivatives should yield the isotropic shifts induced by cobalt substitution. In the case of signals which belong to non metal bound residues, the isotropic shift is only dipolar in origin and can be used for locating the magnetic susceptibility tensor of the cobalt(II) ion. Within this frame, the already performed NOESY and TOCSY experiments on the $Cu(I)_2Zn(II)_2SOD$ sample have been further analyzed to provide as many as possible reference shifts in diamagnetic protein. Besides the already reported assignment of imidazole protons (Bertini et al. 1991), the observed connectivities inside the region of alipathic protons and between NH protons and aliphatic protons have allowed us to assign both Hβ2 protons of *His*-48. The connectivity between Hβ2 of *His*-48 at 3.13 and the NHδ1 of *His*-48, independently assigned at 12.54 has been detected through experiments performed in H_2O; then 2D experiments in D_2O solution allowed us to identify the connectivity between Hβ2, at 3.13 ppm, and Hβ1, at 3.26 ppm. Furthermore, the NHε2 of *His*-71 which in $Cu(I)_2Co(II)_2SOD$ gives rise to NOE to peak M, shows a sizeable connectivity in the $Cu(I)_2Zn(II)_2SOD$ with a signal at 4.18 ppm, i.e. the region of the α protons. On this basis, we assign the signal at 4.18 ppm in the $Cu(I)_2Zn(II)_2$ derivative to the Hα1 of *Gly*-138 and, through both NOESY and TOCSY connectivity, the Hα2 of *Gly*-138 at 4.08 ppm.

A further comment must be made on the similarity observed in the shifts of the residues in the cobalt site in $Cu(I)_2Co(II)_2SOD$ and $Cu(II)_2Co(II)_2SOD$ (Table 1). In particular, among the signals of the coordinated histidines rings (signals B–H and Z), the largest difference (20%) is observed for Hδ2 of *His*-63, which is bridging to copper(II) in the oxidized enzyme. It appears that oxidation of copper does not alter significantly the cobalt site, both from the point of view of the cobalt contribution to the isotropic shifts and of possible contributions from copper. Indeed, with the exception of *His*-63 which can experience some contact contribution from copper(II), the dipolar shift induced by copper(II) on the other cobalt-coordinated histidines are predicted, with simple calculation, to be very small.

Dipolar tensor on cobalt(II)

The NOE experiments provide the unambiguous assignment of *His*-63 and *His*-48 resonances and allow a tentative assignment of several other proton resonances which are collectively reported in Table 1.

In the case of signals not belonging to cobalt coordinated residues that have been also assigned in the $Cu(I)_2Zn(II)_2$ derivative, the dipolar shift induced by Co(II) ion can be determined. This is the case for four *His*-48 signals and for the *His*-120 Hε1 proton resonance, which can thus be used for obtaining information on the magnetic susceptibility tensor of the system. These protons are all in the same region of the active cavity (i.e. the copper site), and therefore they do not provide a satisfactory data set for the calculation. On the other hand, cobalt coordinated residues are obviously affected

by the contact contribution to the isotropic shift, which cannot be easily factorized out. For these reasons we have chosen to introduce the Hα protons of *Gly*-138 in the data set for calculating the magnetic susceptibility tensor. The choice was dictated by the relatively good reliability of their assignment, as well as by their sizeable dipolar shifts. Their chemical shifts in $Cu(I)_2Zn(II)_2SOD$ are also known (Table 1).

The limited data set prevents us from performing a really good computation of the magnetic susceptibility tensor (Emerson and La Mar 1990), but the analysis represents a further criterion for checking the internal consistency of the proposed assignment. We find that there is a unique orientation of the χ tensor which satisfactorily reproduces the dipolar shifts of the seven protons. In this orientation the z axis of the magnetic susceptibility tensor is in the direction of the peptide NH of *His*-46, almost bisecting the angle of the direction of the *His*-63 to Co(II) bond and *Asp*-83 to Co(II) bond. The x axis is approximately directed toward the Hβ2 proton of *His*-80 and the y-axis toward the *His*-63 NH proton. The calculated magnetic susceptibilities anisotropies are $\Delta\chi_{ax} = -1.9 \times 10^{-8}$ m^3 mol^{-1} and $\Delta\chi_{eq} = 0.9 \times 10^{-8}$ m^3 mol^{-1}. Values of $\Delta\chi_{ax}$ of the same sign and magnitude and orientations of the z axis along a pseudo-C_2 axis have already been found in pseudo tetrahedral cobalt(II) complexes (Horrocks Jr. and Greenberg 1971). The $\Delta\chi_{eq}$ value indicates a marked rhombicity of the susceptibility tensor of cobalt(II) in $Cu(I)_2Co(II)_2SOD$, consistent with the actual low symmetry of the chromophore. The above orientation of the χ tensor predicts an upfield shift of about 10 ppm for Hβ2 of *His*-71, in agreement with our assignment of Hβ2 of *His*-71 at -6.5 ppm. A dipolar shift of about 5 ppm upfield is predicted for the NHε2 of *His*-46, inconsistent with its possible assignment as signal J, which is slightly downfield shifted with respect to the NHε2 of *His*-46 in the $Cu(I)_2Zn(II)_2SOD$. On the other hand, several exchangeable protons inside a 6 Å sphere from cobalt ion experience, in the calculated orientation of the susceptibility tensor, a downfield shift. Among these, the best candidate for the assignment of signal J is the *His*-80 NH peptide proton, which, being at 4.5 Å from Co(II), would experience a sizeable downfield shift.

Concluding remarks

The ^{1}H NMR spectra of $Cu(I)_2Co(II)_2$ and $Ag(I)_2Co(II)_2$-SOD represent a stimulating challenge to NMR spectroscopists. There are broad and far shifted signals belonging to the cobalt domain which have been assigned through ^{1}H NOE. The other histidines, including those of the copper domain, have been assigned through ^{1}H NOE and NOESY with reference to the fully diamagnetic system. Finally, some signals of non-coordinated residues in the cobalt domain have been assigned. Among the signals of the latter two classes, several experience pseudocontact shift which has allowed us to work out the orientation of the magnetic susceptibility tensor and its anisotropy.

Acknowledgements. This work was supported by CNR – Progetto Finalizzato Chimica Fine II.

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