Long-distance charge redistribution upon Cu,Zn-superoxide dismutase reduction:

significance for dismutase function

François Dupeyrat\textsuperscript{a}, Claude Vidaud\textsuperscript{b}, Alain Lorphelin\textsuperscript{b} and Catherine Berthomieu\textsuperscript{a*}

\textsuperscript{a}CEA/Cadarache, Laboratoire de Bioénergétique Cellulaire, UMR 6191 CNRS-CEA-Aix-Marseille II,
Univ.-Méditerranée CEA 1000, Bât. 156, F-13108 Saint-Paul-lez-Durance, Cedex, France.

\textsuperscript{b}CEA/Valrhô, DSV-DIEP, Service de Biochimie post-génomique et Toxicologie Nucléaire, 30207
Bagnols sur Cèze, France.

* corresponding author : catherine.berthomieu@cea.fr
Phone : 33 4 42 25 43 53
Fax : 33 4 42 25 47 01, e-mail :

Running title: Effect of Cu,Zn-SOD reduction on the backbone and active site

Key words: recombinant chloroplastic SOD, protein electrochemistry, FTIR spectroscopy,
methylimidazole Cu ligand, \textsuperscript{15}N-labeling.
SUMMARY

Cu,Zn superoxide dismutase (Cu,Zn-SOD) is an ubiquitous enzyme with essential role in antioxidant defense. To better understand structural factors at the origin of the highly efficient superoxide dismutation mechanism, we analyzed the consequence of copper reduction on the electronic properties of the backbone and individual amino acids using electrochemistry coupled to Fourier transform infrared spectroscopy. Comparison of data recorded with bovine erythrocyte and recombinant chloroplastic Cu,Zn-SOD from Lycopersicon esculentum, expressed as a functional tetramer in Escherichia coli and 14N- or fully 15N- labeled, demonstrated that the infrared changes were dominated by reorganizations of peptide bonds and histidine Cu ligands. Two main infrared modes of histidine side-chain, markers of metal coordination, were identified using Cu- and Zn- methylimidazole models: the ν(C_4,C_5), at 1605-1594 cm\(^{-1}\) or =1586 cm\(^{-1}\) for Nτ or Nπ coordination, and the ν(C_5,Nτ), at = 1113-1088 cm\(^{-1}\). These modes, also evidenced in Cu,Zn-SOD using 15N-labeling, showed that the electronic properties of the histidines Nτ ligands of Cu are mostly affected upon Cu reduction. A striking conclusion of this work is that peptide groups from loops and β-sheet largely participate in charge redistribution upon Cu reduction while, in contrast, electronic properties of polar and charged amino acids of the superoxide access channel remain unaffected. This is notably shown for the strictly conserved Arg143 by site-directed mutagenesis on chloroplastic Cu,Zn-SOD. Charge compensation by the peptide backbone and preserved electronic properties of the superoxide access channel and docking site upon Cu reduction may be determinant factors for the high reaction kinetics of superoxide with both reduced and oxidized Cu,Zn-SOD.
Introduction

Superoxide dismutases (SOD) play a determinant role in protection against the toxic effects of oxidative stress by the scavenging of superoxide radicals and their conversion into oxygen and hydrogen peroxide \( (I) \). Four different classes of SOD have been distinguished depending on the metal at the active center, Mn, Fe, Cu and Zn, or Ni \( (2-4) \). Cu,Zn-SOD are ubiquitous. They appear as homodimers in the cytosol, the nucleus and peroxisome of eucaryotic cells and the inter membrane space of mitochondria. They exist as homotetramers in the chloroplast of higher plants and as monomers, in the periplasm of some gram negative bacteria \( (3) \).

The monomer fold and the active site are highly conserved within Cu,Zn-SOD according to structural data \( (5-10) \) and high homologies of protein sequences \( (11-13) \). The monomer consists in a β-barrel and the active site defined by two loops (IV and VII) at the surface of the barrel. The active site comprises the redox active Cu with square planar coordination to four histidines (Scheme 1A), one of each is a bridging ligand of Cu and Zn. The other Zn ligands are two histidines and a monodentate aspartate \( (5, 7, 8) \). A channel across the protein leads to the Cu, towards a fifth axial coordination position, occupied by a water molecule in the oxidized enzyme, which is available for inhibitors and possibly for the superoxide substrate (Scheme 1B, \( 14-16 \)).

SOD and particularly Cu,Zn-SOD are characterized by a very high reaction rate \( (2\times10^9 \text{ M}^{-1} \text{s}^{-1}) \), near the diffusion rate of superoxide which is pH independent between 4.8 and 9.7 \( (3, 17) \). Experimental data demonstrated the role of a positive electrostatic gradient formed by charged residues on the superoxide access channel, including a strictly conserved arginine (Arg141, bovine erythrocyte SOD numbering - corresponds to Arg143 for plants and human Cu,Zn-SOD) to steer highly efficiently the anionic superoxide towards the buried Cu \( (18-20) \). Up to now, the high reaction rate prevented the elucidation of reaction intermediates in superoxide dismutation. Another specificity of these enzymes is their equivalent efficiency for both reactions of superoxide oxidation and reduction \( (21, 22) \). These reactions are coupled with the reduction and oxidation of the Cu,
respectively. Structural factors at the origin of this specific and efficient dismutase mechanisms are not fully understood.

Thorough analyses at the molecular level of structural changes that accompany Cu reduction have been undertaken to detail the active site properties at the origin of the efficient superoxide dismutation and of the protonation events associated to its reduction into hydrogen peroxide (3, 5, 10, 23-34). A main result concerns the rupture of the interaction between reduced Cu(I) and the His63 bridging ligand, as deduced from NMR (23, 24, see also 10, 25), EXAFS (26, 27) and Raman spectroscopy (28-30) for Cu,Zn-SOD in solution. Three-dimensional crystallographic structures of reduced Cu,Zn-SOD presented heterogeneity of the active center (16, 31-33), but a recent high-resolution structure provided convincing evidence for three coordinated Cu(I) (34), in agreement with other spectroscopic data.

This rupture of the Cu-His61 bond upon Cu reduction was the base of the generally accepted reaction mechanism of Tainer et al. (35), in which His61 provides one of the protons necessary for superoxide reduction into hydrogen peroxide at Cu(I). Indeed, NMR data paralleled the loss of Cu,Zn-SOD activity at high pH with His61 deprotonation (36). Also, coordination of His61 to Zn would determine the large pH independence of Cu,Zn-SOD activity (37).

Binding of superoxide as a ligand of Cu(II) and Cu(I) is debated, so as inner or outer sphere electron transfer mechanisms to reduce (oxidize) superoxide (35, 38), and the specific role of Arg141 as a docking site for superoxide in reduced Cu,Zn-SOD was proposed (16, 38, 39).

Experimental data demonstrated thus a crucial role for Arg141 and for charged and polar residues building an electrostatic field in the superoxide access channel for the efficient function of Cu,Zn-SOD (18-20, 38-41) as a dismutase. Since this electrostatic field was predicted from calculations to be contributed not only by charged side chains but also by the Cu and polypeptide backbone (42), we decided to monitor directly the changes in electronic properties of highly conserved amino acids and of peptide bonds upon Cu reduction using Fourier transform infrared (FTIR) spectroscopy. A former study comparing second derivatives of infrared absorption spectra of oxidized or reduced Cu,Zn-SOD
concluded that conformational changes occur at the level of loop and β-sheet structures upon copper reduction (43). In this work, we used electrochemically-induced FTIR difference spectroscopy (44, 45, for reviews on the technique) to detect, on the same sample, minute perturbations of the electronic properties or hydrogen bonding interactions of both amino acid side chains and of the polypeptide backbone.

We analyzed the redox-induced infrared changes detected on dimeric bovine erythrocyte (BeSOD) and tetrameric chloroplastic (ChSOD) Cu,Zn-SOD of Lycopersicon esculentum, which differ at the level of charged and polar residues of the superoxide access channel (3, 12, 42, 46). Recombinant ChSOD was expressed in E. coli and 15N labeled to assign with confidence the vibrational modes from histidine Cu ligands, peptide groups, or conserved amino acid side chains. Site directed mutants at the strictly conserved Arg143 and Thr137 (plant numbering), located in the superoxide access channel were performed to precise their contribution to the electrostatic field reorganization upon Cu reduction in ChSOD.

Experimental Procedures

Samples preparation

BeSOD was purchased from Sigma. Gel electrophoresis on different batches of samples showed that the commercial preparation was sufficiently pure and could be used directly for the FTIR spectroscopic studies (Supplementary Figure 1A). The BeSOD concentration was estimated by the absorbance at 680 nm using $\varepsilon_{680} = 300 \text{M}^{-1}\text{cm}^{-1}$ and the specific activity determined spectrophotometrically, by the measure of the inhibition of cytochrome c reduction by xanthine oxidase, as described in (47). The BeSOD samples had specific activities of $\approx 3000 \text{ U / mg}$. Samples were concentrated to 2 mM either in 50 mM Tris/HBr pH 7.6 or in 50 mM Ches pH 9.3. Except otherwise mentioned, all electrochemical experiments were performed in the presence of 100 mM KBr as supporting electrolyte.
For \( ^1\text{H}_2\text{O}/^2\text{H}_2\text{O} \) exchange, the buffers in \( ^2\text{H}_2\text{O} \) were dried using a speed vacuum system and dissolved in \( ^2\text{H}_2\text{O} \) two times subsequently. The concentrated protein solution in \( ^1\text{H}_2\text{O} \) (10 µL) was diluted in 400 µL of deuterated buffer, maintained for two hours at room temperature and concentrated to =10 µL using a microcon 10 (Millipore) system. This sample was diluted again in 400 µL deuterated buffer, incubated for two weeks at 10°C for extensive \( ^1\text{H}/^2\text{H} \) exchange, and washed again by concentration / dilution steps in \( ^2\text{H}_2\text{O} \)-buffer before analysis by FTIR spectroscopy.

**Expression of chloroplastic Cu,Zn-SOD**

The cDNA gene of \( \text{ChSOD} \) from \( \text{Lycopersicon esculentum} \) was kindly provided by Dr. Perl-Treves (46) on the pGEM2 plasmid (Promega). A pair of restriction-enzyme sites (NdeI at the 5’ end and BamHI at the 3’ end, bold faced characters) were incorporated flanking the cDNA gene of \( \text{ChSOD} \) by PCR amplification using the oligonucleotide primers:

\[
5’-\text{GTCCAGTTCATATGGCTACTAAGAAAGCTGTTG}-3’ \quad \text{and} \quad 5’-\text{ATCTGGATCCTCA-TATTGGAGTCAAACCAACCACA}-3’. 
\]

The 480 bp PCR amplified fragment was purified, digested by NdeI and BamHI, and cloned into the NdeI/BamHI- digested pet11-a expression plasmid from Stratagene (Stratagene, La Jolla, USA) using the \( \text{E. coli} \) strain DH5α. The resulting plasmid pChSOD-1 was used to transform \( \text{E. coli} \) BL21 plys DE3 strain, allowing IPTG induced expression of the recombinant \( \text{ChSOD} \). All digestions, ligations, transformations and other manipulations were performed using standard protocols (48). The DNA sequence was verified by sequencing.

The pChSOD-1 transformed \( \text{E. coli} \) BL21 plys DE3 cells were grown in minimal M9 medium in the presence of thiamin, ampicillin and chloramphenicol at 10 µg/mL, 100 µg/mL and 25 µg/mL respectively. Induction was triggered by the addition of IPTG at 1 mM/L when the cultures reached an optically density (\( A_{600} \)) of 0.5. The cells were harvested 4 hours after induction.

For the production of uniformly \( ^{15}\text{N} \) labeled \( \text{ChSOD} \), the same supplemented M9 medium was used except that \( ^{15}\text{N} \) labeled ammonium chloride (1 g/L) was used as exclusive nitrogen source. Both overnight culture and cultures in 50 mL Erlenmeyer used to inoculate the 1 L flasks were performed.
with the fully $^{15}$N-labeled M9 medium. The extend of $^{15}$N-labeling of the ChSOD was controlled by matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectroscopy. Simultaneous injection of $^{14}$N- and $^{15}$N- labeled ChSOD showed a mass difference of 200 Da between the $^{14}$N- and $^{15}$N-labeled ChSOD, which agreed with a fully $^{15}$N labeled protein (Supplementary Figure 2).

Site directed mutagenesis of ChSOD

The site directed mutants were obtained by two steps of PCR using the oligo nucleotides 1 and 2 previously described and the following oligo nucleotides 3 and 4 including the mutation (bold faced characters), 5'-'ATGCTGTTGGACAAATTGGCATGC-3' and 5'-GCATGCCAAATTGTCCACCAGCAT-3' for the Arg143Gln mutant and 5'-CAGCTTTACCCAGAAATGCTG-3' and 5'-CAGCATTGCCCTTGGTAAGACTG-3' for the Thr137Gln mutant. For each mutant, in a first step, two PCR were performed with oligos 1 and 4 and with oligos 2 and 3. The PCR products from this first step were purified, mixed at a 1 to 1 ratio and used to perform the second PCR step to amplify the whole gene using the initial oligonucleotides 1 and 2.

ChSOD purification.

Typically, the cells from 8 liters culture were harvested by centrifugation for 10 min at 7000 g. The cells suspended in K-phosphate 10 mM at pH 7.8, KCl 8 mM were passed twice through a French press (at 14 MPa). The suspension of soluble proteins was obtained after centrifugation at 5000 g for 10 min at 4 °C followed by centrifugation at 200000 g for 1 hour at 4 °C to discard unbroken cells and membranes. This cell-free extract was loaded onto a DEAE column (80 mL) equilibrated with K-phosphate 5 mM at pH 7.8, KCl 8 mM. The column was developed using a rapid KCl gradient from 8 to 200 mM. The ChSOD containing fractions were selected using a rapid liquid test, based on the SOD-inhibition of light-induced riboflavin reduction of nitroblue tetrazolium (NBT) in the presence of the electron donor methionine. The ChSOD fractions were pooled and applied in six independent runs to a Resource column (6 mL) (Pharmacia). The active enzyme eluted with (14-16 volumes of column) K-
phosphate 10 mM at pH 7.8, KCl 8 mM. The specific activity of this fraction was determined at 3300 U.mg⁻¹, using the Xanthine oxidase/xanthine/cytochrome c method described in (47). Protein gel- electrophoresis in denaturing conditions showed that the ChSOD fractions obtained after the two-steps purification procedure contained only modest contributions from contaminant proteins at 40 and 28 kDa (Supplementary Figure 1B). The gel electrophoresis performed in non-denaturating conditions showed the main band at = 60 kDa indicating that the tetrameric form of ChSOD could be preserved throughout the purification procedure. Using this purification procedure and expression in minimal medium, 1.75 mg tetrameric ChSOD were obtained per culture liter.

**Spectra of model compounds**

FTIR spectra were recorded with solutions of MeIm and CuCl₂ with MeIm to Cu ratios ranging from 1 to 20. Upon increasing concentration of CuCl₂, the IR modes of MeIm in solution were substituted by IR modes characteristic of methylimidazole (MeIm) bond to Cu. These signals were maximized with a Cu to MeIm ratio of 1 to 4. Thus, the FTIR spectra of Cu(II) and Zn(II) - MeIm complexes were recorded in solution at pH 8 with methylimidazole to CuCl₂ or ZnCl₂ at metal to MeIm ratio of 1 to 4.

**Sample preparation for FTIR spectroscopy**

For spectro-electrochemistry, the Cu,Zn-SOD concentration was comprised between 0.8 and 2 mM. Electrochemistry was performed in the optical transparent thin layer electrochemical cell developed by D. Moss and W. Mäntele (49), using a 4 µm thick gold mesh as working electrode (Buckbee Mears, Minnesota, USA). The working electrode was surface-modified by dipping it for 5 min in a 5 mM pyridine-3-carboxaldehyde thiosemicarbazone (PATS-3, purchased from Lancaster) solution heated to 80-90°C. Excess PATS-3 was thoroughly removed with 18 MΩ Millipore water, and the gold grid was dried on CaF₂ window. PATS-3 binds irreversibly to the gold surface through the sulfur atom and interacts with the proteins in solution through the pyridine and amine groups (50). The reference electrode consisted in an Ag/AgCl/KCl(3M) system (Eₘᵋ = 208 mV/NHE). Thereafter,
all redox potentials are given versus NHE. A platinum counter electrode at the periphery of the cell was used to compensate for the potential drop caused by current. Electrochemistry was performed at 4 °C (6 °C in $^2$H$_2$O) in the presence of the electrochemical mediators ferricyanide ($E_m = 430$ mV), pBQ ($E_m = 280$ mV), DCIP ($E_m = 217$ mV), methylviologen ($E_m = -440$ mV), each at 100 to 300 µM final concentration. Several of the usually used redox mediators are not efficient for Cu,Zn-SOD reduction (oxidation) because the copper site is deeply buried in the protein. We selected only mediators which were shown to accelerate Cu,Zn-SOD reduction (oxidation) (51).

The potential was applied to the electrochemical cell using an EG&G (model 362) potentiostat, triggered by the FTIR spectrometer. The redox titration of the Cu,Zn-SOD Cu center was performed by monitoring the potential-dependent UV-Vis absorption spectra, using the same electrochemical cell and conditions as for FTIR. UV-Vis spectra were recorded with a Varian Cary 50 spectrophotometer. The data were fitted with a Nernst curve using the Erithacus software Grafit.

Infrared spectroscopy

FTIR spectra were recorded at 4 cm$^{-1}$ resolution, with a Bruker 66 SX spectrometer equipped with a KBr beam splitter and nitrogen-cooled MCT-A detector. The absorption maximum of the sample was optimized at 0.85 - 0.9 a.u. at 1645 cm$^{-1}$. Single beam spectra recorded before and after a change of the applied redox potential were subtracted to calculate the redox-induced difference spectrum. Typically, 300 scans were accumulated for each spectrum recorded for one electrochemical cycle. The results from 10 to 40 independent redox cycles were averaged to improve the signal to noise.

Results

Electrochemistry of Cu,Zn-SOD

Electrochemical reduction (oxidation) of the Cu active center of BeSOD was performed at a gold working electrode with PATS-3 as surface modifier (see materials and methods) and ferricyanide, p-benzoquinone, DCIP and MV as mediators (51). A cyclic voltammogram recorded at 10 mV s$^{-1}$ in the thin-layer electrochemical cell used for FTIR spectroscopy (49) showed both anodic and cathodic peaks, which defined a midpoint potential of $= 120$ mV/NHE (Figure 1-A). Reduction of BeSOD
could also be obtained by direct electrochemistry at the PATS-3 modified electrode but its reoxidation
necessitated the presence of redox mediators. The redox dependence of the UV-Vis absorption of
BeSOD was analyzed in the 500 to –400 mV range, as shown in Figure 1-B. Potential steps of 25 to
50 mV were performed and the sample was equilibrated during 12 min at each potential before
collecting the UV-Vis spectrum. The absorption changes at 265 nm could be fitted with a Nernst curve
corresponding to a one electron reduction, with a midpoint potential of 130 ± 20 mV (vs NHE) at
pH 7.4.

A large range of midpoint potentials (120 - 525 mV) has been reported for Cu,Zn-SOD (51,
52-58). In particular, midpoint potentials ranging from 260 to 525 mV/NHE have been obtained for
Cu,Zn-SOD using cyclic voltammetry at high sweep rates, depending on the electrode surface
modifier, 1,2-bis(4-pyridyl)-ethene (53-54), cysteine- or 3-mercaptopropionic acid (55-56), bis(4-
pyridyl)disulfide (4,4’-PySSPy), or His or Arg solutions (57). These differences in midpoint potential
may correspond to interactions formed by the protein at the modified electrode surface. In the
reductive titration of BeSOD reported here, we performed a total reduction of BeSOD and measured
the midpoint potential of spectroscopically detected BeSOD which is not adsorbed at the gold
electrode. In these conditions, the midpoint potential was determined at ≈ 130 mV. This E_m was close
to that obtained by Verhagen et al. (58) at the same pH, by chemical reduction or oxidation of Cu,Zn-
SOD using dithionite or ferricyanide in the presence of redox mediators. These authors suggested that
the high midpoint potential of 320 mV/NHE at pH 7.2 reported by Lawrence & Sawyer (51) may be
explained by an interaction between oxygen and high concentrations of ferricyanide used for the
titration (200 mM) for Cu,Zn-SOD samples at 0.7-1 mM concentration (58).

UV-Vis difference spectra corresponding to the complete electrochemical reduction (Figure 1-
C, bold line) or oxidation (thin line) of BeSOD were recorded applying oxidizing and reducing
potentials of 500 and –550 mV (vs. NHE) respectively. In these conditions, a complete reduction
(oxidation) of BeSOD was obtained within 8 minutes, with a high reversibility. Thus 10 to 40
consecutive electrochemical cycles could be performed to record the FTIR difference spectra associated with copper reduction or oxidation (Figure 2).

**FTIR difference spectra associated to Cu,Zn-SOD reduction**

The FTIR difference spectrum associated with copper reduction is shown in Figure 2-A thick line for BeSOD in H₂O. Positive bands are associated to the reduced Cu(I) state, while negative lines correspond to the oxidized Cu(II) BeSOD. Comparison of this spectrum with that obtained upon Cu reoxidation (thin line) demonstrated that only reversible redox-dependent IR modes were recorded. IR modes from peptide bonds and amino acid side chains sensitive to the Cu redox-state contribute in the FTIR spectra of Figure 2-A. We verified that reduced – minus- oxidized spectra recorded either in absence or in presence of redox mediators were almost identical, demonstrating that the mediators do not contribute significantly to the spectrum (not shown).

Fixation of fluoride and chloride at an anion binding site, involving Arg143 in the superoxide access channel of Cu,Zn-SOD was reported notably by NMR (59, 60). FTIR difference spectra obtained using 100 mM NaF, KCl, KBr, or NaClO₄ as supporting electrolytes presented however only very small differences (not shown) and all spectra discussed below were recorded with 100 mM KBr.

The largest bands of the FTIR difference spectra of Figure 2 were observed at 1686/1672/1651/1639 cm⁻¹ and at 1556/1540/1530/1515/1495 cm⁻¹. In these regions, dominating contributions from peptide ν(C=O) Amide I and ν(CN)+δ(NH) Amide II modes are expected (61-64). As compared to the maximum absorption of the protein sample, estimated at = 0.14 a.u. at 1648 cm⁻¹ after subtraction of the buffer absorption (not shown), the largest signals at 1672, 1651, and 1639 cm⁻¹ had amplitudes of 0.001 to 0.0025 a.u. (Figure 2). The total intensity changes thus concerned 1-2 % of the absorption of the whole carbonyl groups. For isolated ν(C=O) modes, this would correspond to the absorption of 2-3 carbonyls per BeSOD monomer. Actually, the Amide I difference bands resulted from minute perturbations of a larger number of coupled peptide ν(C=O) modes within secondary structures. In particular, the signal at 1639 cm⁻¹ is at a frequency typical for peptide bonds involved in β-sheet structures (61, 64). The intensity changes and frequency shifts of the Amide I IR modes
corresponded to changes in hydrogen bond strength or electrostatic interactions, and in orientation of the peptide dipoles, within the β-barrel and at the level of unordered or loop structures. These changes within the peptide backbone seemed a dominant contribution to the electrostatic reorganization accompanying Cu reduction in BeSOD.

IR signals from peptide groups may superimpose with those from amino acid side chains, for arginine, histidine or aspartate and glutamate (65-67). Also, spectra of Figure 2-A presented signals outside the Amide I and Amide II regions, at 1602(-), 1590(+),1233(+), 1223(-) or at 1112(+), 1097(-) cm⁻¹. To identify the respective contributions from the Cu histidine ligands, amino acid side chains, and from the polypeptide backbone to the reorganizations induced by Cu reduction, we analyzed the effect on the FTIR spectra of H₂O/²H₂O exchange and of the ¹⁵N-labeling of ChSOD. Indeed these labeling are expected to induce specific frequency downshifts for each type of IR mode.

**Effect of H₂O/²H₂O exchange**

The reduced – minus – oxidized spectra recorded with BeSOD in H₂O (thin line) and in ²H₂O (bold line) are superimposed in Figure 2-B. In the 1700-1630 cm⁻¹ region, only small changes were induced by the H₂O/²H₂O exchange. The main band at 1651 cm⁻¹ in H₂O remained unchanged, while the peaks at 1672 and 1639 cm⁻¹ were in part slightly downshifted to 1670 (-2) and 1634 (-5) cm⁻¹ in ²H₂O. Downshifts up to 15 cm⁻¹ are expected upon H₂O/²H₂O exchange for peptide carbonyls (68) involved in α-helices, loops, unordered structures and β-sheets (10, 69, 70), while downshifts of 60 cm⁻¹ are characteristic of arginine side chain modes (66, 67, 71). The signals described above were thus best interpreted as contributions from peptide carbonyls. The absence of shift on the largest band at 1651 cm⁻¹ indicated that a large fraction of peptide groups sensitive to the change in redox state of the Cu did not experience H/²H exchange. The bands at 1704(+) and 1693(-) cm⁻¹ in H₂O were downshifted by = 10 cm⁻¹ to 1694(+) and = 1684(-) cm⁻¹ upon H₂O/²H₂O exchange. These signals were thus tentatively assigned to one peptide v(C=O) IR mode, the frequency of which up-shifted from 1693 to 1704 cm⁻¹ upon BeSOD reduction. These spectra indicated that arginine side-chain modes did not contribute significantly to the redox-induced vibrational changes in BeSOD.
A downshift by about 100 cm\(^{-1}\) is expected for the Amide II mode upon deuteration of the peptide bonds (68). In the 1560-1500 cm\(^{-1}\) region, only a fraction of the signals at 1540(+), 1530(-) and 1515(+) cm\(^{-1}\) was perturbed upon H\(_2\)O/\(^2\)H\(_2\)O exchange (Figure 2), corresponding to the amide II mode of peptides groups with the NH group sensitive to deuteration. The effect of H/\(^2\)H exchange on the signals at 1560-1490 cm\(^{-1}\) did not increase significantly for BeSOD incubated during two weeks at 10 °C in deuterated buffer. The limited spectral changes observed upon H\(_2\)O/\(^2\)H\(_2\)O exchange both in the Amide I and Amide II regions suggested a partial deuteration of the peptide NH groups in the sample.

These results are in line with data obtained by NMR spectroscopy on human Cu,Zn-SOD, which showed that 85 amide protons out of 187 were insensitive to H/\(^2\)H exchange (10). The alpha helical structure at the periphery of the protein was the most easily exchangeable part of the protein. Other peptide groups which might exchange rapidly are those in contact with water in the active site cavity, such as the peptide group of the bridging His61 (34). Part of the 1540(+), 1530(-) and 1515(+) cm\(^{-1}\) bands and the bands at 1704(+) and 1693(-) cm\(^{-1}\), sensitive to H\(_2\)O/\(^2\)H\(_2\)O exchange, might be due to peptide groups of this \(\alpha\) helix or at His61.

The vibrational changes of peptide groups of the \(\beta\)-barrel upon Cu reduction may be mediated by the three histidine Cu ligands that are anchored in the barrel (5-8). We therefore analyzed the contribution from histidine Cu ligands in the redox-induced FTIR spectra recorded with BeSOD, using IR markers of histidine - metal coordination identified in spectra of metal - methylimidazole models.

**IR markers of imidazole -metal interactions**

The methylimidazole (MeIm) IR modes have been precisely described (72-76) and are reported in Table I. Two modes can be used to distinguish the two MeIm tautomeric forms presented in Scheme 2-a and 2-b. The \(\nu(C_4C_5)\) mode is observed at 1594 and 1575 cm\(^{-1}\) and the ring \(\nu(C_5N\tau)\) mode at 1104 and 1086 cm\(^{-1}\) for 5- and 4-MeIm, respectively (Figure 3-A). The shifts experienced by these \(\nu(C_4C_5)\) and \(\nu(CN\tau)\) modes upon H/\(^2\)H exchange are also specific for 5- and 4-MeIm (73).
(Figure 3-A thick line, Table I). As detailed below, these modes are also sensitive to metal coordination.

Literature data on Raman spectroscopy show that a number of vibrational modes of histidine side chains are sensitive to metal coordination. This was precisely described on His-Cu and His-Zn models of known structure, as well as on peptides modeling the Cu binding site of the prion protein, or the Zn/Cu binding site of amyloid β-peptides (28, 77-79). The ν(C=C) mode occurs at 1590-1580 cm⁻¹ for histidine coordinated with Nπ to Cu or Zn (Scheme 2-d) and at higher frequency, 1606-1594 cm⁻¹, for histidine coordinated with Nτ (28, 77-79) (Scheme 2 and Table I). Also the shift experienced by the ν(C=C) mode upon ¹H/²H exchange is specific of the mode of coordination to the metal, with a downshift by 20-24 cm⁻¹ for the Nτ interaction versus 7-9 cm⁻¹ for the Nπ coordination (77).

Some of the vibrational modes observed using Raman spectroscopy are not detected by FTIR, while intense IR bands, not reported or observed with small amplitudes in the Raman spectra may be sensitive IR markers of the coordination of MeIm or histidine to a metal (73-76). We therefore recorded the spectra of Cu- and Zn- MeIm complexes in solution using Attenuated Total Reflection (ATR) -FTIR spectroscopy. Comparison of the IR spectra of MeIm with those of MeIm in solutions containing Cu²⁺ or Zn²⁺ at MeIm/metal ratios of 4 (Figure 3B to 3D) showed that the formation of Cu-4(5)MeImH and Zn-4(5)MeImH complexes induced an upshift of the ν(C=C) mode of methylimidazole, in total agreement to that reported with Raman spectroscopy (28, 77-79). Thus, the IR bands at 1605 cm⁻¹ in Figure 3-B and 3-D were assigned to Cu- and Zn-Nτ coordinated histidine and the weaker band at 1586-1587 cm⁻¹ to a fraction of Nπ coordinated Me-MeIm complexes, by comparison with the Raman data. The data recorded for Cu –MeIm in ²H₂O solution also confirmed the downshift by 20 cm⁻¹ of the ν(C=C) mode from 1605 cm⁻¹ to 1586 cm⁻¹ of Nτ coordinated MeIm reported by Raman spectroscopy (Figure 3-C).

The other larger IR modes observed in Figure 3-B and 3-D were not reported in the Raman spectra of metal-histidines complexes. Figure 3B and 3D show that the ring ν(CN) IR mode at 1490 cm⁻¹ for 4(5)-MeImH is upshifted to 1505-1507 cm⁻¹ upon metal complexation. More
importantly, the ring ν(C₅Nτ) IR mode at 1104-1086 cm⁻¹ (Figure 3-A) appeared as a large band at 1109 cm⁻¹ for the Cuᴵᴵ-MeIm complex, corresponding to the Cu-Nτ-MeIm complex, as deduced from the ν(C₅C₅) mode frequency (Figure 3-B). This mode appeared at 1113 cm⁻¹ for the Zn-complexes (Figure 3-D), with at least twice larger intensity than for isolated 4-MeImH. The 1088 cm⁻¹ mode observed in Figure 3-D could correspond to a fraction of Zn-Nπ complexes. This ν(C₅Nτ) IR mode has already been identified as an IR marker of histidine ligands of Fe and Mg in different proteins (80-85) and in protoporphyrin IX-methylimidazole model compounds (84). This typical IR mode is insensitive to H₂/H exchange, as shown also in the Cu-MeIm complex in Figure 3-C. Other metal-binding sensitive modes are observed at 1263 (+6) and 1230 (-3 to +6) cm⁻¹ (Figure 3-B, D). The typical IR markers of metal coordination experimentally determined here corresponded nicely to frequency shifts predicted in theoretical calculations based on density functional theory (DFT) (86) upon MeIm coordination to hydrated Zn.

**IR modes of histidine Cu-ligands in BeSOD**

Comparison of the redox-induced FTIR difference spectra obtained with BeSOD with the spectra of model compounds supported the assignment of signals at 1112(+) and 1097(-) cm⁻¹ in Figure 2-A to the ν(C₅Nτ) mode of histidine Nτ ligands of the copper at the BeSOD active site. This mode was insensitive to H₂O/H₂O exchange, as previously noted for His Nτ ligands of Fe (Figure 2-B thick line) (82, 84). This band presented some structure and probably corresponded to more than one residue. In BeSOD, two histidines, His118 and His46², are coordinated through the Nτ to Cu. The spectrum showed that Cu reduction induced a significant upshift by 15 cm⁻¹ of this mode, that may be assigned to a lesser electronegative character of the histidine ring for reduced BeSOD. Contribution of the bridging His61 ligand for oxidized BeSOD was not expected at 1097 cm⁻¹ but rather near 1045 cm⁻¹. A very small signal at ≈1045 cm⁻¹ could correspond to the mode of bridging His61 in oxidized BeSOD (28, 86).
No clear signal was observed at \(\approx 1088\) cm\(^{-1}\), sensitive to \(\text{H}_2\text{O}/^2\text{H}_2\text{O}\) exchange, that could be assigned to the His N\(\pi\) ligand of the copper, suggesting that the imidazole side chain in this residue (His44) was less affected by Cu reduction. Also, there was no indication from the FTIR difference spectra presented here, that the Zn His N\(\pi\) ligands (His69 and His78) were significantly affected by Cu reduction.

The signals at 1608 and 1592 cm\(^{-1}\) (reduced BeSOD, Figure 2-A and 2-B) and at 1618 and 1602 cm\(^{-1}\) (oxidized BeSOD) were in the frequency range, where the \(v(C_4C_5)\) mode of His ligands are expected to contribute. Moreover, these signals were sensitive to \(\text{H}_2\text{O}/^2\text{H}_2\text{O}\) exchange (Figure 2), and a downshift of the 1618 cm\(^{-1}\) band to 1592 cm\(^{-1}\) was totally consistent with the \(-26\) cm\(^{-1}\) downshift of the \(v(C_4C_5)\) mode of a N\(\tau\) coordinated His upon \(\text{H}_2\text{O}/^2\text{H}_2\text{O}\) exchange (28, 86). These signals were thus assigned to the His N\(\tau\) ligands of Cu His118 and His46.

**Redox-induced infrared changes in chloroplastic SOD**

BeSOD and ChSOD have 68% of sequence homology. As compared to BeSOD, ChSOD lacks some charged residues in the superoxide access channel and is functional as a tetramer. Comparison of BeSOD and ChSOD was performed to evaluate the respective role of these residues and of the peptide fold and Cu histidine ligands in the redox-induced vibrational changes detected by FTIR difference spectroscopy. ChSOD from *Lycopersicon esculentum* was expressed in *E. coli* using the pet-11 /Bl21 IPTG inducible expression system from Stratagen as described in Materials and Methods. Expression in *E. coli* grown in minimal medium led to the production of about 1.7 mg/L of ChSOD. A purification procedure using ion exchange chromatography was developed to obtain the ChSOD as a functional tetramer with 3300 U/mg specific activity. The expression and purification systems allowed the uniform \(^{15}\)N labeling of ChSOD, using \(^{15}\)NH\(_4\)Cl as unique nitrogen source. The heterologous expression system was also used to produce site directed mutants at the level of two strictly conserved residues of the superoxide access channel, Thr137 and Arg143.
Figure 4-A shows the reduced-minus-oxidized FTIR difference spectrum recorded with ChSOD in Ches buffer at pH 9.3. Large similarities between this spectrum and that recorded with BeSOD were underlined by the superimposition of the reduced-minus-oxidized spectra recorded with the two samples in Figure 4-B. Main signals of the Amide I region, at 1685/1673/1651/1640 cm\(^{-1}\) were almost identical in both enzymes, at the exception of the positive band at 1640 cm\(^{-1}\) which had smaller intensity for ChSOD. In the Amide II region, the same band pattern was also observed for both Cu,Zn-SOD with an overall upshift by 5 cm\(^{-1}\) of the modes at 1560/1545/1535/1518 cm\(^{-1}\) for ChSOD as compared to BeSOD (Figure 4-B). A slightly larger contribution was observed for oxidized ChSOD at 1560 cm\(^{-1}\) and at 1540 cm\(^{-1}\) for reduced BeSOD.

Other conserved spectral features concerned signals at 1600(-), 1590(+) and =1097-1095(-) cm\(^{-1}\) proposed to account for histidine Cu ligands (Figure 4-B). The broad difference band centered at 1200 cm\(^{-1}\) in the spectrum recorded with ChSOD was due to a redox mediator and was not further studied (Figure 4-A).

**Contributions from amide groups and histidine side-chains**

\(^{15}\text{N}\)-labeled ChSOD was analyzed since typical and distinct shifts were expected upon \(^{15}\text{N}\)-labeling for the IR modes of peptide amide groups and histidine side chains (87, see below). The reduced-minus-oxidized FTIR difference spectra recorded with \(^{14}\text{N}\) (thin line) and \(^{15}\text{N}\) (bold line) ChSOD were superimposed in Figure 5-A. The largest effect was a clear collective downshift by 10-15 cm\(^{-1}\) of the main IR signals at 1560/1545/1535/1515 cm\(^{-1}\) in the \(^{14}\text{N}\) sample to 1545/1530/1519/1505 cm\(^{-1}\) upon \(^{15}\text{N}\) labeling. This shift is typical for peptide v(CN)+\(\delta\)(NH) Amide II IR modes and was also observed in the IR absorption spectrum of labeled ChSOD (not shown, 87). Thus the \(^{15}\text{N}\)-labeling experiment demonstrated the dominant contribution of peptide Amide II modes at 1560-1515 cm\(^{-1}\) in the redox-induced FTIR difference spectrum. A negative shoulder remained at 1560 cm\(^{-1}\) in the spectrum recorded with \(^{15}\text{N}\)-ChSOD, which corresponded to an other mode, less sensitive to \(^{15}\text{N}\) labeling. This band appeared at a frequency consistent with the v(C\(_4\)C\(_3\)) IR mode of the bridging His63 imidazolate for oxidized Cu,Zn-SOD (28, 86).
In contrast to the large $^{15}$N labeling effect at 1560-1500 cm$^{-1}$, the two spectra of Figure 5-A were almost superimposed in the 1700-1630 cm$^{-1}$ region. Shifts by at most 1-2 cm$^{-1}$ are expected for peptide v(C=O) modes. The difference spectrum calculated from spectra with $^{15}$N -minus- $^{14}$N ChSOD displayed in Figure 5-B showed a tiny signal at 1698 cm$^{-1}$ and a sharp signal at 1655/1648 cm$^{-1}$ which were compatible with the small downshifts expected on v(C=O) peptide modes. Only two small and broader bands, at 1680(-) and 1664(+), cm$^{-1}$ might originate from other chemical groups. These signals were the only ones that could account for the v$_{\text{as}}$(guanidium) IR mode of Arg143 side-chain (71, 88) (see below). These bands were observed however at 1674 and 1664 cm$^{-1}$ in the $^{15}$N –minus- $^{14}$N difference spectrum calculated from spectra recorded in $^2$H$_2$O and the modest frequency shift induced by the H$_2$O/$^2$H$_2$O exchange on these signals was not in agreement with that expected for an arginine side chain mode (66, 67, 71).

The $^{15}$N labeling experiment demonstrated without ambiguity that the IR changes induced by Cu,Zn-SOD reduction resulted from dominating electrostatic reorganization of peptide dipoles. Moreover, the $^{15}$N labeling showed that most of the other IR signals could be assigned with confidence to side chain modes of the histidine Cu ligands.

Indeed, downshifts by 3 to 18 cm$^{-1}$ were expected for histidine side-chain IR modes upon $^{15}$N labeling, as inferred from data on $^{15}$N-imidazole (84) or on $^{15}$N-photosystem II intermediates (80, 82, 83). Comparison of the reduced – minus - oxidized FTIR spectra recorded with $^{14}$N- and $^{15}$N labeled ChSOD in H$_2$O in the 1615-1560 cm$^{-1}$ region (Figure 5-A inset) showed that the IR signals at 1610(-), 1589(+), 1581(-) and 1575(+) cm$^{-1}$ were downshifted by about –3 cm$^{-1}$ to 1597(-), 1586(+), 1578(-) and 1571(+) cm$^{-1}$ upon $^{15}$N-labeling. The $^{15}$N- minus $^{14}$N difference spectrum of Figure 5-B confirmed the slight shift of the v(C$_4$C$_5$) IR modes at =1580 cm$^{-1}$ upon $^{15}$N-labeling. Also, the spectra recorded in $^2$H$_2$O with $^{14}$N and $^{15}$N labeled ChSOD showed these small but significant shifts on the typical $^2$H$_2$O IR bands at 1584(-) and 1574(+), cm$^{-1}$, downshifted to 1581(-) and 1571(+) cm$^{-1}$ (Figure 5-C). The signals at 1584/1574 cm$^{-1}$ in $^{14}$N-ChSOD in $^2$H$_2$O and at 1600/1584 cm$^{-1}$ in H$_2$O were assigned to the IR modes of N$\tau$ His ligands.
Further, the positive band at 1111 cm\(^{-1}\) was downshifted to 1102 (-9) cm\(^{-1}\) and the 1095 cm\(^{-1}\) signal to 1086 cm\(^{-1}\) (-10 cm\(^{-1}\)). These shifts are in agreement with the \(\approx 7\) cm\(^{-1}\) downshift observed for the histidine \(\nu(CN\tau)\) upon \(^{15}\)N- labeling (80, 82-84). The bands at 1111 and 1102 cm\(^{-1}\) for reduced \(ChSOD\) and at 1095 and 1086 cm\(^{-1}\) for oxidized \(ChSOD\) presented some structure corresponding to more than one chemical group. Finally, a small band at 1225 cm\(^{-1}\), downshifted by 5 cm\(^{-1}\) upon \(^{15}\)N-labeling may also be due to histidine ring vibration (84).

**Identification of arginine and threonine side-chain IR modes**

**Arginine 143**: The side-chain of the strictly conserved Arg143 is located in the superoxide access channel of Cu,Zn-SOD, at \(\approx 6\) Å of the active site Cu (5, 10, 34, 35). Its positive charge is thought to play determinant role in superoxide attraction and to greatly enhance its reaction kinetics with Cu (18, 19, 41). To identify the IR side-chain modes of Arg143, we performed the Arg143Gln mutation on recombinant \(ChSOD\), using the heterologous expression system described above. In this mutant, the activity towards superoxide scavenging was largely impaired, with about 10 % of the activity of WT \(ChSOD\) (380 U.mg\(^{-1}\)). This result is in line with literature data on Arg143 mutants with neutral side chains of human Cu,Zn-SOD (Ile and Ala, 18, 19, 41).

Comparison of the reduced – minus - oxidized FTIR spectra recorded with WT (thin line) and Arg143Gln mutant (bold line) is shown in Figure 6-A. The spectra present overall similar shape with a large number of conserved bands. Reproducible changes are displayed more precisely in the mutant – minus - WT difference spectrum of Figure 6-B. They may be due to the side-chain modes of Arg, Gln or to other amino acids perturbed by the mutation.

Arginine side-chain is characterized by two intense IR modes, the \(\nu_{as}\) and \(\nu_{s}\) (C\(_2\)N\(_3\)H\(_5\)) guanidium modes at 1675-1670 cm\(^{-1}\) (\(\epsilon = 460\) L.mol\(^{-1}\).cm\(^{-1}\)) and 1640-1630 cm\(^{-1}\) (\(\epsilon = 340\) L.mol\(^{-1}\).cm\(^{-1}\)) in H\(_2\)O (66). These modes are downshifted by 65 and 47 cm\(^{-1}\) in \(^2\)H\(_2\)O, respectively (71), and by \(= 9\) cm\(^{-1}\) upon \(^{15}\)N- labeling (65). For glutamine side chain, the \(\nu(C=O)\) and \(\delta(NH_2)\) modes contribute at 1684-1668
(ε = 380 L.mol⁻¹.cm⁻¹) cm⁻¹ and 1611-1586 (ε = 220 L.mol⁻¹.cm⁻¹) cm⁻¹ in H₂O, respectively, and at 1654-1635 cm⁻¹ and 1163 cm⁻¹ in 
^2H₂O (66, 88).

The signals at 1687, 1671 and 1661 cm⁻¹ in Figure 6-B are in the frequency range of the arginine νₐs (C₂N₃H₅) and glutamine or peptide ν(C=O) IR modes. ¹⁵N-labeling of WT ChSOD showed however that no IR mode sensitive to ¹⁵N labeling appeared at 1687 and 1671 cm⁻¹ (Figure 5-B) and ruled out contribution from the arginine side chain at these frequencies. This points to the contribution of the Gln ν(C=O) side chain in the mutant at 1687/1671 cm⁻¹ in Figure 6-B. The assignment of the signals at 1687/1671 cm⁻¹ to Gln side chain were also supported by a large effect of H₂O/^2H₂O exchange on signals at these frequencies in the mutant (not shown). From the comparison of the absorption coefficients of Arg and Gln side-chain modes (66), bands even larger than those assigned to the ν(C=O) Gln side chain mode at 1687/1671 cm⁻¹ were expected for the νₐs(C₂N₃H₅) arginine mode, if Arg143 were sensitive to Cu reduction. We conclude that while the side chain of Gln is sensitive to the redox state of the Cu, the arginine side-chain in WT Cu,Zn-SOD remains unaffected by the reduction (oxidation) of Cu.

The mutant – minus – WT difference spectrum of Figure 6-B presents small changes at 1648, 1538, 1526, and 1512 cm⁻¹ that correspond to peptide ν(C=O) and ν(CN)+δ(NH) modes. They could arise from a peptide hydrogen bonding network around Arg143, that is perturbed upon Arg143Gln mutation. Finally the bands at 1100 and 1093 cm⁻¹ were assigned to one histidine ligand of the Cu, whose electronic properties are perturbed by the Arg143Gln mutation.

**Thr137** : Thr137 is a strictly conserved amino acid that determines the size of the superoxide access channel together with Arg143 (5, 35). Mutation of Thr137 into a Gln was undertaken and the FTIR difference spectra recorded with the WT and Thr137Gln mutant were superimposed in Figure 6-C. These spectra are almost identical showing that there is no structural effect of the mutation. The small perturbations isolated in the WT – minus - mutant difference spectrum of Figure 6-D consisted in a decreased intensity of the negative bands at 1696 and 1686 cm⁻¹ for the mutant, and in the signal
at 1664/1649 cm\(^{-1}\) that may be assigned to the Gln side chain ν(C=O) mode. No clear signal in this spectrum could be assigned to Thr137 side chain, showing that this strictly conserved amino acid at the level of the superoxide access channel was not sensitive to the change of redox state of the copper.

**Discussion**

By coupling electrochemistry and FTIR difference spectroscopy, we recorded directly vibrational changes at the level of amino acid side chains and of the peptide backbone induced by the change in redox state of the active site copper. Data recorded with WT or mutated ChSOD, comparison of spectra obtained with Be and ChSOD, so as experiments performed with \(^{15}\)N labeled ChSOD demonstrated that most of the vibrational modes perturbed by the copper reduction are due to the histidine Cu ligands and to reorganizations at the level of peptide bonds.

**Infrared modes of histidine ligands**

Infrared studies on protoporphyrin-methylimidazole model compounds and on isolated cytochrome b559 (84) or on the non heme iron of photosystem II coordinated by four histidine (80-82) showed an histidine IR mode at \(\approx 1100 \text{ cm}^{-1}\) characteristic for histidine ligands of the iron. This signal, assigned to a His ν(C\(_5\)N\(_\text{t}\)) ring mode by normal mode calculations (86) was also observed for the FeHis\(_4\)Cys superoxide reductase center of *Desulfoarcus baarsii* (85) and for the histidine ligands of the primary donor chlorophylls of photosystem I (83). The experiments performed here on MeIm Cu or Zn complexes confirm that this mode is enhanced upon metal coordination and is a sensitive infrared marker of histidine coordination to a metal through N\(_\text{t}\). This mode was clearly identified and assigned to the His120 and His48 N\(_\text{t}\) ligands of Cu in Cu,Zn-SOD. The upshift by \(\approx 15 \text{ cm}^{-1}\) observed upon Cu reduction is proposed to account for the lesser electronegative character of these histidine N\(_\text{t}\) ligands in reduced Cu,Zn-SOD. This correlation between the frequency of the ν(C\(_5\)N\(_\text{t}\)) ring mode and the electronegative character of the histidine side chain resides on the observation that the ligand
methyl-imidazolate contributes at lower frequency (at 1099 cm\(^{-1}\)) than the ligand methyl-imidazole (at 1103 cm\(^{-1}\)) in the protoporphyrin IX-methylimidazolate model compounds (84). Such a downshift is also predicted for N\(\tau\) methylimidazolate ligands by DFT calculations (86).

In this work, we also show that the \(v(C=C)\) IR mode of histidine side-chains can be used in metalloenzymes, as a marker of coordination to a metal. We identified the \(v(C=C)\) IR mode of histidine ligands of the Cu, on the basis of a comparison with Raman data (28, 77-79), of the shifts induced by H\(_2\)O/\(\text{D}_2\)O exchange and \(^{15}\)N labeling. Signals at 1618 and 1602 cm\(^{-1}\) for oxidized BeSOD (1600 cm\(^{-1}\) for ChSOD) and at 1608 and 1590 cm\(^{-1}\) for reduced BeSOD (1590 cm\(^{-1}\) for ChSOD) are best interpreted as an effect of Cu reduction on the two His N\(\tau\) ligands of the Cu. A frequency downshift is consistent with a slightly weaker bond strength between His and Cu(I) (86).

Several of the modes identified by Raman spectroscopy for oxidized Cu,Zn-SOD were assigned to the His63 bridging ligand (28-30). In particular, the \(v(C=C)\) mode of bridging His63 imidazolate was assigned at 1567-1555 cm\(^{-1}\) (28-30, 79). No signals were assigned to the other histidine ligands of the copper using UV-Raman. In the present work, we tentatively assigned a small band at 1560 cm\(^{-1}\) to the bridging imidazolate in oxidized ChSOD.

The new low-temperature structure of reduced BeSOD, at 1.15 Å resolution showed that Cu reduction induces a displacement by 1.3 Å of the Cu relative to its position in oxidized BeSOD which together with the rupture of the imidazolate bridge between Cu and Zn increases the distance between Cu and Zn from ~6 Å for oxidized BeSOD to 6.9 Å for reduced BeSOD (34). The movement also involves His118 (N\(\tau\)) which maintains the usual distance to the copper. The FTIR data suggest that the electronic properties of the His118 ring are affected by the Cu reduction together with that of His46.

**Contribution from peptide amide I and II modes**

One striking result of the present FTIR study is that most of the infrared changes detected upon Cu reduction in both ChSOD and BeSOD are due to the polypeptide backbone of the superoxide dismutases. The comparison of the reduced-minus-oxidized spectra recorded with \(^{14}\)N- and \(^{15}\)N-
labeled ChSOD clearly demonstrated the dominating contribution from peptide Amide II modes in the 1560-1500 cm\(^{-1}\) spectral range. The minute changes observed upon \(^{15}\)N-labeling and H\(_2\)O/\(^2\)H\(_2\)O exchange in the 1700-1630 cm\(^{-1}\) region also corroborated a dominating contribution from peptide \(\nu(C=O)\) IR modes in that region. It is known that particular secondary structural motives like \(\alpha\)-helices or \(\beta\)-sheets are characterized by specific carbonyl vibration frequencies (61, 64). The frequency of the main bands at 1674-1672 and 1652-1651 cm\(^{-1}\) correspond to peptide groups involved in loops, \(\alpha\) helices or unordered structure. The frequency of part of the \(\nu(C=O)\) modes, at 1640-1639 cm\(^{-1}\) accounts best for peptides carbonyl groups involved in \(\beta\) sheet structure. The \(\nu(C=O)\) IR mode results from collective interactions within the sheet or the helix. Therefore, the changes observed in IR cannot be assigned to a particular group, that would experience a large change upon copper reduction, but correspond to the overall and slight modification of the electrostatic properties of the connected peptide groups (61, 89).

Dong et al. (43) compared FTIR absorption spectra of chemically oxidized or reduced BeSOD and emphasized an intensity redistribution between two \(\beta\)-sheet related bands at 1633 and 1625 cm\(^{-1}\) upon change in oxidation state of the copper. These results were obtained by comparison of absorption spectra obtained with different samples. With electrochemically induced FTIR difference spectroscopy, we have a very accurate picture of the structural changes recorded on the same sample. It confirms the participation of \(\beta\)-sheet peptide groups, but also demonstrates that these changes are largely contributed by other structural elements of Cu,Zn-SOD. We also showed that most of the amide modes perturbed upon copper reduction are not accessible to H\(_2\)O/\(^2\)H\(_2\)O exchange. By reference to results obtained by Banci et al. (10), this effect points to amide protons located on the \(\beta\)-barrel and on loops including loop VII forming part of the superoxide access channel. The implication of the peptide backbone in the reorganization of the electrostatic field upon Cu reduction corresponds to charge delocalization at large distance from the copper. This mechanisms may be relevant to the specific functioning as a superoxide dismutase i.e. with the comparable efficiency of oxidized and reduced Cu,Zn-SOD in superoxide oxidation and reduction (17, 22).
Contribution of Arg143, polar and charged amino acids in the superoxide access channel

Charged Glu (Glu130 and 131) and Lys (Lys120 and 134) of the superoxide access channel in BeSOD are substituted in ChSOD by uncharged residues (5, 7, 46). The FTIR difference spectra recorded with BeSOD and ChSOD showed overall similarity, meaning that these residues do not contribute significantly in the spectrum recorded with BeSOD. Mutation of these residues in human Cu,Zn-SOD (20) demonstrated that they are involved in a hydrogen bond network which determines an electrostatic network, favouring superoxide guidance towards the Cu (20). Mutations increasing the positive charges but disturbing the network showed impaired reaction rates with superoxide (20). Moreover, in spite of differently localized charged residues at proximity of the superoxide access channel in BeSOD or human Cu,ZnSOD and in ChSOD, electrostatic fields calculated based on the structure of the two types of enzymes are similar (42). This was interpreted as a dominating role of the peptide fold in the establishment of these electrostatic fields. The FTIR results presented in this study are in line with these calculations. They also show that the Cu,Zn-SOD fold is optimised for the peptide backbone to compensate the charge upon Cu reduction without perturbation of the electrostatic charge at the active site Arg143 implicated in both superoxide attraction and stabilization.

Indeed, the FTIR data obtained with recombinant ChSOD $^{14}$N and $^{15}$N labeled, and with the Arg143Gln mutant show that only one very small IR signal might be assigned to the Arg143 side chain at $\approx$1680 cm$^{-1}$ for oxidized ChSOD. Since the IR modes of Arg side chains are intense (66) and since these modes are sensitive to changes in the electrostatic properties of the guanidium group, induced by the proximity of anions for example (65), any perturbation at the level of Arg143 side chain upon reduction of the active-site Cu in ChSOD would have been detected. Thus we conclude that the Arg143 side chain is insensitive to the redox state of the Cu.

Mutants at Arg143 have shown the determinant role of this residue to maintain a high enzyme activity within a large pH range (18, 19, 41, and this work). The positively charged Arg143 side-chain plays an electrostatic role in steering efficiently superoxide towards the copper. However Arg143Lys mutants also had impaired activity (18, 19, 41). These data, and the three dimensional structure of
Cu,Zn-SOD (34, 35) led to the conclusion that the hydrogen bonding network involving Arg side-chain constrains the guanidium group, ideally oriented as a docking site for superoxide. The FTIR data presented here show that the properties of the Arg143 side chain are maintained whatever the redox state of the copper, which is not the case of the substituted glutamine side chain. The fact that the overall fold of the access channel stabilizes the properties of Arg143 side chain whatever the redox state of the copper may be crucial for the function of Cu,Zn-SOD as a dismutase, with high attractive power towards superoxide of both reduced and oxidized Cu.

In conclusion, the long distance charge redistribution observed at the level of peptide groups upon Cu reduction and the preserved electrostatic properties of the superoxide access channel may constitute a specific mechanism of Cu,Zn-SOD that contrasts to the reorganizations observed for the amino acid side chains near the Fe active site of superoxide reductase (85) and may be a key factor of the superoxide dismutase function.

**Acknowledgments**

We gratefully acknowledge Perl Treaves for the gift of the pGEM plasmid bearing the cDNA of the ChSOD from *Lycopersicon esculentum*, Isabelle Dany for the mass-spectroscopy experiments performed on the $^{14}$N and $^{15}$N labeled ChSOD, Rainer Hienerwadel and Alain Boussac for critical reading of the ms and André Verméglio for his constant support to this project. F.D. acknowledges a Ph.D. fellowship from the CEA and the PACA (Provence Alpes côte d’Azur) region. Part of this work was financed by the CEA- Program Toxicologie Nucléaire.

**Footnotes**

1. Abbreviations used in this ms include: (Be or Ch)SOD, (bovine erythrocyte or chloroplastic) Cu,Zn-superoxide dismutase; Tris, tris(hydroxymethyl) aminomethane; Ches, 2-(N-cyclohexylamino)ethane sulfonic acid; PATS-3, pyridine-3-carboxaldehydethiosemicarbazone; pBQ, 1,4-pbenzoquinone; DCIP, 2,6-dichlorophenol-indophenol; MV, methylviologen; NBT, nitrobluetetrazolium; MeIm,
methylimidazole; IPTG, isopropyl-beta-D-thiogalactopyranoside; EXAFS, extended X-rays absorption fluorescence spectroscopy; NMR, nuclear magnetic resonance; MALDI-TOF, matrix-assisted laser desorption ionization time of flight; ATR, attenuated total reflection; (FT)IR, (Fourier transform) infrared; ν, stretching vibration; δ, bending vibration; UV-Vis, UV-visible; RR, resonance Raman; DFT density functional theory.

2. Amino acid numbering is given according to human or plant Cu,Zn-SOD except to describe scheme 1 in the introduction and in the Results and discussion section concerning BeSOD, where BeSOD numbering is used: Arg141, Thr135, His61, His118, His46, and His44 in BeSOD correspond to Arg143, Thr137, His63, His120, His48 and His46, respectively in plant and human SOD.
REFERENCES

1. McCord, J.M., and Fridovich, I. (1969) *J. Biol. Chem.* **244**, 6049-6055.

2. Miller, A.-F., and Sorkin, D. L. (1997) *Comments Mol. Cell. Biophys.* **9**, 1-48.

3. Bertini, I., Mangani, S., and Viezzoli, M. S. (1997) *Adv. Inorg. Chem.* **45**, 127-250.

4. Youn H. D., Kim E. J., Roe J. H., Hah Y. C., and Kang S. O. (1996) *Biochem J.* **318**, 889-896.

5. Tainer, J. A., Getzoff, E. D., Beem, K. M., Richardson, J. S., and Richardson D. C. (1982) *J. Mol. Biol.* **160**, 181-217.

6. Redford, S. M., McRee, D. E., Getzoff, E. D., Steinman, H. M., and Tainer, J. A. (1990) *J. Mol. Biol.* **212**, 449-451.

7. Kitagawa, Y., Tanaka, N., Hata, Y., Kusunoki, M., Lee, G.-P., Katsube, Y., Asada, K., Aibara, S., and Morita, Y. (1991) *J. Biochem.* **109**, 477-485.

8. Djinovic, K., Gatti, G., Coda, A., Antolini, L., Pelosi, G., Desideri, A., Falconi, M., Marmocchi, F., Rotilio, G., and Bolognesi, M. (1992) *J. Mol. Biol.* **225**, 791-809.

9. Pesce, A., Capasso, C., Battistoni A., Folcarelli, S., Rotilio, G., Desideri, A., and Bolognesi, M. (1997) *J. Mol. Biol.* **274**, 408-420.

10. Banci, L., Bertini, I., Cramaro, F., Del Conte, R., and Viezzoli, M. S. (2002) *Eur. J. Biochem.* **269**, 1905-1915.

11. Bannister, W. H., Bannister, J. V., Barra, D., Bond, J., and Bossa, F. (1991) *Free Radic. Res. Commun.* **12-13**, 349-361.

12. Bordo, D., Djinovic, K., and Bolognesi, M. (1994) *J. Mol. Biol.* **238**, 366-386.

13. Bowler, C., Van Camp, W., Van Montagu, M., and Inze, D. (1994) *Critic. Rev. Plant Sci.* **13**, 199-218.

14. Djinovic-Carugo, K., Politcilli, F., Desideri, A., Rotilio, G., Wilson, K. S., and Bolognesi, M. (1994) *J. Mol. Biol.* **240**, 179-183.

15. Djinovic-Carugo, K., Battistoni, A., Carri, M. T., Politcilli, F., Desideri, A., Rotilio, G., Coda, A., and Bolognesi M. (1994) *FEBS Lett.* **349**, 93-98.

16. Ferraroni, M., Rypniewski, W. R., Bruni, B., Orioli, P., and Mangani, S. (1999) *J. Biol. Inorg. Chem.* **3**, 411-422.

17. Klug, D., Rabani, J., and Fridovich, I. (1972) *J. Biol. Chem.* **247**, 4839-4842.

18. Beyer, W. F. Jr, Fridovich, I., Mullenbach, G. T., and Hallewell, R. (1987) *J. Biol. Chem.* **262**, 11182-11187.
19. Banci, L., Bertini, I., Luchinat, C., and Hallewell, R. A. (1988) *J. Am. Chem. Soc.* **110**, 3629-3633.
20. Getzoff, E. D., Cabelli, D. E., Fisher, C. L., Parge, H. E., Viezzoli, M. S., Banci, L., and Hallewell, R. A. (1992) *Nature* **358**, 347-351.
21. Klug-Roth, D., Fridovich, I., and Rabani, J. (1973) *J. Am. Chem. Soc.* **95**, 2786-2790.
22. Fielden, E. M., Roberts, P. B., Bray, R. C., Lowe, D. J., Mautner, G. N., Rotilio, G., and Calabrese, L. (1974) *Biochem. J.* **139**, 49-60.
23. Bailey, D. B., Ellis, P. D., and Fee, J. A. (1980) *Biochemistry* **19**, 591-596.
24. Bertini, I., Capozzi, F., Luchinat, C., Piccioli, M., and Viezzoli, M. S. (1991) *Eur. J. Biochem.* **197**, 691-697.
25. Banci, L., Benedetto, M., Bertini, I., Del Conte, R., Piccioli, M., and Viezzoli, M. S. (1998) *Biochemistry* **37**, 11780-11791.
26. Murphy L. M., Strange, R. W., and Hasnain, S. S. (1997) *Structure* **5**, 371-379.
27. Ascone, I., Castaner, R., Tarricone, C., Bolognesi, M., Stroppolo, M. E., and Desideri, A. (1997) *Biochem. Biophys. Res. Commun.* **241**, 119-121.
28. Hashimoto, S., Ono, K., and Takeuchi, H. (1998) *J. Raman Spectrosc.* **29**, 969-975.
29. Zhao, X., Wang, D., and Spiro, T. G. (1998) *Inorg. Chem.* **37**, 5414-5415.
30. Wang, D., Zhao, X., Vargek, M., and Spiro, T. G. (2000) *J. Am. Chem. Soc.* **122**, 2193-2199.
31. Rypniewski, W. R., Mangani, S., Bruni, B., Orioli, P. L., Casati, M., and Wilson K. S. (1995) *J. Mol. Biol.* **251**, 282-296.
32. Ogihara, N. L., Parge, H. E., Hart, P. J., Weiss, M. S., Goto, J. J., Crane, B. R., Tsang, J., Slater, K., Roe, J. A., Valentine, J. S., Eisenberg, D., and Tainer, J.A. (1996) *Biochemistry* **35**, 2316-2321.
33. Hough, M. A., and Hasnain, S. S. (1999) *J. Mol. Biol.* **287**, 579-592.
34. Hough, M. A. and Hasnain, S. S. (2003) *Structure* **11**, 937-946.
35. Tainer, J. A., Getzoff, E. D., Richardson, J. S., and Richardson D. C. (1983) *Nature* **306**, 284-287.
36. Banci, L., Bertini, I., Luchinat, C., and Viezzoli, M. S. (1993) *Inorg. Chem.* **32**, 1403-1406.
37. Ellerby, L. M., Cabelli, D. E., Graden, J. A., and Valentine, J. S. (1996) *J. Am. Chem. Soc.* **118**, 6556-6561.
38. Hart, P. J., Balbirnie, M. M., Ogihara, N. L., Nersissian, A. M., Weiss, M. S., Valentine, J. S., and Eisenberg, D. (1999) *Biochemistry* **38**, 2167-2178.
39. Leone, M., Cupane, A., Militello, V., Stroppolo, M. E., and Desideri, A. (1998) *Biochemistry* **37**, 4459-4464.
40. Getzoff, E. D., Tainer, J. A., Weiner, P. K., Kollman, P. A., Richardson, J. S., and Richardson, D. C. (1983) *Nature* **306**, 287-290.

41. Fisher, C. L., Cabelli, D. E., Tainer, J. A., Hallewell, R. A., and Getzoff, E. D. (1994) *Proteins: Struct. Funct. and Gen.* **19**, 24-34.

42. Marino, M., Galvano, M., Cambria, A., Politicelli, F., and Desiredi, A. (1995) *Protein Eng.* **8**, 551-556.

43. Dong, A., Huang, P., and Caughey, W. S. (1995) *Arch. Biochem. Biophys.* **320**, 59-64.

44. Mäntele, W. (1993) *Trends Biochem. Sci.* **18**, 197-202.

45. Zscherp, C., and Barth, A. (2001) *Biochemistry* **40**, 1875-1883.

46. Perl-Treves, R., Nacmias, B., Aviv, D., Zeelon, E. P., and Galun, E. (1988) *Plant Mol. Biol.* **11**, 609-623.

47. McCord, J.M. (1999) Analysis of superoxide dismutase activity, in *Current Protocols in Toxicology* 7.3.1-7.3.9, John Wiley & Sons, Inc.

48. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

49. Moss, D., Nabedryk, E., Breton, J., and Mäntele, W. (1990) *Eur. J. Biochem.* **187**, 565-572.

50. Hill, H. A. O., Page, D. J., Walton, N. J., and Whitford, D. (1985) *J. Electroanal. Chem.* **187**, 315-324.

51. Lawrence, G. D., and Sawyer, D. T. (1979) *Biochemistry* **18**, 3045-3050.

52. Strong St. Clair, C., Gray, H. B., and Valentine, J. S. (1992) *Inorg. Chem.* **31**, 925-927.

53. Borsari, M., and Azab, H. A. (1992) *Bioelectrochem. Bioenerg.* **27**, 229-233.

54. Azab, H. A., Banci, L., Borsari, M., Luchinat, C., Sola, M., and Viezzoli, M. S. (1992) *Inorg. Chem.* **31**, 4649-4655.

55. Ge, B., Scheller, F. W., and Lisdat, F. (2003) *Biosensors and Bioelectronics* **18**, 295-302.

56. Tian, Y., Shioda, M., Kasahara, S., Okajima, T., Mao, L., Hisabori, T., and Ohsaka, T. (2002) *Biochim. Biophys. Acta* **1569**, 151-158.

57. Wu, X.-Q., Meng, X.-Y., Wang, Z.-S., and Zhang, Z.-R. (1999) *Bioelectrochemistry and Bioenergetics* **48**, 227-231.

58. Verhagen, M. F. J. M., Meussen, E. T. M., and Hagen, W. R. (1995) *Biochim. Biophys. acta* **1244**, 99-103.

59. Banci, L., Bertini, I., Luchinat, C., Scozzafava, A., and Turano, P. (1989) *Inorg. Chem.* **28**, 2377-2381.

60. Mota de Freitas, D., Ming, L.-J., Ramasamy, R., and Silverstone, J. S. (1990) *Inorg. Chem.* **29**, 3512-3518.

61. Krimm, S., and Bandekar, J. (1986) *Adv. Protein Chem.* **38**, 181-364.
62. Susi, H., and Byler, D. M. (1986) *Methods. Enzymol.* **130**, 290-311.
63. Surewicz, W. K., and Mantsch, H. H. (1988) *Biochim. Biophys. Acta* **952**, 115-130.
64. Venyaminov, S. Y. U., and Kalnin, N. N. (1990) *Biopolymers* **30**, 1259-1271.
65. Braiman, M. S., Brierecheck, D. M., and Krieger, K. M. (1999) *J. Phys. Chem. B* **103**, 1243-1257.
66. Venyaminov, S. Y., and Kalnin, N. N. (1990) *Biopolymers* **30**, 1243-1257.
67. Barth, A. (2000) *Prog. Biophys. & Mol. Biol.* **74**, 141-173.
68. Susi, H. (1969) in *Structure and stability of biological macromolecules* (Tunasheff, S.N., and Fasman, G.D., Eds) Vol 2, pp. 575-663, Marcel Dekker, New York.
69. Susi, H., and Byler, D. M. (1987) *Arch. Biochem. Biophys.* **258**, 465-469.
70. Harris, P. I., and Chapman, D. (1995) *Biopolymers* **37**, 251-263.
71. Chirgadze, Y. N., Fedorov, O. V., and Trushina, N. P. (1975) *Biopolymers* **14**, 679-694.
72. Majoube, M., Millié, Ph., and Vergoten, G. (1995) *J. Mol. Struct.* **344**, 21-36.
73. Hasegawa, K., Ono, T.-A., and Noguchi, T. (2000) *J. Phys. Chem. B* **104**, 4253-4265.
74. Cordes de N. D., Marcia, and Walter C.S.C., J.L. (1967) *Spectrochimica Acta* **24A**, 237-252.
75. Gallouj, H., Lagant, P., and Vergoten, G. (1997) *J. Raman Spectrosc.* **28**, 909-916.
76. Ashikawa, I., and Itoh, K. (1979) *Biopolymers* **18**, 1859-1876.
77. Miura, T., Satoh, T., Hori-i, A., and Takeuchi, H. (1998) *J. Raman Spectrosc.* **29**, 41-47.
78. Miura, T., Hor-i, A., Mototani, H., and Takeuchi, H. (1999) *Biochemistry* **38**, 11560-11569.
79. Miura, T., Suzuki, K., Kohata, N., and Takeuchi, H. (2000) *Biochemistry* **39**, 7029-7031.
80. Hienerwadel, R., and Berthomieu, C. (1995) *Biochemistry* **34**, 16288-16297.
81. Berthomieu, C., and Hienerwadel, R. (2001) *Biochemistry* **40**, 4044-4052.
82. Noguchi, T., Inoue, Y., and Tang, X.-S. (1999) *Biochemistry* **38**, 399-403.
83. Breton, J., Xu, W., Diner, B. A., and Chitnis, P. R. (2002) *Biochemistry* **41**, 11200-11210.
84. Berthomieu, C., Boussac, A., Mäntele, W., Breton, J., and Nabedryk, E. (1992) *Biochemistry* **31**, 11460-11471.
85. Berthomieu, C., Dupeyrat, F., Fontecave, M., Vermeglio, A., and Niviere, V. (2002) *Biochemistry* **41**, 10360-10368.
86. Hasegawa, K., Ono, T.-A., and Noguchi, T. (2002) *J. Phys. Chem. A* **106**, 3377-3390.
87. Haris, P. I., Robillard, G. T., van Dijk, A. A., and Chapman, D. (1992) *Biochemistry* **31**, 6279-6284.
88. Rahmelow, K., Hubner, W., and Ackermann, T. (1998) *Anal. Biochem.* **257**, 1-11.

89. Barth, A., and Zscherp, C. (2002) *Q. Rev. Biophys.* **35**, 369-430.
FIGURE LEGENDS

Figure 1-A) Cyclic voltammogram recorded with BeSOD at 10 mV.s⁻¹; 1-B) Potential titration of the absorption at 265 nm of oxidized BeSOD. The data are fitted with a Nernst curve, giving a midpoint potential of 130 mV/NHE; 1-C) UV-Vis difference spectra obtained by applying oxidizing and reducing potentials of 500 and −500 mV vs. NHE, respectively, reduced – minus – oxidized (bold line) and oxidized – minus - reduced (thin line) spectra.

Figure 2-A) Reduced – minus – oxidized (bold line) and oxidized – minus – reduced (thin line) FTIR difference spectra recorded with BeSOD in Tris pH 7.4. 9000 scans, corresponding to an average of 30 electrochemical cycles; 2-B) superimposition of the reduced –minus- oxidized FTIR spectra recorded with BeSOD in Ches pH 9.3 in H₂O (thin line) and ²H₂O (bold line).

Figure 3 MeIm IR modes sensitive to metal-coordination. FTIR absorption spectra of 3-A) MeIm in H₂O (thin line) and in ²H₂O (bold line), 3-B) Cu(II) - MeIm solutions at a one to four molecular ratio in H₂O and 3-C) in ²H₂O; 3-D) absorption spectrum of Zn(II) -MeIm solutions at a Zn(II) to MeIm ratio of one to four. Spectra were obtained after subtraction of the buffer absorption. 500 scans per spectrum.

Figure 4-A) Reduced – minus- oxidized FTIR spectrum recorded with ChSOD in Ches pH 9.3, 4-B) superimposition of reduced – minus - oxidized spectra recorded with BeSOD (thin line) and ChSOD (bold line).

Figure 5-A) Superimposition of reduced – minus - oxidized FTIR spectra recorded with ¹⁵N-ChSOD (bold line) and ¹⁴N-ChSOD (thin line) in Ches pH 9.3 in H₂O; 5-B) difference spectra calculated from spectra in Figure 5-A, ¹⁵N- minus – ¹⁴N; 5-C) superimposition of the reduced – minus – oxidized FTIR spectra recorded with ¹⁵N-ChSOD (bold line) and ¹⁴N-ChSOD (thin line) in ²H₂O.
Figure 6 Arg143Gln and Thr137Gln mutants of ChSOD. A) reduced - minus - oxidized FTIR spectra recorded with Arg143Gln mutant (bold line) and WT ChSOD (thin line) in Ches pH 9.3; B) Arg143Gln – minus – WT difference spectrum calculated from spectra of Figure 6-A; C) reduced - minus - oxidized FTIR spectra recorded with Thr137Gln mutant (bold line) and WT ChSOD (thin line) in Ches pH 9.3; D) Thr137Gln – minus – WT difference spectrum calculated from spectra of Figure 6-C.
|      | 5-MeIm | 4-MeIm | Cu-MeIm | Zn-NtMeIm | Zn-NtMeIm |
|------|--------|--------|---------|-----------|-----------|
| v(C\_C\_s) | 1594\textsuperscript{a} | 1575 | 1574 | 1570 | 1605\textsuperscript{d} | 1586\textsuperscript{d} |
| v(C\_N) | 1492\textsuperscript{a} | 1492\textsuperscript{b} | 1485\textsuperscript{a,b} | 1505 | 1500 | 1507 |
| δ(NH) + v(CN) | 1451 | 1451 | 1462 | 1455 | 1450 | 1447 |
| v(C\_N) | - | 1303\textsuperscript{b} | 1303\textsuperscript{b} | 1307 | 1307 |
| v(CN), v(CC) | - | 1264\textsuperscript{a} | - | 1268\textsuperscript{b} | 1268\textsuperscript{b} |
| δ(CH) + v(CN) | 1230\textsuperscript{a,c} | 1230\textsuperscript{a,b,c} | 1223\textsuperscript{a,c} | 1236 | 1236 | 1227 |

|      | BeSOD, H\_2\textsuperscript{2}O oxidized | 1\textsuperscript{52}NChSOD, H\_2\textsuperscript{2}O oxidized | 1\textsuperscript{52}NChSOD, H\_2\textsuperscript{2}O oxidized |
|------|-----------------|-----------------|-----------------|
| v(C\_C\_s) | 1602 \textbf{1592} | 1600 \textbf{1584} | 1597 \textbf{1581} |
| v(C\_C\_s) | 1590 \textbf{1580} | 1589 \textbf{1573} | 1586 \textbf{1570} |
| δ(CH) + v(CN) | 1223 | 1225 | 1560 \textbf{1560} |
| v(C\_N) | 1097 \textbf{1097} | 1095 \textbf{1095} | 1086 \textbf{1086} |

Table I: Main IR modes marker of histidine coordination to a metal in Cu- and Zn- MeIm models and in BeSOD and ChSOD.

(a) Berthomieu et al, 1992 (83); (b) Majoube et al, 1995 (71); (c) Hasegawa et al, 2000 (72); (d) Miura et al, 1999 (77); (e) Hasegawa et al, 2002 (85); (f) Miura et al, 2000 (78); (h) Hashimoto et al, 1998 (28).
Scheme 1: A- active site of CuZnSOD from bovine erythrocyte. Determined from the three-dimensional structure (Tainer et al., 1982). B- Superoxide access channel.
Scheme 2: Representation of Methylimidazole (MeIm) and Metal-MeIm complexes: Nτ-protonated form (5-MeIm) (a), Nτ-protonated form (4-MeIm) (b), Nτ-Cu-bound, Nπ-protonated (NτCu-5MeIm) (c), Nπ-Cu-bound, Nτ-protonated (NτCu-4MeIm)
Figure 1
Figure 3
Figure 4
Figure 5

Wavenumber (cm$^{-1}$)

1700 1600 1500 1400 1300 1200 1100 1000

1584 1571 1550 1545

1581 1560 1550

1545 1535 1520

1505 1500

1095 1111 1102

1066 1086 1045

1680 1664 1655

1648 1650 1655

1600 1590 1586

1581 1578 1574

1560 1535 1500

1545 1535 1510

1095 1086 1045

1x10$^{-3}$ a.u.
Figure 6

Wavenumber (cm$^{-1}$)

A

B

C

D

1x10$^{-3}$ a.u.

1800 1700 1600 1500 1400 1300 1200 1100 1000

1696 1686 1640 1672 1671 1648 1687 1661 1623

1559 1534 1519 1526 1538 1512

1096 1111 1111 1093 1096 1111

1649 1695 1508 1519 1504 1518 1526 1538 1508

1644 1651 1615 1649 1651 1644 1687 1661 1519

1x10$^{-3}$ a.u.
Supplementary Figure 1

A: Denaturing protein gel electrophoresis. Columns 1 and 3: Broad range protein marker (New England Biolabs), columns 2 and 4: deposition of 10 µg of two different batches of commercial BeSOD.

B: Protein gel electrophoresis. Column 1 protein marker (Amersham Biosciences Low Weight), column 2 ChSOD in non denaturating condition, column 3 ChSOD in denaturating condition. This gel was performed on a Phast System (Amersham Biosciences) with 50 ng protein per sample.
Supplementary Figure 2:
MALDI-TOF spectrum recorded with a sample containing $^{14}$N- and $^{15}$N- labeled ChSOD. The analysis was performed in the presence of cytochrome c and apomyoglobin, as calibrating proteins with respective molecular masses of 12362 Da and 16952 Da, using a matrix composed of sinapinic acid at saturation in 30% acetonitrile and 70% trifluoroacetic acid.
The mass were observed at 15861 and 16061 Da for $^{14}$N- and $^{15}$N-ChSOD, respectively (note that only one main peak was observed for the $^{14}$N or $^{15}$N ChSOD sample, not shown).
The mass difference of 200 kDa corresponds to that expected for a full $^{15}$N labeling of ChSOD.
Long distance charge redistribution upon Cu,Zn-superoxide dismutase reduction: Significance for dismutase function
François Dupeyrat, Claude Vidaud, Alain Lorphelin and Catherine Berthomieu

J. Biol. Chem. published online August 24, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M402728200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2004/09/16/M402728200.DC1

This article cites 0 references, 0 of which can be accessed free at
http://www.jbc.org/content/early/2004/08/24/jbc.M402728200.citation.full.html#ref-list-1