Investigation of the active site of *Escherichia coli* Cu,Zn superoxide dismutase reveals the absence of the copper-coordinated water molecule. Is the water molecule really necessary for the enzymatic mechanism?

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Abstract The active site of the Cu,Zn superoxide dismutase from *Escherichia coli* in the oxidized Cu(II) state has been studied by nuclear magnetic relaxation dispersion (NMRD), optical and nuclear magnetic resonance spectroscopy. The orientation of some metal ligands is different with respect to all the other Cu,Zn superoxide dismutases. Moreover, NMRD measurements demonstrate the lack of a copper-coordinated water molecule. In spite of these differences the enzymatic activity is still high. Azide also binds copper with normal affinity and induces modifications in the active site comparable to those previously observed in the eukaryotic enzymes. Our results suggest that, in this enzyme, the copper-coordinated water molecule appears not necessary for the enzymatic reaction. A role for the copper-coordinated water molecule is discussed in the light of recent crystallographic studies.

Key words: Superoxide dismutase; Cu,Zn superoxide dismutase; Water coordination; Active site; *Escherichia coli*

1. Introduction

Cu,Zn superoxide dismutases (Cu,Zn-SODs) are ubiquitous enzymes that play a key role in the mechanisms of cells protection against the toxic effects of reactive oxygen species [1]. These enzymes are characterized by a flattened β-barrel and a unique active site arrangement with two metal ions linked together by the imidazole of His-61. Three other histidines (His-44, 46 and 118) coordinate the catalytic copper ion, which is cyclically oxidized and reduced during the enzymatic reaction [2]. Two histidines (His-69 and 78) and an aspartic residue (Asp-81) are the zinc ligands. A water molecule is also weakly coordinated to copper in all the natural eukaryotic and prokaryotic species studied so far [3–8].

While all eukaryotic Cu,Zn-SODs possess the same global dimeric fold and the same arrangement of the active site ligands, the prokaryotic enzymes have distinct features including a different quaternary structure and a modified organization of the active site channel [9,10]. A case of interest is the SOD from *Escherichia coli* (ESOD), which is the only natural monomeric variant purified so far [11]. The activity of the enzyme is similar to that observed for the bovine enzyme, nevertheless some spectroscopic (electron paramagnetic resonance and optical) and biophysical (denaturation and pH behavior) properties are different [12]. The X-ray structure of the oxidized form of this enzyme showed a different arrangement of the ligands around the copper and zinc ion [10], which, however, has been subsequently rationalized in terms of copper reduction by the high X-ray photon flux of the synchrotron source used in the crystallographic work [13]. To elucidate the structural features of the active site of the monomeric enzyme in the oxidized Cu(II) state and to compare them with the information available on dimeric variants, we have performed a characterization of its active site in solution. Nuclear magnetic relaxation dispersion (NMRD) measurements on the Cu,Zn enzyme and nuclear magnetic resonance (NMR) studies of the Cu,Co enzyme show that ESOD does not have a water molecule coordinated to the copper ion and that its active site structure differs from that of the other Cu,Zn-SODs studied so far. These results clearly indicate that the water molecule coordinated to copper is not necessary for highly efficient catalysis in this Cu,Zn-SOD.

2. Materials and methods

2.1. Preparation of Cu,Co-ESOD

ESOD was purified from *E. coli* QC871 cells, as previously described [12]. The metal-free enzyme was obtained by extensive dialysis against EDTA at pH 3.8, as already described [12]. The Cu,Co enzyme was prepared by titration of the apo-enzyme with sub-stoichiometric amounts of $CoCl_2$ in 50 mM acetate buffer, pH 5.5. After the addition of a stoichiometric amount of cobalt the enzyme was further incubated at room temperature for 2 h and then a stoichiometric amount of $CuCl_2$ was added to obtain the Cu,Co derivative. The reconstituted enzyme was subsequently dialyzed against 10 mM phosphate buffer, pH 6.5. Catalytic measurements confirmed that also in this Cu,Zn-SOD variant, the substitution of zinc with cobalt does not affect the enzyme activity.

2.2. NMR spectroscopy

NMRD profiles, i.e. plots of solvent water proton relaxation rates as a function of the applied magnetic field, were measured on a Stelar Spinmaster-FFC field cycling spectrometer (Stelar, Mede, Italy), operating in a field range from 1.85×10^{-4} to 0.29 T (proton Larmor frequencies from 8 kHz to 12 MHz). The temperature was changed by using a built-in temperature controller and actually measured in the probehead with a mercury thermometer after temperature stabiliza-

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tion. The relaxometer is able to switch the magnetic field strength in a ms-time scale and works under complete computer control. The reliability of the measured profiles was checked separately with standard solutions having T_1 values comparable to those being measured and it has been shown to correspond to an absolute uncertainty in $1/T_1$ of ± 1%, on average. Reported paramagnetic contributions are obtained by subtracting the diamagnetic water relaxation rate from the observed relaxation rates, at every temperature.

NMR spectra were recorded at 25°C on a Bruker AM400 operating at a frequency of 400 MHz. Sample concentrations were about 2 mM: at higher protein concentrations significant aggregation was observed. One-dimensional spectra were recorded by using a WEFT pulse sequence [14]. Longitudinal relaxation times (T_1) were obtained using an inversion recovery pulse sequence. Two-dimensional dipolar-correlated spectra, to observe connectivities between the isotropically shifted resonances, were recorded using a normal NOESY sequence [15]. The mixing times were 1, 3, 5, 7, 9, 11 and 15 ms. Scalar-correlated two-dimensional spectra were recorded by using a TOCSY sequence, with a modified DIPSY sequence [16] to suppress dipolar effects. Spin-lock times employed were 5, 10, 15, and 20 ms. In all the cases the optimization of the spectral parameters take into account the fast relaxation rate of the observed resonances [17]. Data were processed using TRITON software [18].

3. Results and discussion

The NMRD profile of ESOD is reported in Fig. 1A and shows lower relaxation rate values in the whole frequency range with respect to that of bovine Cu,Zn-SOD (BSOD). The addition of an excess of NaN_3 does not change the relaxivity profile of ESOD, except for slight differences observed at proton Larmor frequencies lower than 1 MHz. As previously reported [19], the relaxivity profile of BSOD is heavily perturbed by the addition of NaN_3, and in the presence of an excess of this anion it resembles the profile of ESOD.

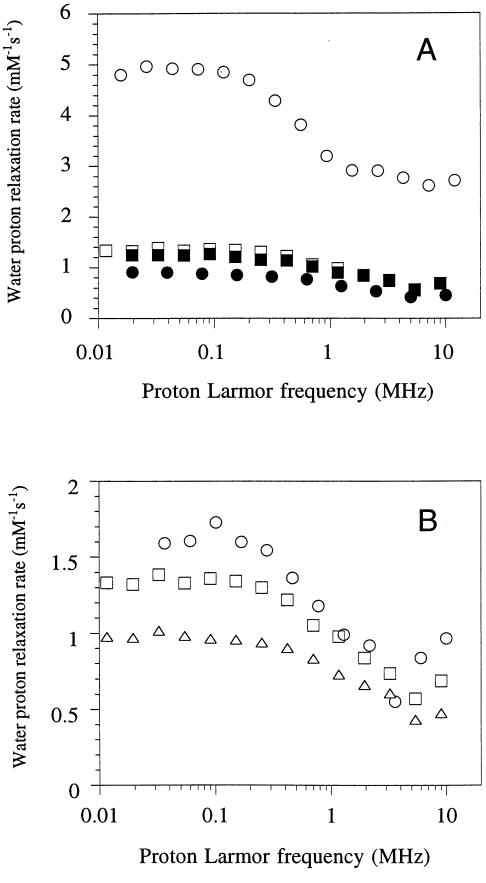

Fig. 1. A: Water proton relaxation rate of Cu(II)Zn(II)BSOD in the absence (○) and in the presence (●) of saturating amounts of azide and of Cu(II)Zn(II)ESOD in the absence (□) and in the presence (■) of saturating amounts of azide. B: Water proton relaxation rate of Cu(II)Zn(II)ESOD at different temperatures: 12°C (○); 25°C (□) and 37°C (△). The copper concentration was 1 mM.

Different hypotheses might explain this observation: (A) the small paramagnetic relaxation contribution observed in ESOD could be due to a very long residence lifetime of the water molecule coordinated to the Cu(II) ion, which is no longer able to promote the relaxation of the bulk water molecules; (B) the different relaxivity observed in the case of ESOD could be due to a different contribution of the correlation time in the dimeric BSOD and the monomeric ESOD; (C) ESOD could lack the coordinated water molecule which, in BSOD, is displaced upon azide binding [19]. In this case, a residual paramagnetic contribution could be due to the occurrence of water molecules in the proximity of the paramagnetic center: these water molecules are stabilized by weak interactions among each other or with hydrophilic sidechains of neighboring amino acids, in a way similar to that observed for myoglobins [20]. The presence of azide in the inner coordination sphere of Cu(II) in ESOD restricts the volume of the hydrophilic cavity, thus removing the contribution from second-sphere water molecules [21].

In order to investigate the possibility of a long residence lifetime, NMRD profiles of ESOD have been recorded at different temperatures. Fig. 1B shows the paramagnetic contributions to the water relaxation rate from a sample of millimolar concentration at different temperatures and different proton Larmor frequencies. As the temperature increases, the relaxivity decreases at every Larmor frequency, thus indicating the occurrence of a fast exchange process. The occurrence of a slow exchanging directly coordinated water molecule would give low relaxivity values, and the dependence of relaxivity on temperature should be the opposite to that observed.

Moreover, it has been reported that the NMRD profile of wild type human Cu,Zn-SOD (dimeric) and of an engineered monomeric mutant are identical [22] and, in both cases, indicate the presence of a copper-coordinated water molecule. Although ESOD and BSOD differ for both molecular mass (determining the reorientational correlation time) and metal coordination environment (determining electron relaxation time), we believe that a difference in the electronic relaxation properties would not affect the NMRD profile in such way; actually, the NMRD profile of ESOD looks like 'scaled down', suggesting that the large differences in the relaxivity of ESOD and BSOD cannot be attributed to a different contribution of the correlation time.

Therefore, ESOD appears to be the first Cu,Zn-SOD isolated from a natural source that does not contain a copper-coordinated water molecule.

The study of the structural features of the active site of ESOD was carried out by a spectroscopic characterization of the Cu,Co derivative, in which the spectroscopically silent zinc ion was replaced by cobalt. As in the case of the bovine enzyme, the electronic spectrum of the oxidized *E. coli* Cu,Co-SOD derivative is characterized by a cobalt absorption spectrum which is split in three bands, typical of a cobalt ion coordinated in a tetrahedral geometry (not shown). However,

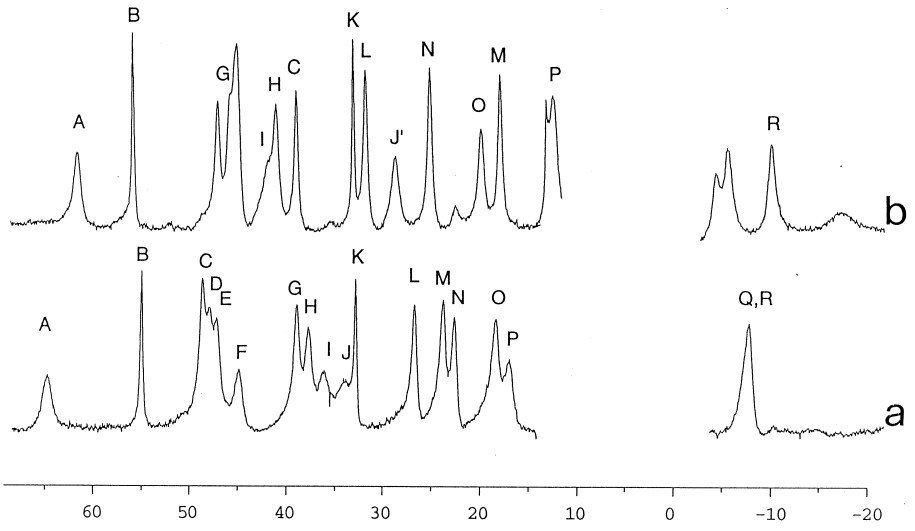

Fig. 2. ^{1}H-NMR spectra of (a) Cu(II)Co(II) BSOD and (b) Cu(II)Co(II) ESOD. The region of the isotropically shifted resonances is reported and the labelling is that previously reported [24]. Only the assigned resonances (see Table 1) are labelled in spectrum (b).

the relative intensities of the peaks centered at 598 and 567 nm are different in the two enzymes, thus suggesting that some rearrangement in the orientation of the cobalt ligands occurs in the monomeric *E. coli* enzyme. As in the case of the bovine enzyme, the reduction of the copper ion in the *E. coli* Cu,Co enzyme is followed by a modification of the cobalt absorption spectrum characterized by a reduction in the intensity of the 598 nm peak together with a shift to lower

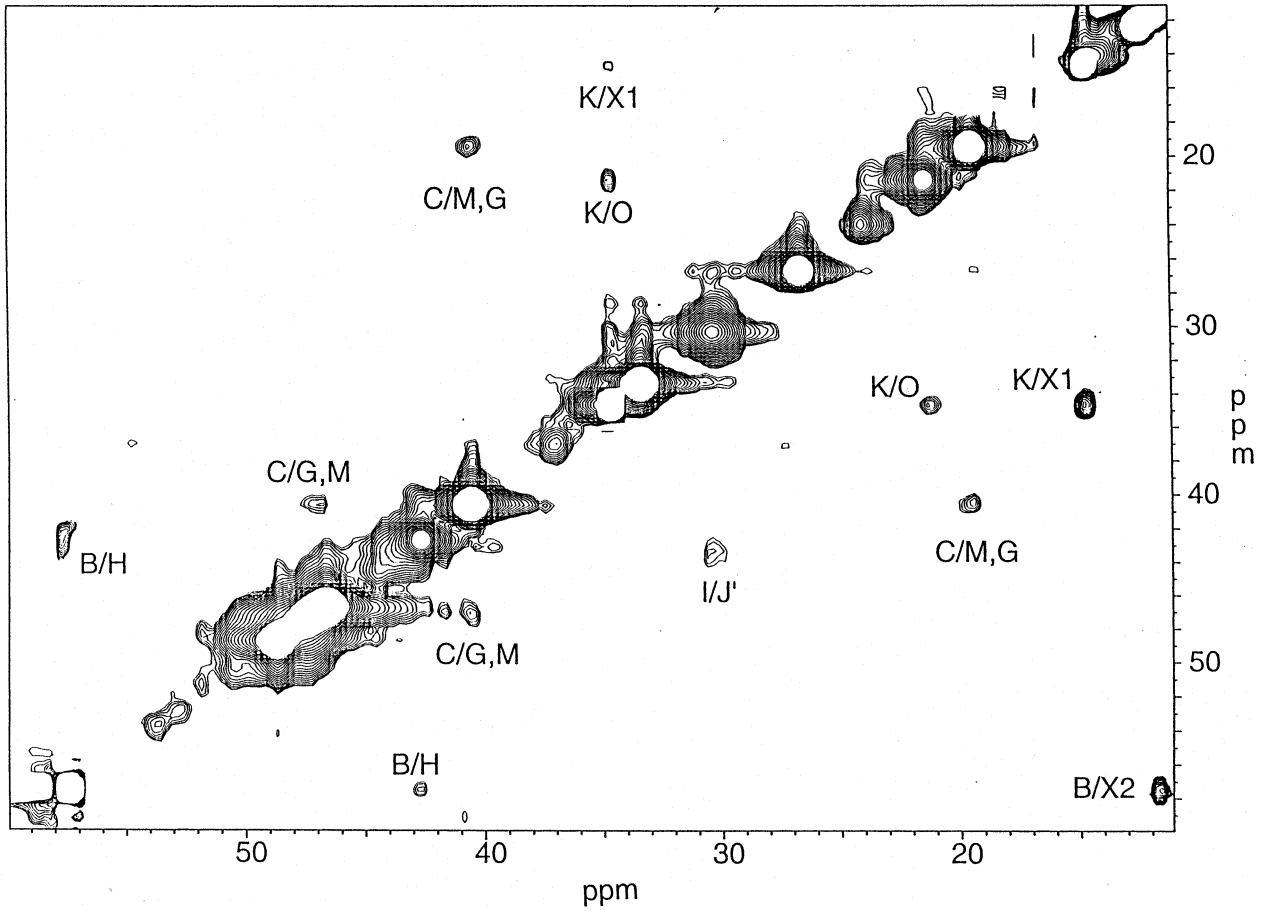

Fig. 3. Two-dimensional NOESY spectrum of Cu(II)Co(II) ESOD showing the connectivities between the isotropically shifted resonances. Only the region in which connectivities are observed is reported. The mixing time was 5 ms.

wavelengths (not shown). By analogy with the bovine enzyme, such a spectral modification may be interpreted as the consequence of the release of the bridging imidazole from the copper upon reduction.

The NMR spectrum of Cu,Co-ESOD is reported in Fig. 2 along with the spectrum of the bovine enzyme obtained in the same conditions. The two spectra show the same number of resonances, indicating that there is not an additional ligand to the copper ion that replaces the water molecule and that the same magnetic coupling between the two metal ions exists in the *E. coli* SOD. In particular, resonance A at 62.6 ppm is characteristic of His61CD2 (the amino acid numbering refers to the bovine enzyme), i.e. of a proton of the bridging His-61 when the copper is in the oxidized form [23–25].

The general shape of the spectrum is different from that observed in bovine, yeast and human enzymes that are very similar to each other, thus suggesting a different arrangement of some ligands.

Since protons belonging to resonances due to cobalt-coordinated ligands have longitudinal relaxation times shorter than protons belonging to copper-coordinated ligands [26], T_1 values have been measured to discriminate the signals due to ligands bound to the two metals. Resonances labelled B and K in the *E. coli* spectrum have chemical shifts, line widths and longitudinal relaxation times, very similar to those observed in the eukaryotic enzymes and disappear when the spectrum is recorded in deuterium oxide. For these reasons we assumed that they belong to NH imidazole protons of His118ND1 and His46ND1, respectively, i.e. like in the bovine enzyme [23–25], and we used them as the starting point for the assignment of the other resonances.

Two-dimensional experiments [17] allowed us to assign the resonances belonging to the copper-ligated histidines (see Fig. 3). Table 1 reports the assignment of the resonances and the comparison of their chemical shifts with those of the resonances of the spectroscopically characterized bovine enzyme [23–25]. Starting from resonances B and K in the NOESY spectra, we assigned the resonances belonging to the CE1 protons, as well as the resonances due to the β-protons. Cross-peaks in TOCSY spectra between the methylene geminal pairs confirmed the assignments of the β-protons. The resonances of the CD2 protons (H and L for His-118 and His-46, respectively) were assigned on the basis of their behavior in the presence of azide (not shown), because their distance with its ND1 or CE1 protons is too large to give a detectable NOE. The remaining resonances of the copper-ligands should belong to His-44. His-44 is coordinated via its ND1 nitrogen to the copper ion, instead of His-46 and His-118, and this allows the observation of NOEs between the NE2 proton and the CD2 and the CE1 ones, while the NH protons of His-46 and His-118 only give NOEs with their CE1 protons. This allowed us to assign the signal at 40.4 ppm to the NE2 proton of His-44 but did not allow the discrimination between CE1 and CD2 of the same histidine. In any case, the chemical shifts of the NE2 and of CE1 or/and CD2 are very different from the bovine enzyme. Also, the chemical shift of the β-protons of His-44 (assigned by a strong symmetric peak in a NOESY spectrum recorded with a mixing time of 1 ms) shows a difference with respect to the bovine enzyme (see Table 1). The limited solubility of the enzyme prevented us from observing interresidue NOEs, useful to locate the relative orientation of the copper-ligands.

Table 1
Assignment of the isotropically shifted resonances belonging to the metal ligands of Cu,Co-ESOD.

Assignment	Label	BSOD ppm (ms)	ESOD ppm (ms)
His-61CD2	A	66.2 (2.3)	62.6 (2.2)
His-118ND1	B	56.5 (10.9)	57.2 (9.7)
His-44NE2	C	50.3 (5.5)	40.4 (5.3)
His-69CD2	D	49.4[a]	
His-78CD2	E	48.8[a]	
His-78NE2	F	46.7 (2.0)	
His-44CD2	G/M	40.6 (4.9)	46.9[a]
His-118CE1	H	39.0 (2.9)	42.6 (1.9)
Asp-81β1	I	37.4[a]	44.1 (1.9)
His-69NE2	J	35.6[a]	
Asp-81β2	J′	35.4[a]	30.3 (1.9)
His-46ND1	K	34.5 (11.6)	34.7 (11.4)
His-46CD2	L	28.4 (5.7)	33.4 (4.5)
His-44CE1	M/G	25.3 (3.4)	19.5 (3.3)
His-118CD2	N	24.1 (3.4)	26.6 (3.0)
His-46CE1	O	19.6 (2.7)	21.5 (2.3)
His-44β1	P	18.7 (2.6)	13.5[a]
His-69β2	Q	−6.2 (3.3)	
His-44β2	R	−6.2 (3.3)	−8.7 (3.2)
His-46β2	X1	12.4	14.7
His-118β2	X2	10.9	11.7
His-118β1	f	3.1	2.4
His-118α	k	4.5	4.1
His-46β1	n	6.3	6.3

For comparison purposes the assignments of BSOD are reported. In parentheses the values of the longitudinal relaxation times (T_1) for the isotropically shifted resonances are indicated. Resonances are labelled as previously reported [24].
[a]Not measured because it is under a complex envelope.

The similar chemical shifts of the resonances belonging to His-46 and His-118 to those observed in the bovine enzyme [23–25] indicates that the corresponding protons have a similar magnetic environment and suggests that the structural arrangement of these two histidines is the same in the two enzymes. Small differences in chemical shift exists for resonance L, belonging to His46CD2 but, in this case, the observation of intraresidues NOEs with the β-protons located at similar chemical shifts of the bovine enzyme [27], suggests that the conformation of this residue is not very different. On the contrary, the protons of His-44 are in a different position with respect to the eukaryotic enzymes, suggesting a different orientation of this ligand. Further, the signal belonging to the His44NE2 proton readily disappears in the presence of deuterium oxide (not shown), at variance with that reported for the bovine enzyme [26], indicating a greater exposure to the solvent of this proton.

The remaining signals are due to protons of the cobalt-ligands and their position in the spectrum shows that some differences exist with respect to the eukaryotic enzymes. This is consistent with the differences observed between the optical spectra of the ESOD and BSOD in the electronic spectrum of the cobalt ion. These resonances of cobalt ligands have shorter relaxation times than the signals of copper-ligands and their signals have larger linewidths [26]. Thus, the corresponding crosspeaks are too weak to be detected in NOESY spectra. An exception is the cross-peak observed between the methylene geminal pair of Asp-81: these two protons are located at a very short distance (1.77 Å) and their corresponding cross-peak then shows a larger intensity. Therefore, the cross-peak between 44.1 and 30.3 ppm was ascribed to the β-protons of Asp-81, because these are the only geminal protons that have

been observed in this region. Also in this case the chemical shifts of these protons show a significant difference with respect to the bovine enzyme (Table 1).

Thus, the data obtained suggest differences in both the copper and the cobalt ligands: three of the four copper-ligands, His-61, His-46 and His-118, have an orientation similar, although not identical, to the bovine enzyme, while significant differences exist for His-44; Asp-81, and, perhaps, other cobalt-ligands, show a different orientation.

Hence, the lack of the copper-coordinated water molecule results in a different geometry of the active site.

Despite these differences, a titration with azide revealed that the resonances in the NMR spectrum of Cu,Co-ESOD follow the same trend observed for the bovine enzyme, where the resonances belonging to His-46 shift toward the diamagnetic region and those belonging to His-44 experience a larger isotropic shift. Also, an affinity constant of 150 M^{-1}, identical to the value observed for the Cu,Co derivative of the bovine enzyme [28], was obtained. Similar to the bovine enzyme, the resonances belonging to the cobalt ligands are weakly affected by azide. This result indicates that the two enzymes bind azide with similar modalities and that, upon binding of this substrate analog, His-46 undergoes a rotation of its imidazole ring. Also, the activity of the Cu,Co derivative was similar to that observed for the bovine enzyme.

Our studies on ESOD provide novel hints to understand the role of the copper-coordinated water molecule. This water molecule is present in all the other natural Cu,Zn-SODs so far studied. Several studies have questioned its possible involvement in the correct disposal of the substrate and/or in the efficient proton transfer during catalysis. In fact, in the second step of the enzymatic mechanism, two protons are required to convert the superoxide anion to hydrogen peroxide. One proton comes from the imidazole NE2 of His-61 when it reforms its bond with copper, while the second one is probably obtained from solvent molecules. ESOD, which lacks the copper-coordinated water molecule has a catalytic activity comparable to that of bovine enzyme [11]. The above reported azide binding studies suggest that the catalytic reaction proceeds via the same mechanism in both the enzymes. Therefore, it may be argued that, at least in the *E. coli* enzyme, the copper-coordinated water molecule is not necessary for efficient delivery of the second proton. Moreover, it should be noticed that a similar result was suggested for eukaryotic enzymes on the basis of a study of the human Thr137Ile mutant, whose catalytic activity and affinity for anions are unchanged, at least at pH values close to neutrality, in spite of the lack of the copper-coordinated water molecule [29].

An alternative role for the water molecule coordinated to copper is suggested by recent crystallographic studies on Cu,Zn-SODs from different sources [30–32]. These studies have shown that a well defined water network is present both in the oxidized and reduced enzyme. Hough and Hasnain have proposed that a hydrogen-bonding network which involves the water ligand could be important to stabilize the catalytically crucial Arg-141, whose position in the active site is not affected by the copper oxidation state [31]. The importance of Arg-141 stabilization is demonstrated by poor catalytic activity of a monomeric human Cu,Zn-SOD mutant, where subunit dissociation induces significant mobility in several loops leading to rearrangements of the copper site, including a high disorder of the Arg-141 residue [32]. In ESOD the ordered conformation of the Arg-141 side chain is ensured by a salt-bridge, found uniquely in this Cu,Zn-SOD, with the nearby residue Glu-56 [10]. In this case a network of hydrogen bonding water molecules is not necessary to reduce the disorder of the guanidinium group, thus explaining why this enzyme may possess a high catalytic activity despite the lack of the copper-coordinated water ligand. In conclusion, our studies on ESOD and the analysis of the available structural data allow us to suggest that the main role of the water ligand could be that of stabilizing the Arg-141 residue in the correct orientation for optimal catalysis.

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