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Mapping the copper ligands of Cu,Zn superoxide dismutase by nuclear Overhauser enhancement of the isotropically shifted ^{1}H-NMR lines of the Cu,Co derivative

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Transient nuclear Overhauser effect (NOE) experiments were made with bovine Cu,Co superoxide dismutase on the hyperfine shifted resonances of protons of the imidazole groups bound to the Cu,Zn cluster of the native enzyme. Assignments of lines based on the observed magnetization transfers were satisfactorily obtained only by altering the arrangement of the ligands around the catalytically active copper shown by X-ray crystallography.

Cu,Zn superoxide dismutase; Nuclear magnetic resonance; Nuclear Overhauser enhancement; Active site; Geometry

1. INTRODUCTION

A recent approach to the study of the active site of Cu,Zn superoxide dismutase has been NMR spectroscopy of the Cu,Co derivative in which the magnetic coupling between the adjacents Co(II) and Cu(II) allows the isotropically shifted ^{1}H NMR lines of the histidines bound to the oxidized metal cluster to be detected. A tentative assignment of the spectrum has been proposed on the basis of the relaxation times T_1 and T_2, which can discriminate the protons of the domains of the copper and cobalt [1], of the exchange H_2O/D_2O which identifies the NH protons [2], and of the chemical shift changes upon titration with anions [1,2], which are known to interact with the copper ion [3]. A more direct step in this direction is the investigation of the magnetization transfer between protons of the metal-binding histidines which may give rise to enhancement of the NMR lines (nuclear Overhauser enhancement or effect, NOE). The intensity of the observed effect is a function of interproton distances [4] and can therefore be related to the spatial coordinates of the active site derived from the high resolution X-ray structure of the bovine enzyme [5]. This paper reports the NOEs observed in Cu,Co superoxide dismutase between active site protons: assignments could satisfactorily be obtained only assuming a spatial arrangement for the copper-coordinated imidazole groups which deviates from that observed in the crystals.

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2. MATERIALS AND METHODS

Bovine Cu,Zn superoxide dismutase was isolated and its Cu,Co derivative was prepared as previously described [6]. 400 MHz ^{1}H NMR spectra were recorded in 10–15% deuterated solution at pH 7.00 with a Bruker AM400 spectrometer. The spectra were obtained and the resonances were labelled as previously reported [2,6]. NOE spectra were recorded by subtracting the spectrum obtained by selective excitation at the frequency of the resonance of interest from a reference spectrum obtained by irradiation made exactly in the same conditions in an empty spectral region. Transient NOE was preferred to steady state NOE [4,7] to avoid spin diffusion effects due to the fast relaxation of the active site protons in Cu,Co superoxide dismutase, which is related to the short relaxation time of the Co(II) ion [2,3]. Experiments were carried out by inserting a selective 180° pulse within the DEFT pulse sequence [8], the power of which was carefully adjusted to avoid spill-over effects and to give a rather narrow excitation bandwidth, and the duration of which was chosen as to be compatible with the spin-lattice relaxation time (T_1) of the resonances. During the experiments a cycling over the excitation frequencies as well as over the delay times was applied in order to minimize instrumental drifts or artifacts due to the long experimental time.

Molecular modelling of the enzyme active site was carried out with an Evans and Sutherland PS390 graphics system linked to a Microvax II Digital Equipment Corp. computer. The software employed was the last available version (5.2) of the Sybyl program from Tripos Associates. Minimization of the molecule was obtained by running the MAXIMIN 2 program which takes into account the Amber force field [9]. Stereoviews of the enzyme's active site were generated from the coordinates from the Protein Data Bank [10].

3. RESULTS AND DISCUSSION

3.1. *NOE spectra of Cu,Co superoxide dismutase*

NOE experiments (figs 1–3) were carried out on the 9 lines, among the 18 of the 400 MHz ^{1}H-NMR spectrum of Cu,Co superoxide dismutase (fig.1a), which permitted most selective irradiation. Evident NOEs were ob-

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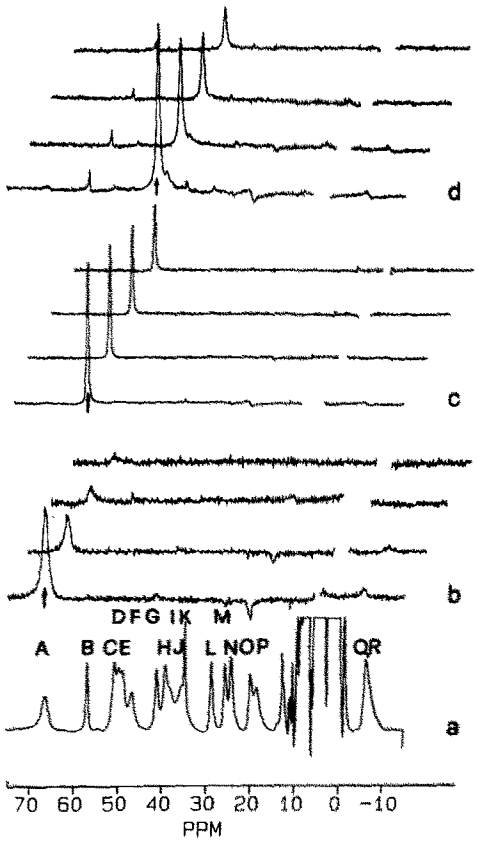

Fig.1. NOEs of hyperfine shifted lines of Cu,Co superoxide dismutase. (a) NMR spectrum of 1.4 mM enzyme in 20 mM Tris-Mops buffer at pH 7.00. (b–d) correspond to irradiation of the resonances marked with an arrow (A, B and G). The four different traces in each group correspond to 1, 3, 5 and 7 ms delay time respectively, starting from the bottom trace.

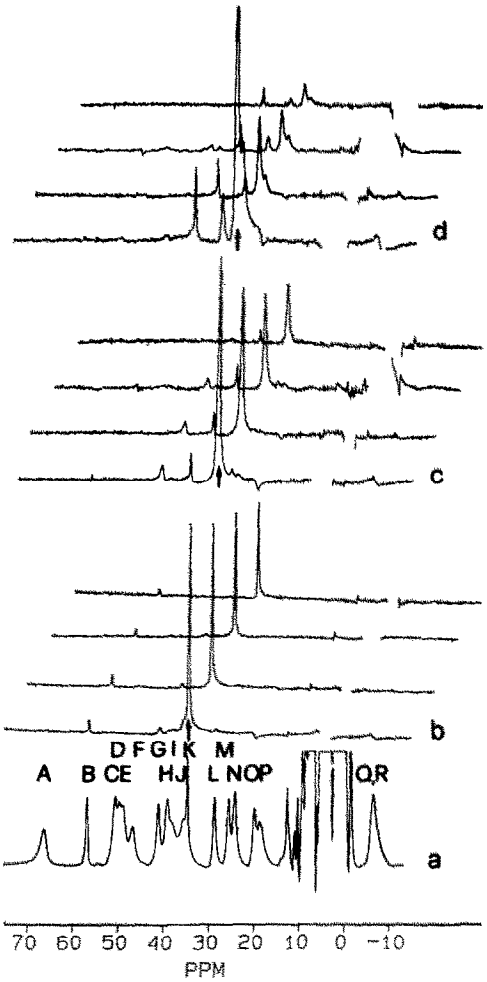

Fig.2. The same as fig.1, but for K, L and M lines.

tained in spite of the low intensity of the peaks in the difference spectrum, due to the short relaxation times of both the irradiated resonance and of the resonance receiving the magnetization. Increasing the delay time the relaxation proceeds and the intensity of the NOE peak further decreases.

3.2. *Interpretation of the NOE data according to the crystal X-ray structure*

In order to assign the resonances, the interproton distances obtained from the crystal structure (table 1) were used for a tentative fit of three qualitative classes of NOEs, namely strong (s), medium (m) and weak (w) NOEs (table 2). Resonances B and K, which give mutual NOEs, are established to arise from exchangeable NH protons [2]. Moreover they have been assigned to copper-bound imidazoles [1,2] on the basis of their relaxation times [1], and their large chemical shift changes [2] upon addition of anions that are known to coordinate only to the copper in the Cu,Co enzyme [3]. The only two NH groups at a distance lower than 0.5 nm (table 1), such as to give mutual NOEs [4], are, however, those belonging to His^{69}, a cobalt ligand, and His^{44}, a copper ligand [5]. This fact indicates that either substitution of Co(II) for the Zn(II) induces a rearrangement of the active site structure in spite of the identical activity of the two derivatives [3], or the active site geometry may change in solution with respect to the crystal. Therefore caution should be taken when using the crystallographic structure to assign the NOEs. This is confirmed by further discrepancies arising from assignments that could be attempted starting from B and K as protons of His^{69} or His^{44} (table 2). In this procedure K, which gives rise to more high intensity NOEs than B, has been assigned to His^{44}-NE2, which has a higher number of short interproton distances, and B to His^{69}-NE2. Excitation of either B or K gives a strong NOE with O. K gives a strong NOE also with G, which gives a strong transfer of magnetization to both K and B. G and O can therefore be assigned to His^{69}-CE1 and His^{61}-CE1 which are within 0.5 nm from His^{44}-NE2 and His^{69}-NE2. Because of the NOE between A and O and of the assignment of A to His^{61} in view of its relaxation times indicative of the Cu(II)–Co(II) bridging residue [1], O can be assigned to His^{61}-CE1 and G to His^{69}-CE1. From the NOE of O on N, the latter should

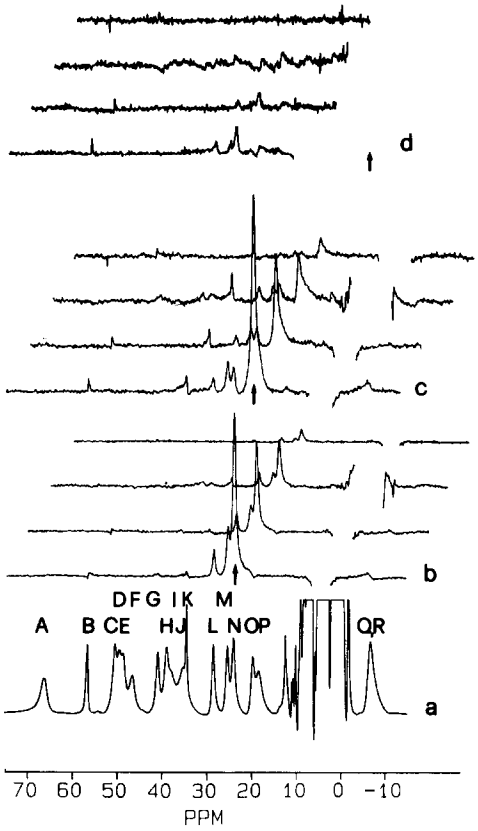

Fig.3. The same as fig.1, but for N, O and Q-R lines.

be the only proton at less than 0.5 nm from His^{61}-CE1 i.e. His^{78}-CE1, another cobalt-bound imidazole. However O, G and N are established, on the basis of their relaxation times, to belong to residues coordinated only to copper [1,2] and the observed mutual NOEs between A and B are in contrast with the too long distance (0.7 nm) between the two proposed corresponding protons. Resonances L and K have a strong mutual NOE, L and M have also a mutual NOE and both have a strong NOE on K. This suggests the same imidazole (i.e. His^{44}) as the source of K, L and M, even though only K and L move in the same direction in the presence of anions [2,11]. Since L and M give rise to a strong and a weak NOE, respectively, with G, and G gives rise to a NOE with L but not with M, L could be assigned to His^{44}-CE1 and M to His^{44}-CD2.

3.3. *Alternative structure of the site by computer graphics*

In addition to its internal inconsistencies the procedure described above involves unlikely magnetization transfers between the fast relaxing protons of residues bound to Co(II) and the relatively slow relaxing protons of Cu(II)-bound histidines. Therefore other possible active site conformations were constructed by computer graphics in order to generate a network of NOE-compatible distances including only protons of imidazole rings bound to the copper. The essential step was to put two NH groups of the three available from the copper-coordinated histidines (His^{46}, His^{44} and His^{118}) at a distance lower than 0.5 nm by rotating their side chains without altering the backbone orientations, and taking into account the Van der Waals interactions and the surrounding hydrogen bands. The direction of the C_α-C_β and C_β-C_γ bond of His^{46} with respect to those of His^{44} and His^{118} did not allow the NH group of His^{46} to be placed at a distance lower than 0.5 nm from the NH groups of the other histidines. On the other hand, clockwise rotation of 26° and 38° on χ_1 and χ_2 respectivaly of His^{44} and of 14° and 132° on χ_1 and χ_2 respectively of His^{118}, reduced the His^{118}-ND1-His^{44}-NE2 distance, after several cycles of minimization of the overall molecular structure, from 0.795 to 0.408 nm. The final energy was lower (500 kcal/mol) than that of the crystallographic molecule (2000 kcal/mol). In the new model (fig.4 and table 2), K is His^{118}-ND1 and B His^{44}-NE2. A is His^{61}-CE1, since His^{61}-CD2 is excluded by the NOE between A and B. The NOE between A and O indicates His^{44}-CD2 or His^{44}-CE1 or His^{61}-CD2 as possible candidates for O. His^{61} is excluded because of the relaxation time of O [1] which is typical of a Cu-bound residue; His^{44}-CD2 would not explain the NOEs between L and O and G and O; then O is His^{44}-CE1.

His^{61}-CD2 could be P because of its fast relaxation [1]. Since K and L (see above) are likely to belong to the same residue, His^{118}-CD2 or His^{118}-CE1 are good candidates for L in the new site. If L = His^{118}-CD2: G = His^{118}-CE1 from the NOE between O and G; M = His^{46}-CE1 from the NOE between L and M; N = His^{44}-CD2 from the NOE between the M-N pair (which cannot be resolved on excitation) and K. If L = His^{118}-CE1: G = His^{118}-CD2, M = His^{44}-CD2 because of the NOE between O and M; N = His^{46}-CE1 from the NOE between the pair M, N and G. In either case H = His^{46}-CD2 as it is the only resonance of the groups bound to the copper that has not yet been assigned, besides C, which is the exchangeable NH of His^{46}-ND1 [2].

4. CONCLUDING REMARKS

A large number of well defined NOEs has been obtained with hyperfine shifted proton lines of Cu,Co superoxide dismutase. All evidence points to copper-coordinated residues as the source of the NOEs observed, which were satisfactorily assigned only altering the active site geometry based on X-ray coordinates [5]. It should be kept in mind that the proposed model is not the only one that can explain the NOE results, but it was preferred because it was obtained without producing strong alterations of the overall structure. Work in progress on the crystal structure of Cu,Co superoxide dismutase will substantially help clarify this point. In any case, the present work suggests a different pattern

Table 1

Distances between the active site protons of Cu,Zn superoxide dismutase

His	46ND1	46CD2	46CE1	44NE2	44CD2	44CE1	118ND1	118CD2	118CE1	61CD2	61CE1	69NE2	69CD2	69CE1	78NE2	78CD2	78CE1
His 46ND1		4.2 (4.2)	2.4 (2.5)	9.6 (9.5)	9.0 (9.3)	8.0 (7.8)	7.9 (7.7)	7.7 (7.3)	5.4 (5.7)	3.6 (4.3)	7.2 (7.7)	12.0(12.8)	12.8(13.5)	9.7(10.3)	9.7(10.6)	11.0(12.2)	7.8 (8.8)
46CD2	4.2 (4.2)		4.2 (4.2)	7.1 (7.1)	5.9 (6.0)	6.3 (6.3)	7.4 (6.1)	5.1 (7.3)	5.5 (3.8)	3.0 (2.6)	6.1 (5.8)	10.5(10.6)	12.0(11.7)	8.0 (7.8)	10.8 (9.8)	11.4(10.6)	8.7 (7.8)
46CE1	2.4 (2.5)	4.2 (4.2)		7.9 (7.4)	7.8 (7.7)	5.9 (5.4)	6.1 (6.2)	6.1 (4.9)	3.6 (4.2)	3.0 (3.6)	5.4 (5.8)	10.1(10.7)	11.0(11.5)	7.9 (8.3)	9.2 (9.9)	10.7(11.6)	6.9 (7.7)
44NE2	9.6 (9.5)	7.1 (7.1)	7.9 (7.4)		2.4 (2.4)	2.7 (2.5)	7.9 (4.1)	4.8 (4.7)	7.6 (4.5)	6.5 (7.2)	3.3 (4.4)	4.0 (6.2)	6.4 (8.3)	2.5 (4.6)	10.1(11.7)	10.3(12.3)	8.0 (9.2)
44CD2	9.0 (9.3)	5.9 (6.0)	7.8 (7.7)	2.4 (2.4)		4.2 (4.1)	8.8 (4.5)	5.0 (6.4)	8.1 (4.4)	5.7 (6.5)	4.1 (4.7)	5.9 (6.9)	8.0 (9.0)	3.9 (4.8)	10.2(11.4)	10.0(11.5)	8.2 (9.1)
44CE1	8.0 (7.8)	6.3 (6.3)	5.9 (5.4)	2.7 (2.5)	4.2 (4.1)		5.5 (4.3)	3.3 (2.8)	5.1 (3.9)	5.6 (5.8)	2.8 (3.2)	5.3 (6.4)	7.2 (8.0)	3.7 (4.6)	10.1(10.3)	10.9(11.4)	7.7 (7.8)
118ND1	7.9 (7.7)	7.4 (6.1)	6.1 (6.2)	7.9 (4.1)	8.8 (4.5)	5.5 (4.3)		4.2 (4.3)	2.5 (2.4)	7.9 (7.2)	7.5 (6.8)	10.4(10.1)	12.1(12.0)	9.1 (8.1)	13.9(13.7)	15.2(14.6)	11.3(11.2)
118CD2	7.7 (7.3)	5.1 (7.3)	6.1 (4.9)	4.8 (4.7)	5.0 (6.4)	3.3 (2.8)	4.2 (4.3)		4.2 (4.3)	6.1 (6.9)	5.5 (5.6)	8.3 (8.8)	10.5(10.2)	6.7 (7.3)	12.6(12.0)	13.3(13.5)	10.1 (9.5)
118CE1	5.4 (5.7)	5.5 (3.8)	3.6 (4.2)	7.6 (4.5)	8.1 (4.4)	5.1 (3.9)	2.5 (2.4)	4.2 (4.3)		5.7 (4.9)	6.3 (5.4)	10.1 (9.7)	11.6(11.3)	8.4 (7.3)	12.0(11.8)	13.4(12.7)	9.5 (9.3)
61CD2	3.6 (4.3)	3.0 (2.6)	3.0 (3.6)	6.5 (7.2)	5.7 (6.5)	5.6 (5.8)	7.9 (7.2)	6.1 (6.9)	5.7 (4.9)		4.2 (4.3)	8.9 (9.3)	9.9 (9.9)	6.5 (6.6)	7.8 (7.5)	8.7 (8.6)	5.7 (5.4)
61CE1	7.2 (7.7)	6.1 (5.8)	5.4 (5.8)	3.3 (4.4)	4.1 (4.7)	2.8 (3.2)	7.5 (6.8)	5.5 (5.6)	6.3 (5.4)	4.2 (4.3)		4.7 (5.1)	6.0 (6.1)	2.5 (2.6)	7.4 (7.3)	8.1 (8.2)	5.0 (4.8)
69NE2	12.0(12.8)	10.5(10.6)	10.1(10.7)	4.0 (6.2)	5.9 (6.9)	5.3 (6.4)	10.4(10.1)	8.3 (8.8)	10.1 (9.7)	8.9 (9.3)	4.7 (5.1)		2.6 (2.5)	2.5 (2.8)	9.2 (9.0)	9.3 (9.2)	7.6 (7.3)
69CD2	12.8(13.5)	12.0(11.7)	11.0(11.5)	6.4 (8.3)	8.0 (9.0)	7.2 (8.0)	12.1(12.0)	10.5(10.2)	11.6(11.3)	9.9 (9.9)	6.0 (6.1)	2.6 (2.5)		4.2 (4.3)	8.0 (7.6)	8.2 (7.9)	6.9 (6.5)
69CE1	9.7(10.3)	8.0 (7.8)	7.9 (8.3)	2.5 (4.6)	3.9 (4.8)	3.7 (4.6)	9.1 (8.1)	6.7 (7.3)	8.4 (7.3)	6.5 (6.6)	2.5 (2.6)	2.5 (2.8)	4.2 (4.3)		8.0 (7.8)	8.2 (8.1)	6.1 (5.7)
78NE2	9.7(10.6)	10.8 (9.8)	9.2 (9.9)	10.1(11.7)	10.2(11.4)	10.1(10.3)	13.9(13.7)	12.6(12.0)	12.0(11.8)	7.8 (7.5)	7.4 (7.3)	9.2 (9.0)	8.0 (7.6)	8.0 (7.8)		2.5 (2.8)	2.6 (2.5)
78CD2	11.0(12.2)	11.4(10.6)	10.7(11.6)	10.3(12.3)	10.0(11.5)	10.9(11.4)	15.2(14.6)	13.3(13.5)	13.4(12.7)	8.7 (8.6)	8.1 (8.2)	9.3 (9.2)	8.2 (7.9)	8.2 (8.1)	2.5 (2.8)		4.2 (4.4)
78CE1	7.8 (8.8)	8.7 (7.8)	6.9 (7.7)	8.0 (9.2)	8.2 (9.1)	7.7 (7.8)	11.3(11.2)	10.1 (9.5)	9.5 (9.3)	5.7 (5.4)	5.0 (4.8)	7.6 (7.3)	6.9 (6.5)	6.1 (5.7)	2.6 (2.5)	4.2 (4.4)	

Data are from X-ray [5] and, in brackets, from the structure worked out by computer graphics in order to best fit the NOE data.

Table 2

Listing of NOEs of figs 1–3 and relative assignments according to the X-ray structure (A) or the computer graphics model (B)

Resonance	NOE observed[1]	Assignments	
		A	B
A	O(m), Q(w)	His^{61}-CD2	His^{61}-CE1
B	A(m), K(w), O(s)	His^{69}-NE2	His^{44}-NE2
C			His^{46}-ND1
G	B(s), K(s), L(m), O(s)	His^{69}-CE1	His^{118}-CE1 (His^{118}-CD2)
H			His^{46}-CD2
K	B(s), G(s), L(s), O(s)	His^{44}-NE2	His^{118}-ND1
L	G(s), K(s), M(m), O(m)	His^{44}-CE1	His^{118}-CD2 (His^{118}-CE1)
M	G(w), K(s), L(s)	His^{44}-CD2	His^{46}-CE1 (His^{44}-CD2)
N	G(w), K(w), L(s)[2], Q(w)	His^{78}-CE1	His^{44}-CD2 (His^{46}-CE1)
O	B(w), K(w), L(w), M(m), N(m), Q(w)	His^{61}-CE1	His^{44}-CE1
P			His^{61}-CD2
Q, R	B(m), N(m), L(w), M(w), O(w)		

[1] The peaks are qualitatively grouped according to their intensity: strong (s), medium (m) and weak (w). Blank spaces refer to unirradiated peaks.

[2] Probably due to the overlap with the near M line which generates the same NOE.

of assignments for the hyperfine shifted lines of Cu,Co superoxide dismutase with respect to previous reports [1,11]. In particular, NMR titration with anions capable of replacing the copper-bound water molecule suggested displacement from copper coordination of an imidazole ligand, which was assigned earlier to His^{44} [1] and then to His^{46} [11]. In our model the lines affected by anion binding, in particular K and L [1,11], would rather suggest His^{118}. Further NMR and NOE work is in progress in our laboratory to establish water and anion coordination to copper in the light of the new model presented here.

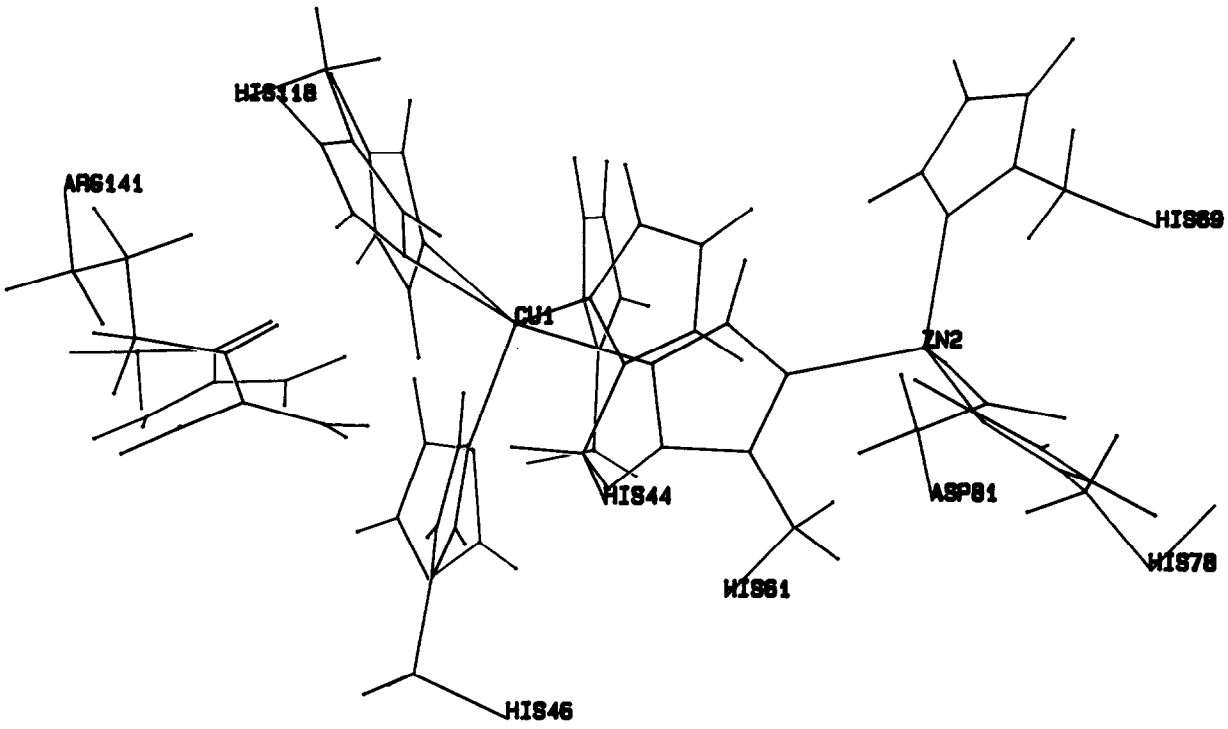

Fig.4. Active site of Cu,Zn superoxide dismutase as obtained from NOE experiments with the Cu,Co enzyme. The computer graphics display of the structure derived from the X-ray analysis [5] is outlined in black, while the structure best-fitting the NOE data is outlined in red.

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