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X-RAY, NMR AND MOLECULAR DYNAMICS STUDIES ON REDUCED BOVINE SUPEROXIDE DISMUTASE: IMPLICATIONS FOR THE MECHANISM

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Summary: Single crystals of the reduced form of Cu, Zn superoxide dismutase (space group $P2_12_12_1$, one dimer per asymmetric unit) have been obtained and their X-ray structure refined at 1.9 Å resolution. The structure shows that the imidazolate bridge is maintained in the present crystalline form. It is confirmed that in solution the bridge is broken and the involved histidine is protonated on the side of copper. Based on the NOE constraints, and with the aid of molecular dynamics calculations, a structural model is proposed for the molecule in solution. Both structures are considered significant as far as the enzymatic mechanism is concerned. © 1994 Academic Press, Inc.

The dimeric enzyme copper,zinc superoxide dismutase (SOD), that catalyzes the dismutation of the superoxide ion into molecular oxygen and hydrogen peroxide (1-6), may exist in an oxidized and in a reduced form (E^0 = 403 mV at pH 7 and 298 K (7)). The oxidized enzyme has been well characterized through X-ray investigation (8-13) and physical method studies (2,14-17) on several isoenzymes and metal substituted derivatives. The

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copper ion, which is essential for catalysis, is coordinated in the oxidized state to four histidines, one of which is bridging copper and zinc (8,18). UV/visible absorption spectroscopy (19), NMR (20-22), and EXAFS data (23) are available for the reduced SOD, which provide evidence for the breaking of the copper-nitrogen bond of the bridging imidazolate and simultaneous protonation of the nitrogen Nε2 of the same residue (20-22).

We have solved the X-ray structure of the reduced SOD and we have found that in its crystalline form the bridge is maintained. We have then collected the ^{1}H NMR spectra in solution and, through NOE measurements and molecular dynamics (MD) simulations, a different solution structure is proposed.

METHODS

X-ray Investigations. Crystals of $Cu(I)_2Zn_2SOD$ were obtained by slowly diffusing polyethylene glycol (PEG6000) in Tris·HCl buffered solutions at pH 7.5 of the native bovine enzyme (Sigma Chem. Company) reduced with sodium dithionite under nitrogen atmosphere. The crystals of reduced SOD were colorless and the EPR spectrum of the crystal used for data collection was featureless before and after exposure to X-rays. Crystals of the oxidized enzyme of comparable size showed the EPR lines typical of copper(II). Crystals sealed in glass capillaries with their mother liquor were stable to reoxidation for several weeks. The crystals were orthorhombic, space group $P2_12_12_1$, with cell constants a=47.66, b=50.90, c=147.42 Å, one dimeric molecule per asymmetric unit (MW 32,000). The crystals were different from those used for the crystal structure of the bovine $Cu(II)_2Zn_2SOD$ (8), but isomorphous to those obtained for the semisynthetic bovine $Cu(II)_2Co_2SOD$ structure (11). Data were collected at the EMBL Hamburg Outstation using the synchrotron radiation source at the X 31 beamline on the DORIS storage ring, with a locally developed image plate system. The experiment was performed twice on different crystals from different enzyme batches in order to check the reproducibility of the results. The positions and the temperature factors of 2197 protein atoms and of 582 solvent water molecules were refined against 28337 unique reflections (97% of the theoretical reflections in the 20.0 to 1.9 Å resolution range). The r.m.s. coordinate shift in the final cycle was 0.009 Å and the deviations from ideal values of the refined model are as follows: bond length 0.015 Å, bond angles 0.045 Å, torsion angles 0.053 Å, planar groups 0.014 Å (3° ≅ 0.07 Å). The starting coordinates for the refinement were those from the isomorphous semisynthetic bovine $Cu(II)_2Co_2SOD$, refined at 2.0 Å resolution to an R factor of 17.6 % (11) and deposited with the Protein Data Bank (24). Structure refinement was performed by restrained least-squares methods, with manual checking and rebuilding on an Evans and Sutherland PS 390 interactive graphics system. The computer programs used were PROLSQ (25) for the least-squares calculations and FRODO for model building (26). The final R factor was 16.1 %. The upper limit for the average error in atomic positions, as estimated from a σ_A plot, is 0.15 Å, but for the metals and their coordinated atoms the error should be substantially less. A comparison between the two zinc geometries in the dimeric unit suggests estimated standard deviations of 0.10 Å and 2.0° for metal-ligand bond lengths and angles, respectively. The model derived for the reduced SOD has been compared with the $Cu(II)_2Co_2SOD$ (11), since the coordinates available from PDB for the native $Cu(II)_2Zn_2SOD$ refer to a less accurate model (R = 25.5 %) (8). Least squares superposition of the two backbones results in a r.m.s. deviation of 0.21 Å. In any case, the present structure is also similar to the monoclinic $Cu(II)_2Zn_2SOD$ (8).

NMR and MD Calculations. NMR experiments were carried out on a Bruker AMX 600 spectrometer operating at 14.1 T. The NOE data were obtained on millimolar water solutions of $Cu(I)_2Zn_2SOD$ in phosphate buffer at pH 5.5 to ensure slow exchange of

histidine NH protons. The pK_a of HNε2 of His 61 has been determined to be above 10 (22). The NOE experiments, as a function of the signal saturation time, were performed with a pulse sequence and a methodology already reported (27). From the analysis of these NOE build-up curves, proton-proton distances were evaluated.

MD calculations have been performed by using the AMBER 4 package (28). Restrained MD trajectories have been calculated for 139.5 ps following the same protocol used for the oxidized form (29). and including the NOE derived constraints by applying a harmonic potential function as defined in the SANDER module of AMBER (28), with a force constant of 133.9 kj mol^{-1}. The *ab initio* Mulliken charges for the active site have been determined with the HONDO (30) program present in the MOTECC-90 package, at the STO-3G level. The partial charges on the atoms present in the active site of the reduced form have been calculated following the same procedure used by Shen *et al.* (31) for the oxidized SOD. At variance with the oxidized form, in the present calculations the Nε2 nitrogen of His 61 is protonated. The partial charge of copper in the reduced form results to be +0.09, to be compared to +0.65 obtained for the oxidized SOD with the same basis set (31), consistent with the reduction of the copper ion.

RESULTS AND DISCUSSION

Crystals grown from reduced SOD solutions belong to the space group $P2_12_12_1$. The structure has been determined at 1.9 Å resolution, as described in the Methods section. The overall structure is practically superimposable to that of the oxidized protein. The copper coordination geometry (Fig. 1A) does not show remarkable differences with respect to the oxidized enzyme except for a trend towards longer coordination bonds (as expected for a reduced copper ion), although the differences are of the order of the estimated standard deviation. Table 1 reports coordination bond lengths around the copper atoms in the oxidized and reduced crystalline forms. It is evident that no detachment of His 61 occurs upon reduction. It can be also noted that the two subunits of the dimeric enzyme have slightly different active site geometries. However, the histidinate bridge is clearly maintained in both subunits.

The present findings are in constrast with: 1) The NMR studies on the reduced form in solution of both bovine and human isoenzymes (20,21); 2) the preliminary results of an X-ray structure determination of reduced bovine enzyme in a different crystal form (space group C2) (32), according to which copper(I) is not coordinated to His 61.

We have repeated the NMR experiments on a sample of reduced recombinant human enzyme with the aim of obtaining as much as possible structural information in solution. From quantitative analysis of the intraresidue NOE data, no internal mobility has been observed in any of the metal ion ligands. Owing to the unfavorable histidine disposition, only six inter-histidine NOEs could be observed. By measuring their intensity as a function of time, as accurate as possible (± 0.2 Å) inter-residue distances have been obtained. These are: His 44 HNε2-His 61 Hε1, 3.5 Å; His 44 Hε1-His 61 HNε2, 3.1 Å; His 44 HNε2-His 69 Hε1, 3.0 Å; His 44 HNε2-His 69 HNε2, 3.7 Å; His 46 Hε1-His 61 Hδ2, 3.7 Å; His 61 Hε1-His 69 Hε1, 2.7 Å. The detection of the His 61 HNε2 proton signal and of the NOEs between the His 61 protons and protons of other residues are consistent with a picture according to which the protonated (formerly bridging) His 61 moves towards the opening of the active site cavity.

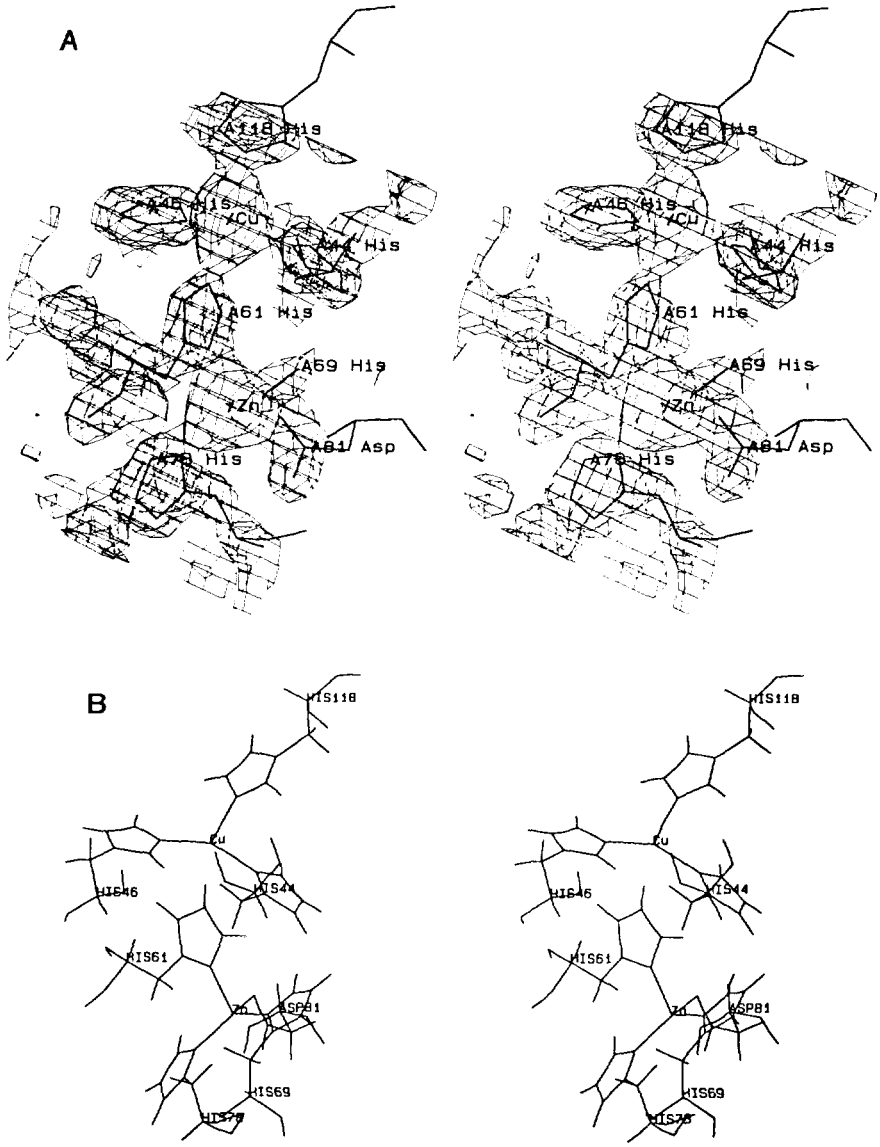


Figure 1. (A) Stereo view of the active site residues of the reduced bovine SOD (monomer A, residues 1 - 153) superimposed to a 2|Fo| - |Fc| electron density map contoured at 1.5 σ, as it results from the X-ray structure. For clarity His 69 is not shown. (B) Stereo view of the active site structure of reduced SOD as obtained from NOE-restrained MD calculations.

The above NOE measurements have been performed at pH 5.5 in order to better detect exchangeable protons. The NMR features, and therefore the structural conclusions, are otherwise absolutely identical up to pH above 9.

Table 1. Bond lengths (Å) around copper in both sites (A and B) of $Cu(I)_2Zn(II)_2SOD$ (red) and $Cu(II)_2Co(II)_2SOD$ (ox) (bovine SOD numbering)

Ligands	Cu (A - red)	Cu (A - ox)[a]	Cu (B - red)	Cu (B - ox)[a]
Nδ1-His 44	2.18	2.10	2.08	2.03
Nε2-His 46	2.30	2.28	2.30	2.18
Nε2-His 61	2.21	2.16	1.93	1.99
Nε2-His 118	2.16	2.13	2.10	2.14
Water	3.10	2.87	2.42	2.37

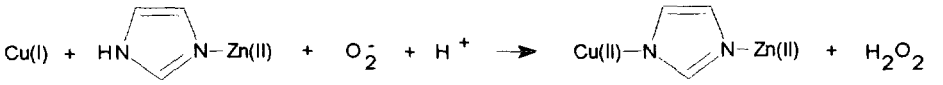
[a]The comparison is made with the oxidized form of the cobalt derivative (11) because its accuracy is comparable with the present structure and higher than that of the native oxidized protein (8). The bond lengths of the latter are the same as those of the cobalt derivative within their standard deviation.

Some of us had succeeded in refining the oxidized SOD structure through MD calculations (29,33). MD calculations are well suitable for the refinement of solution structures and for the obtainement of structural models in solution (34,35). MD calculations have now been performed also on the reduced form, using the active site NOEs as constraints. These calculations indicate that the overall protein backbone structure in solution of the reduced and oxidized forms is almost identical. A picture of the active site of the reduced form, as obtained from the above calculations, is reported in Figure 1B, where it is shown that His 61 moves to a new position, with the protonated Nε2 nitrogen more than 3 Å from the copper ion, while the remaining part of the coordination polyhedron remains essentially unaffected. The active site channel in the MD reduced SOD structure undergoes similar mobility as in the MD oxidized SOD structure (29) (not shown). In particular, both systems experience a large movement of Arg 141 which determines an opening of the active site channel. The overall backbone structures of the oxidized and reduced MD models are essentially the same, as indicated by the r.m.s. deviation values with respect to the starting, X-ray structure (1.6 Å for the oxidized (29) and 2.0 Å for the reduced form).

This is a striking case in which the single crystal X-ray structure dramatically differs from the solution structure. Furthermore, the NMR data have indicated, in both the oxidized (27,36) and the reduced forms (20,21,37,38) of the enzyme, that the two sites are indistinguishable on the NMR time scale, whereas the X-ray structure shows some differences.

The present X-ray results demonstrate that a reduced form with the bridging histidine is close to an energy minimum. This may be significant with respect to the enzymatic mechanism. It is widely accepted that the substrate reduces copper(II) providing a derivative with the broken imidazolate bridge (19,39)

and that the reduced derivative is successively oxidized by another molecule of substrate, according to the following scheme (40,41):



The observed overall catalytic rates are independent of pH in the 5 - 9.5 range.

This complex mechanism is hardly consistent (42) with a maximal turnover of about 10^6 s^{-1} as found for the enzymatic reaction under saturating conditions (42). Indeed, too many chemical bonds have to be broken and formed again. On the contrary, for low concentrations of O_2^- (19,39) (i.e. for slower turnover rates), as it occurs *in vivo*, a mechanism involving the breaking of the imidazolate bridge is quite acceptable. Under the latter conditions the oxidized and reduced species have the same activity (40,41,43), as expected on electrostatic grounds because the total charge of the active site remains the same in both oxidation states. The high turnover rate under saturating conditions would be more consistent with a bridging histidine in both oxidized and reduced forms (42). The needed protons would be directly provided by the solvent. Under these circumstances the mechanism based on a Cu^{2+}-O_2^- intermediate, stabilized by a hydrogen-bond formed by the terminal oxygen and Arg 141, may be also reconsidered (44,45). The Cu^{2+}-O_2^- moiety would react with O_2^- to give O_2, and the remaining Cu+-O_2^- provides H_2O_2 and the oxidized enzyme (44,45). The Cu-Zn bridge breaking would be unnecessary.

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