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# Assignment of imidazole resonances from two-dimensional proton NMR spectra of bovine Cu,Zn superoxide dismutase

## Evidence for similar active site conformation in the oxidized and reduced enzyme

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Two-dimensional $^1$H-NMR spectra were carried out on bovine Cu(I),Zn superoxide dismutase. The ring protons of the single tyrosine and of the 4 phenylalanines were identified from COSY spectra. From NOESY spectra all imidazole C-resonances could be specifically assigned to each of the 8 histidines using the crystal coordinates of the Cu(II),Zn enzyme. Since 6 histidines are involved in the structure of the active site, this result implies nearly identical active site conformations for the two oxidation states of the catalytic cycle of this enzyme, in line with its diffusion-limited rate.

Cu,Zn superoxide dismutase; Nuclear magnetic resonance, 2-dimensional; Active site geometry

## 1. INTRODUCTION

The structure of Cu,Zn superoxide dismutase, and in particular that of its active site containing the Cu and Zn ions, has been determined by high-resolution X-ray diffraction studies of crystals of the oxidized, or Cu(II), enzyme from bovine erythrocytes [1]. Each identical enzyme subunit contains 8 histidines: His 19 and 41 are not involved in metal binding, His 44, 46 and 118 are involved in the binding of Cu, His 69 and 78 coordinate to the Zn, and His 61 is a bridging ligand between the two metals in the oxidized enzyme. A great deal of spectroscopic evidence indicated that the copper-imidazolate bond of this bridge is broken with subsequent protonation of the latter group in the reduced Cu(I),Zn enzyme [2]. This event is relevant to the mechanism of action of the enzyme, which involves alternate reduction and oxidation of the enzyme by $O_2$ to give $H_2O_2$ and $O_2$ as products at a rate approaching diffusion-limited values [2]. Therefore, knowledge of the structure of the active site of the reduced, or Cu(I), form of the enzyme is fundamental to the description of the catalytic mechanism; unfortunately the X-ray analysis of the Cu(I),Zn protein is not yet available. In the present paper, we report Nuclear Overhauser Effect (NOE)-2D correlated spectra (NOESY) of the aromatic residues of bovine Cu(I),Zn superoxide dismutase. We used the coordinates of the oxidized enzyme crystal [1] to assign the $C_2$ and $C_4$ protons of the imidazole groups in the 400 MHz spectrum of the reduced protein. All the histidines resonances could be satisfactorily assigned to specific residues, and this is strong evidence for a similar active site conformation in the oxidized and reduced enzymes. Furthermore, the aromatic protons of the single tyrosine and the 4 phenylalanine residues were identified from the 2D scalar correlated (COSY) spectra.

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## 2. MATERIALS AND METHODS

Cu,Zn superoxide dismutase was isolated from bovine erythrocytes as previously described [3]. The reduced form of the enzyme was prepared by the borohydride method, which is established to be much milder than dithionite or $H_2O_2$ treatments [4]. Small aliquots of a concentrated solution of $NaBH_4$ was added in air to the native enzyme dissolved in 0.001 M sodium borate, pH 10.2, 90% $D_2O$. The extent of reduction of the Cu(II)Zn superoxide dismutase was monitored spectrophotometrically following the decrease of the optical density at 680 nm, typical of the oxidized copper [3]. The solution was then transferred to a Thunberg apparatus and deareated by 5 cycles of equilibration with nitrogen. The excess of $NaBH_4$ was eliminated by lowering the pH to about pH 4.5 by anaerobic addition of small aliquots of 5 M acetic acid. The pH was then adjusted to pH 7.0 by anaerobic addition of 1 M phosphate buffer, pH 7.4. The solution was then transferred by a gas-tight syringe to a rubber-capped NMR tube, kept under nitrogen atmosphere. The final protein concentration was 1 mM in 80% $D_2O$. No change in the oxidation state of the enzyme treated according to this procedure was observed for as long as two days at room temperature, which was the average time required for acquisition of the NMR data.

$^1$H-NMR spectra were obtained with a Bruker AM400 instrument operating at 400.135 MHz. The spectral width was 14 ppm. A number of scans ranging from 128 to 512 was used over 8k data size. The residual water peak was suppressed by preirradiating the water

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resonance for 1.0 s. Resolution enhancement of the spectrum was obtained by using a sine-bell window shifted by $\pi/3$. Chemical shifts were referred to the water resonance at 4.76 ppm.

The NOESY spectra were recorded in the phase-sensitive mode using the time-proportional phase increment (TPPI) method [5]. The best value of the mixing time was set at 150 ms after a number of trials with different values. The spectra consisted of 450 free-induction decays (FIDs) of 2k data points of 128 scans each. The double-quantum filtered two-dimensional correlated (COSY) spectra [6] were performed using TPPI [5]. 512 FIDs were accumulated on 2k data points with 128 scans each.

The data were weighted by a sine-bell shifted by $\pi/3$ in both dimensions. Data were processed on a microVAX II with the 2D NMR software written in FORTRAN 77. The program was kindly provided by Professor R. Kaptein, Dept. of Organic Chemistry, Afd. NMR, University of Utrecht, The Netherlands. A matrix of 1024 × 1024 phase sensitive absorption spectrum was thus obtained with a digital resolution of 6.51 Hz/point. An accurate base line correction was carried out in both dimensions by using a polynomial fit provided by the same program. This procedure minimizes spurious signals by eliminating the base line roll and reducing the $t_1$ and $t_2$ ridges. The phase was corrected as to give the best result, compatible with the large width of the spectral envelope. Therefore only the crosspeaks in the 'unfavorable' upper part of the spectrum were taken into consideration for the assignment.

The X-ray coordinates of bovine Cu(II),Zn superoxide dismutase were obtained from the Brookhaven data bank [7].

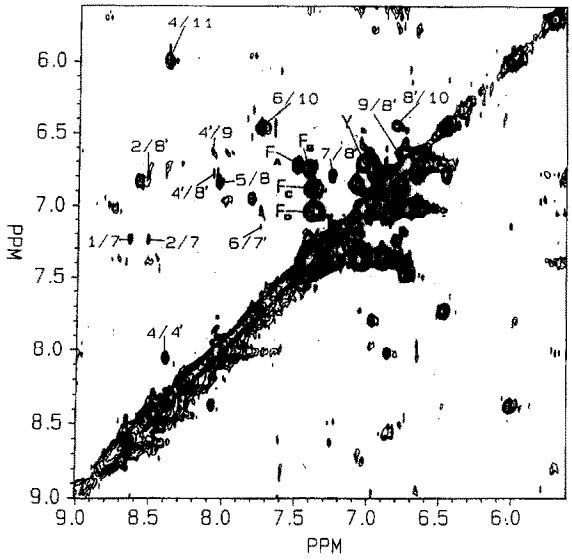


Fig. 2. Aromatic region of the NOESY spectrum of bovine Cu(I),Zn superoxide dismutase. The crosspeaks between the imidazole proton resonances are indicated by the number of the corresponding resonances as in Fig. 1. $F_A$, $F_B$, $F_C$, $F_D$, indicate the spin systems of the 4 phenylalanines. Y indicates the spin system of the single tyrosine.

## 3. RESULTS AND DISCUSSION

Fig. 1 shows the aromatic region of the 400 MHz $^1$H-NMR spectrum of Cu(I),Zn superoxide dismutase. The resonances belonging to histidine imidazole protons are labelled with numbers from 1 to 11 according to previous work done at 270 MHz [8]. At the higher resolution of the present work, 3 more resonances are detected (4', 7' and 8').

The aromatic region of the NOESY spectrum of Cu(I),ZnSOD (Fig. 2) shows several crosspeaks, many of which are to be assigned to residual NH protons not fully exchanged with deuterons. The crosspeaks due to the single Tyr (Tyr 108) and the 4 Phe residues [1,2] are better evidenced in the COSY spectrum (Fig. 3). The symmetrical AA' XX' spin system unambiguously identifies the single Tyr residue, while the 4 different Phe cannot be assigned to specific residues at the present stage.

Most of the previous assignment regarding the imidazole resonances [8] relied on the fact that 6 out of the 8 His are involved in the binding of the active site metals. Resonances 1 and 5 were assigned to the $C_2$ protons of His 41 and 19, respectively; resonances 2 and 4 to $C_2$ protons of two copper-liganding histidines, and

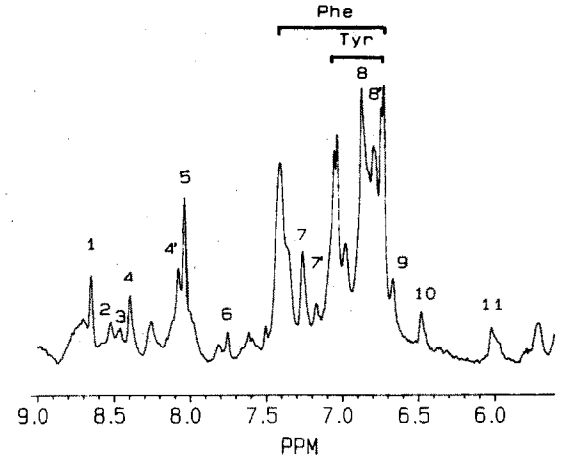


Fig. 1. Aromatic region of the $^1$H-NMR spectrum of bovine Cu(I),Zn superoxide dismutase at 400 MHz. For experimental details, see section 2.

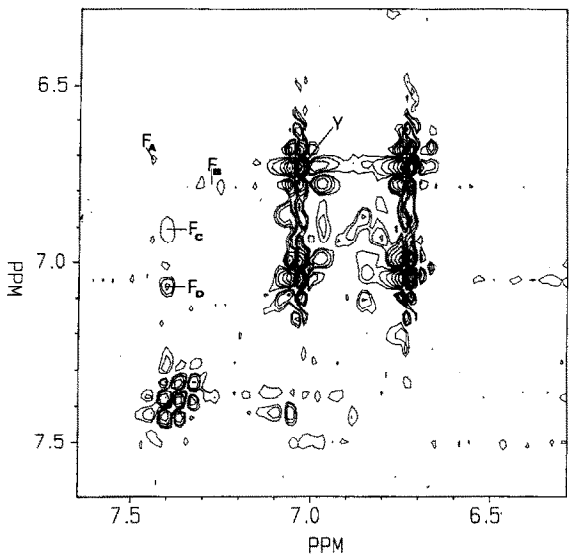


Fig. 3. Aromatic region of the COSY spectrum of bovine Cu(I),Zn superoxide dismutase. Labels as in Fig. 2.

Table I

Interproton distances (Å) at the active site of bovine Cu,Zn superoxide dismutase

| Imidazole groups | Interproton distance (Å) |
|---|---|
| ($C_2$)His69---($C_2$)His61 | 2.5 |
| ($C_2$)His61---($C_2$)His44 | 2.8 |
| ($C_2$)His46---($C_4$)His61 | 3.0 |
| ($C_4$)His61---($C_4$)His46 | 3.0 |
| ($C_2$)His44---($C_4$)His118 | 3.3 |
| ($C_2$)His118---($C_2$)His46 | 3.6 |
| ($C_2$)His69---($C_2$)His44 | 3.7 |
| ($C_2$)His69---($C_4$)His44 | 3.9 |
| ($C_4$)His44---($C_2$)His61 | 4.1 |

Values <4.2 Å are reported

resonances 3, 6 and 10 to $C_2$ protons of 3 zinc-liganding histidines. Moreover, because of their characteristic chemical shifts, resonance 4′ can be confidently assigned to $C_2$ histidine protons, whilst resonances 7, 7′, 8, 9 and 11 fall in the region of $C_4$ protons. Assignments to specific residues can be attempted by taking into consideration the interproton distances from the high-resolution X-ray diffraction of the oxidized enzyme [1]. In Table I are reported the distances between protons on different imidazole groups that are shorter than 4.2 Å, which is the distance between a $C_4$ and $C_2$ proton on the same imidazole.

In order to fit the experimental crosspeaks, the assignment procedure was started from resonance 4 (Fig. 1), which is unequivocally a $C_2$ proton of a copper ligand [8]. Starting from this point, a network of NOEs involving magnetization transfer between 4 different active site residues could be identified (Table II). The 3 histidines known to be bound to the copper in its reduced state (His 44, 46, 118) were then taken into consideration as possible candidates for resonance 4. All the possible assignment schemes are also reported in Table II. Schemes A and C can be ruled out, as neither ($C_4$)His69 nor ($C_4$)His118 have a close $C_4$ proton (Table I) such as to proceed with the assignment beyond the third step. Scheme B is to be ruled out, since it involves resonances 10 and 6 as belonging to copper ligands whilst they have been assigned to zinc ligands [8]. Scheme D is untenable for the same reason as referred to for resonance 6. The only assignment that nicely fits the interproton distances reported in Table I and is in agreement with the previous data [8] is scheme E, which assigns the resonances 4, 6, 4′, 10 to the $C_2$ protons of His 118, 69, 46, 61, respectively, and resonances 7′, 9, 8′ to the $C_4$ proton of His 69, 46, 61, respectively. This scheme leaves unassigned resonances 2 and 3, which arise from $C_2$ protons, and lines 7, 8 and 11, which arise from $C_4$ protons. The former two resonances must belong to the $C_2$ protons of His 44 and His 78 as they are known to belong to a copper and zinc ligand, respectively [8]. Resonance 11 must be assigned to the $C_4$ proton of His 118, because of its crosspeak with line 4, which is due to the corresponding $C_2$ proton (Fig. 2). Resonance 8 must be the $C_4$ proton of the non-liganding His 19 because of its crosspeak with resonance 5, which is due [8] to the corresponding $C_2$ proton (Fig. 2). Resonance 7 is given by the $C_4$ protons of both His 41 and His 44, as deduced from the 1/7 and 2/7 crosspeaks. The resonance of the $C_4$ proton of His 78 cannot be identified because of the absence of any evident crosspeak with resonance 3, which is due to the corresponding $C_2$ proton. The active site is depicted with the relative assignments in Fig. 4.

The expected crosspeaks 10/2 and 8′/4′ (see Table I), which would correspond to the magnetization transfers ($C_2$)His61/($C_2$)His44 and ($C_4$)His61/($C_2$)His46, re-

Table II

Assignment of the $^1$H resonances in the 400 MHz spectrum of Cu(I),Zn superoxide dismutase between 6 and 9 ppm

| Scheme | Sequence |
|---|---|
| | ($C_2$)4-----($C_2$)4′--($C_4$)9-----($C_4$)8′---($C_2$)10-----($C_2$)6--($C_4$)7′ |
| A | ($C_2$)44------($C_2$)69--($C_4$)69 |
| B | ($C_2$)44-----($C_2$)61--($C_4$)61-----($C_4$)46---($C_2$)46-----($C_2$)118--($C_4$)118 |
| C | ($C_2$)46-----($C_2$)118--($C_4$)118 |
| D | ($C_2$)118-----($C_2$)46--($C_4$)46-----($C_4$)61---($C_2$)61-----($C_2$)44--($C_4$)44 |
| E | ($C_2$)118-----($C_2$)46--($C_4$)46-----($C_4$)61---($C_2$)61-----($C_2$)69--($C_4$)69 |

Resonances are labeled as in Fig. 1. In the top line of the Table, a network of experimental crosspeaks is reported (see text). The type of protons is reported in brackets followed by the relative resonance. The length of the connecting lines indicates the approximate magnitude of the NOE (i.e. strong or medium or weak). The sequences A–E represent possible assignments based on the interproton distances reported in Table I. The type of proton is followed by the number of the relative His residue in the linear sequence of the protein [1,2]

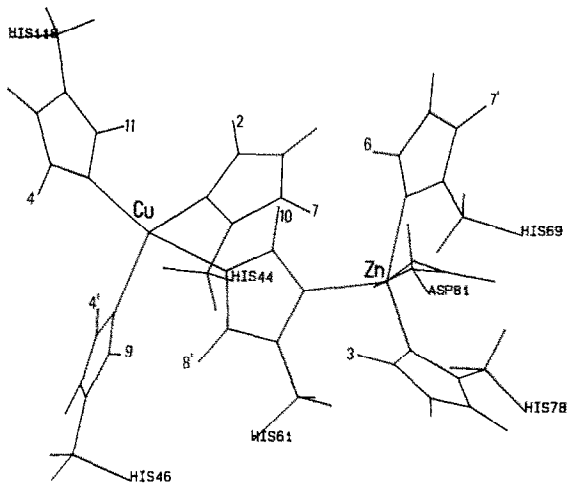


Fig. 4. Schematic view of the active site protons of bovine Cu(I),Zn superoxide dismutase. $C_4$ and $C_2$ protons of imidazole rings are labelled with the numbers relative to the NMR resonances reported in Fig. 1.

spectively, are not detected or just barely detected (Fig. 1), despite the fact that they correspond to protons which are 2.8 and 3.0 Å apart, respectively. However, the detachment of His 61 from copper coordination in the reduced enzyme is likely to alter the distances involved in these interactions. On the other hand, the two weak crosspeaks 2/8′ and 7/8′, which correspond to ($C_2$)His44/($C_4$)His61 and ($C_4$)His44/($C_4$)His61, respectively in our assignment, are observed in spite of having interproton distances of 5.6 and 5.7 Å, respectively, in the oxidized enzyme. However, also this inconsistency involves His 61 and can be related to a small rotation of the side chain, once it is detached from the reduced copper [9].

## 4. CONCLUSIONS

The internal consistency of the assignment to specific histidine residues of the proton resonances of the 400 MHz NOESY spectrum of the reduced Cu,Zn superoxide dismutase on the basis of the X-ray structure of the oxidized enzyme [1], contains a two-fold message: (i) the reduced and oxidized enzymes should have a similar conformation to all the metal-coordinating residues, besides small changes imposed by the cleavage of the imidazolate bridge of His 61 in the reduced protein [9]; (ii) the crystal structure and the structure in solution seem to be very closely in agreement with each other, at variance with the case of the Cu(II),Co(II) enzyme [10]. While the latter result awaits the X-ray structure of the cobalt derivative of the enzyme for its interpretation, the former conclusion is relevant to the mechanism of action of the enzyme. In fact Cu,Zn superoxide dismutase catalysis is characterized by a very high, nearly diffusion-limited, rate of reduction and oxidation of the enzyme-bound copper [2]. Since the protonation of the bridging imidazolate on the copper side was demonstrated to occur also in the catalytic reduction step [11], the rate of the event requires that all the nuclei surrounding the metal cluster undergo just a very slight rearrangement upon copper reduction. The results reported here support this hypothesis.

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