ISAS - INTERNATIONAL SCHOOL
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XAS study of the reconstitution process of Cu,Zn superoxide dismutase in the presence of Cu(I) - GSH complex

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1. INTRODUCTION

An important goal of biological structure studies is to understand the functioning of metallo-proteins. Typically these contain only a few metal atoms (Mn, Fe, Zn, Cu, Mo, etc) among several thousand light atoms (H, C, O and N) and they often have an important enzymatic function centered on the metal atom site. About one-fourth to one-third of proteins and enzymes contain metals or otherwise require metal ions for their biological activity.

The structure of a number of metallo-proteins have been determined by X-ray diffraction techniques. These metallo-proteins have been shown to be highly elaborated coordination complexes whose metal-containing sites, comprising one or more metal atoms and their ligands, are usually the loci of electron transfer, binding of exogenous molecules, and catalysis. Selected active sites whose structure have been deduced from crystallographic investigations are collected in Fig.1 [Ibers and Holm (1980)].

Besides being very complex structurally, many of these systems cannot be crystallized and therefore X-ray diffraction studies are difficult or impossible. Even in the systems that can be crystallized, there is always the concern that the crystalline form is not the natural state of the protein and that the solution of the entire protein structure is needed in order to determine the local environment of the metal sites.

Protein crystals rarely diffract to a resolution better than 1.5 Å. Thus the overall structure is usually well described, but details such as the local environments of metal atoms in metallo-proteins are not as precisely defined as would be expected from low molecular weight molecules. Since the metal sites are recognised as being essential for the activity of the metallo-enzyme, it is important to have a very accurate picture of them.

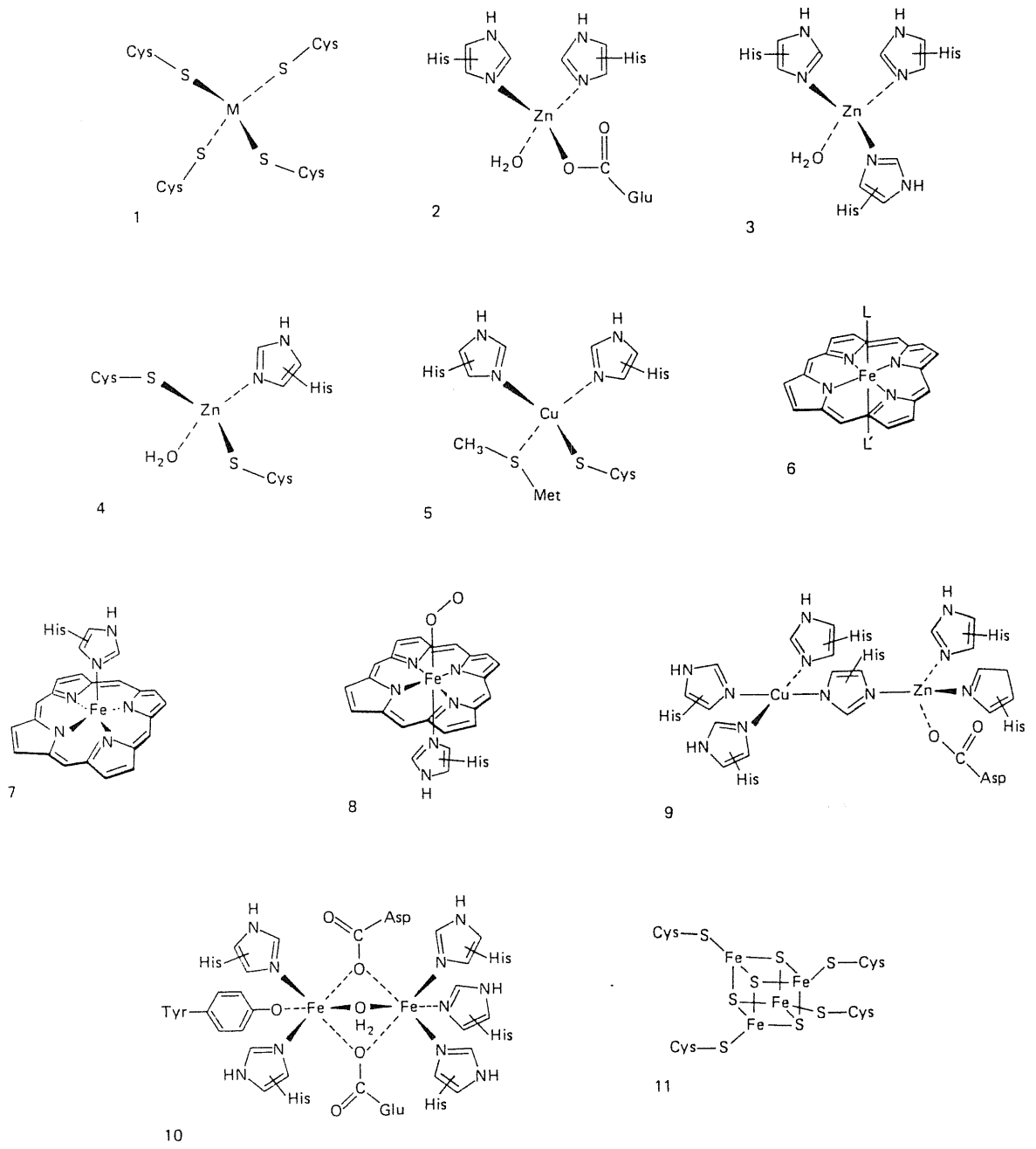


Fig. 1: Examples of some metal ions bound tightly to proteins, with the partial structure of the amino acid side chains to which they are attached.
1, M is either FE, as in rubredoxin, or Zn, as in aspartate transcarbamylase and liver alcohol dehydrogenase. 2, Carboxipepsidase A. 3, Carbonic qnhydrase and insulin. 4, Liver alcohol dehydrogenase. 5, Azurin and plastocyanin. 6, Heme group; L is His and L' is His in cytochrome b_5 or Met in cytochrome c. 7, Deoxy heme groups in myoglobin and hemoglobin. 8, Oxy form of 7. 9, Superoxide dismutase.

A complementary technique of X-ray diffraction in structural studies of metal atoms in biomolecules is X-ray Absorption Spectroscopy (XAS).

XAS has the distinct advantage of probing the metal site directly, greatly simplifying the analysis of the data: the distance information extends within about 3.5-4 Å from the absorber and in favorable cases may distinguish up to four different coordination distances in this bond-length range. This means that one needs only to refine a small number of variables to obtain the structure around the absorbing atom, whereas single-crystal diffraction results depend for accuracy on refining all of the atomic positions in the crystal. The localized nature of the phenomenon gives a major advantage in that local structure of the metal site can be probed with accuracy often approaching that routinely achieved in small atom crystallography: the accuracy in determining the near neighbour distances is usually within ± 0.02 Å [Cramer & Hodgson (1979), Teo (1981)]. This allows more subtle changes in the local environment to be monitored: it is possible to study changes in the local chemistry upon a biochemical reaction. These changes are often quite subtle and within the error limits of crystallographic determination.

Being sensitive to short-range order in atomic arrangements rather than to long-range crystalline order, XAS can be used equally well for systems in solution and in crystalline form. Since XAS does not require crystallinity, it allows the study of proteins in solution under various chemical conditions: thus changes in the metal site can be monitored throughout the stages of chemical reactions in solution and in physiological conditions. XAS can also clearly define the oxidation state of the absorber; at the same time it is possible to investigate the neighbourhood of the metal atom in different oxidation states.

XAS experiments have been performed on a large number of macromolecules of interest to biochemistry: complete and recent reviews on the applications of XAS to biological molecules are those of

Cramer (1989) for all metallo-proteins, Blackburn (1990) for copper proteins and Hedman (1990) for vanadium and molybdenum proteins. Lindley *et al* (1990) discuss the applications of both crystallography and XAS to metallo-proteins.

XAS can be applied to solve different problems. A first kind of experiments involves proteins where the ligands of the metal ion are already known from diffraction studies and XAS is used to obtain more accurate bond length and geometry information, and their extentions to non-crystalline situations. Experiments can also deal with proteins where some or all of the ligands are unknown and the goal is to determine the coordination number, the bond length and ultimately the local structure. A third kind of experiments have developed the utility of XAS as a direct probe of the oxidation state of metal sites in proteins.

In this thesis we have applied XAS to the study of the reconstitution of the native Cu, Zn superoxide dismutase (SOD) from the copper-free protein in the presence of copper-glutathione complexes. The reconstitution process was followed by EPR spectroscopy and by absorption spectroscopy monitoring the formation of the native Cu(II)-protein binding, by NMR monitoring Cu(I)-protein binding [Ciriolo *et al* 1990]. The reduced copper-glutathione complex has been shown to be able to donate Cu(I) to Cu-free, Zn superoxide dismutase while the oxidized complex was not able to full reconstitute the holoenzyme [Ciriolo *et al* 1990]. Evidence was obtained for the occurrence of a Cu(I)•GSH•protein intermediate in the reconstitution process [Ciriolo *et al* 1990]. The main purpose of this XAS study has been to inquire if the formation of the ternary complex takes place by monitoring the copper atom in both the reduced and oxidized state.

2. THE BIOLOGICAL PROBLEM.

2.1 Copper.

Copper is a transition metal essential for life and its metabolism has been widely studied [Cousins 1985 and references therein]. The total amount of copper in an adult human is normally between 100 and 150 mg, with most of it found in the liver, blood, nervous tissue and kidneys. It is required for a variety of functions, including bone formation, proper cardiac function, connective tissue development, keratinization and tissue pigmentation. It is a cofactor in several mammalian enzymes including cytochrome c oxidase, lysyl oxidase, tyrosinase, copper/zinc superoxide dismutase (SOD), dopamine β-hydroxylase, amine oxidase and ceruloplasmine. In most cases the function of copper in metallo-enzymes involves electron transfer and enzymatic binding of molecular oxygen.

Even if copper is an essential trace nutrient, it is toxic when present at inappropriately high concentrations. Several mechanisms have been proposed to describe cellular copper toxicity, but it seems to be a result of the metal ability to catalyze the formation of toxic oxygen species through a series of redox reactions [Samuni *et al* (1981), Goldstein *et al* (1986)]. It has also been shown that cupric ion can bind to protein cysteinyl thiol groups: if oxygen is present, the metal-thiolate bond can be oxidized, forming cystine cross-links and thus irreversibly inactivating the enzyme [Nakamura *et al* (1972)].

Because of its high toxicity it is important to know how copper is transported to the cell and inside the cell. Copper is absorbed from stomach and small intestine, then it is transported in portal plasma, bound principally to albumin and possibly as amino acid complexes. Hepatic uptake occurs via a saturable transport process. Systemic transport of copper from liver is primarily as ceruplasmin, which appears to donate copper to tissues. Two different systems have been shown to be able to answer to elevated and toxic levels of copper in

the cell: metallothionein (MT) [Freedman *et al* 1989 b] and glutathione (GSH) [Freedman *et al* 1989 a].

Even if copper is present in many metallo-proteins, few is known of how and when copper is inserted into the intact apo-enzyme molecule. Copper-metallo-thioneins has been successfully used *in vitro* for reconstitution and reactivation of several copper enzymes [Beltramini *et al* (1982), Morpurgo *et al* (1983), Hartmann *et al* (1983), Brutsch *et al* (1984), Schechinger *et al* (1986), Markossian *et al* (1983)].

2.2 Glutathione.

Glutathione is a three peptide (γ–glutamil-L-cysteinyl-glycine). It is ubiquitous in eucaryotic cells and is implicated in many cellular functions [Meister *et al* 1983]. It is the most prevalent cellular nonprotein thiol [Ziegler *et al* 1985] and the most abundant low-molecular-weight peptide.

Although tissue GSH can change as a function of age or nutritional state, the concentration of reduced glutathione (GSH) is very high and constant relative to its disulfide, the oxidized glutathione (GSSG). In human erythrocytes the physiological concentration of reduced glutathione is 2 to 3 mM [Rabenstein *et al* (1979) and Miyoshi *et al* (1983)] and that of the oxidized form is about hundred or thousand times lower.

The relative levels of reduced and oxidized glutathione are regulated by a series of reactions. GSH reduces H_2O_2 or lipid peroxides via the glutathione peroxidase reaction. NADPH is used to reduce the GSSG via the glutathione reductase reaction. GSSG is also formed by reaction of GSH with free radicals.

Since glutathione may function as an intracellular metal chelator, as well as a substrate for the removal of toxic oxygen species, the relationship between copper, GSH and MT was examined using rat liver hepatoma cell lines resistant to the toxic effects of copper [Freedman *et al* (1986)]. These cells accumulate more copper

than the nonresistant parental cell line and contain elevated levels of MT [Freedman *et al* (1989 b)]. Resistant cells also have up to four times more glutathione than the parental wild type cell line. A majority of the cytoplasmic copper in both wild and resistant cells was found as a GS-Cu complex [Freedman *et al* (1989 a)]. These observations, and the finding that *in vivo*, copper is complexed to GSH before the bounding to MT, suggested that GSH chelates cytoplasmic copper and then transfers the metal to MT for storage [Freedman *et al* (1989 a)]. Also the reverse pathway has been described [Freedman *et al* (1989 c)], demonstrating that the copper bound to metallothionein is not permanently sequestered, but can be incorporated into other copper proteins (Fig. 1).

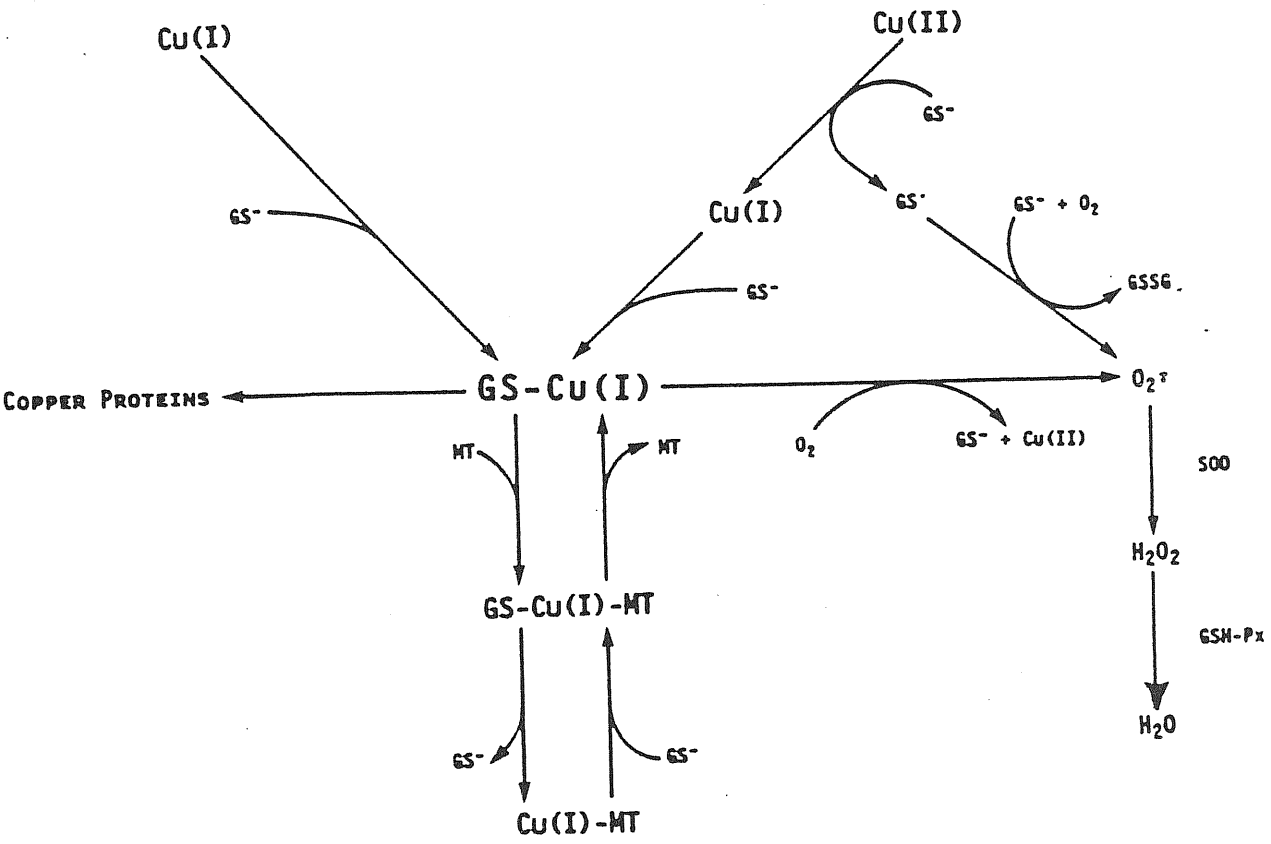


Fig. 1: Schematic diagram of cellular copper metabolism as proposed by Freedman et al 1989 a). The diagram outlines a mechanism for the transfer of copper from GSH to MT. It also indicates that when copper is needed for the synthesis of other metallo-enzymes it can be obtained from the GS-Cu(I) pool.
MT, metallothionein; SOD, superoxide dismutase; GSH-Px, GSH peroxidase.

2.3 The Cu(II)•GSSG complex.

The Cu(II)-oxidized glutathione complex is of biological and chemical interest:

• an inhibitor of opiate receptor binding from human erythrocytes has been identified as a glutathione copper complex [Marzullo *et al* 1977]. While glutathione *per se* has no effect, Cu and other transition metals are potent inhibitors of opiate receptor binding.

• it has been reported that the Cu(II) complex is involved in rheumatoid arthritis and that the inhibitory activity is due to a complex of oxidized glutathione with Cu(II) [Micheloni *et al* 1978].

• the catalytic activity of a Cu(II)-oxidized glutathione complex upon the disproportionation of superoxide radicals has been shown in the pH range 7-9 [Jouini *et al* 1986].

A complex can be obtained by adding $CuSO_4$ to solutions of oxidized glutathione. This complex (Cu(II)-GSSG) has been studied by EPR and visible absorption spectra [Postal *et al* 1985]. The compound has its λ_{max} at 624 nm. The complex seems different in solution and in solid state and its structure have been related to the pH of the solution.

Between pH 6-11 the only Cu(II)-GSSG complex present in aqueous solution is a mononuclear species with the metal bound to the glutamyl carboxyl and amine groups. From these results copper binds to two nitrogen and two oxygen atom in approximately square-planar coordination (Fig.2).

Below pH 5 the glycine group also binds to the metal ion, although coordination of the glycine carboxyl group has not been excluded at other pH values.

The solids products are binuclear with Cu_2GSSG stechiometry. It seems that these solids contain a mixture of mononuclear and binuclear CuGSSG complexes, the proportion of which depends upon the pH of the solution from which they were obtained. Anyway the major solid products obtained from solutions at pH values between 7 and 11 all have the stechiometry Cu_2GSSG.

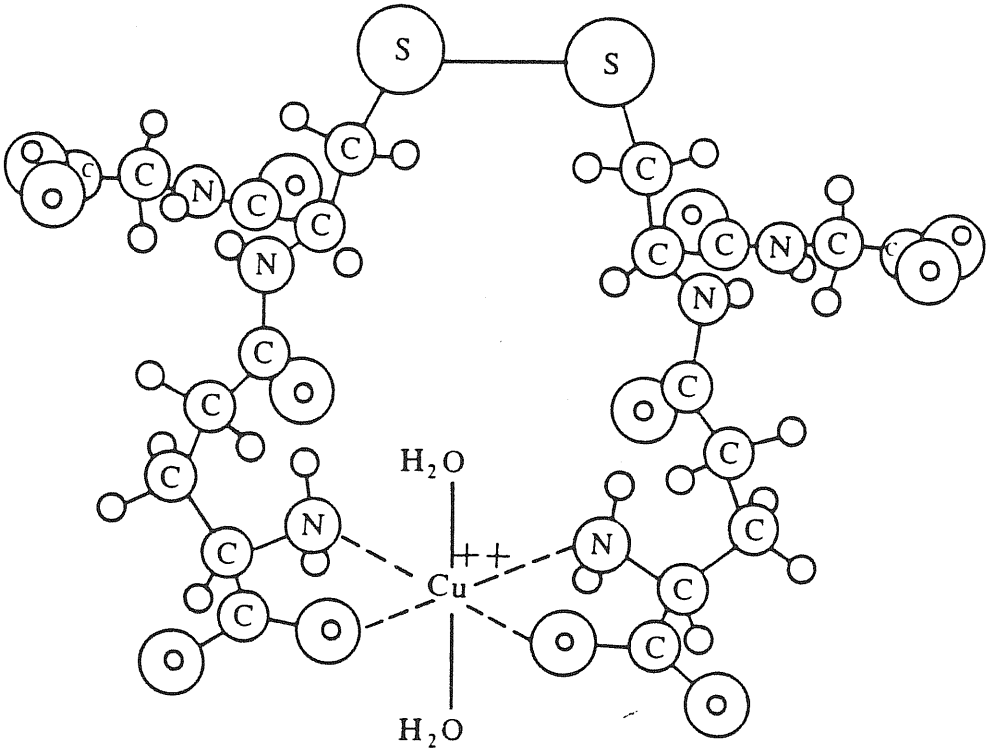


Fig. 3: Suggested conformation for oxidized glutathione-Cu(II) complex from ESR spectroscopy [Huet *et al* (1984)].

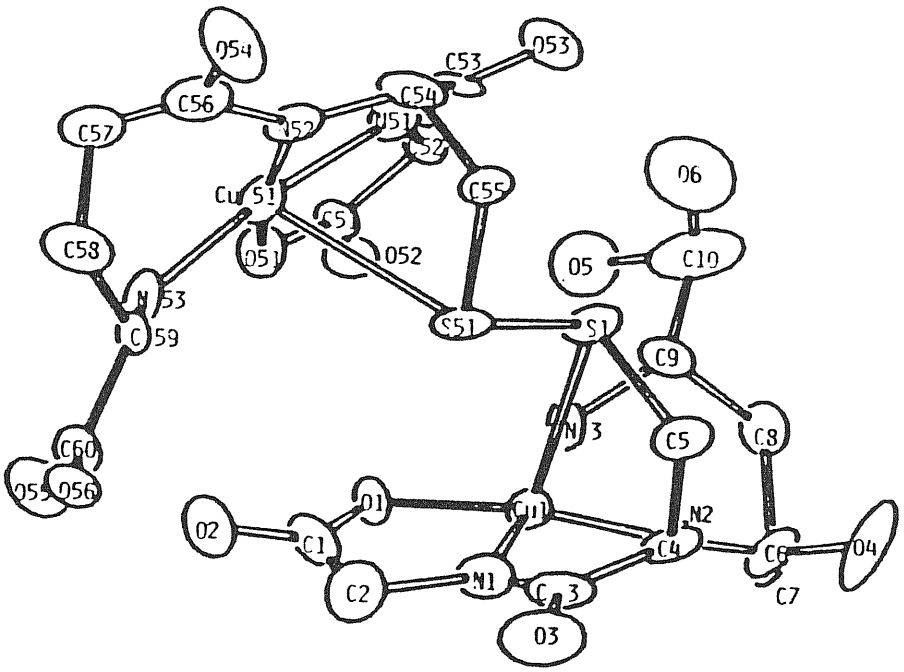


Fig. 2: Proposed molecular structure for the binuclear oxidized glutathione-Cu(II) complex: the crystallographic analysis clarified the presence of two Cu(II) sites via a disulfide bridge [Miyoshi *et al* (1980)].

This is in agreement with the X-ray crystallographic data [Miyoshi *et al* 1980] of a violet glutathione-copper(II) complex from a solution at pH 11.3. The complex is a binuclear Cu_2GSSG with each Cu(II) atom in a distorted square-pyramidal configuration and the Cu(II)-Cu(II) distance of 5.21 Å. Each copper atom is coordinated to three nitrogen atoms, one oxygen and one sulfur (Fig.3). The visible absorption spectrum has its maximum at 593 nm.

2.4 The Cu(I)•GSH complex.

The univalent state of copper is supposed to be important in the cell membrane and within the cell, where the local redox potential is supposed to be considerably lower than in blood plasma. Copper(II) complexes are considerably more toxic than Cu(I)-thiol complexes when they are administered parenterally [Whitehouse *et al* 1978].

A Cu(I)•GSH complex can be obtained by adding $CuSO_4$ to a solution of reduced glutathione in phosphate buffer, either under strictly anaerobic conditions or in air. Cu(II) readily oxidizes glutathione to diglutathione and simultaneously Cu(II) is reduced to Cu(I); GSH then forms a complex with Cu(I): this complex is very stable even in the presence of oxygen. The complex starts to oxidize in air only after 5 h of incubation in a shaking water bath at 37°C. The reoxidation time increases as the GSH:Cu(I) ratios increase.

There are no spectroscopic studies on the Cu(I) complex because Cu(I) is magnetically and optically silent. Equilibrium data were obtained by electromotive force titrations on the Cu(I)-glutathione system at 25° C in 0.5 M $NaClO_4$ medium [Österberg *et al* 1979]. The results show that a mononuclear $Cu(GSH)_2$ species form. It is suggested that the initial phase in copper intoxication involves the following general reaction:

$$2\ Cu(II) + 6\ GSH^- \longrightarrow 2\ Cu(I)(GS)_2^{3-} + GSSG^{2-} + 6\ H^+.$$

2.5 The reconstitution of Cu, Zn-SOD.

Superoxide dismutases are metalloenzymes that play an essential role in the defense of the cell against the potentially toxic derivative of the biological activation of oxygen, the free radical superoxide $O_2^{\cdot-}$. For complete reviews on superoxide dismutase, see Fee (1981), Bannister (1984) and (1987).

The x-ray crystal structure of bovine Cu, Zn superoxide dismutase has been determined to a resolution of 2.0 Å [Tainer *et al* (1982 and 1983)]. The protein exists as a dimer with the two subunits held together by non-covalent interactions. Each subunit is comprised of a flattened β-barrel containing eight anti-parallel strands and in addition three external loops (Fig. 4).

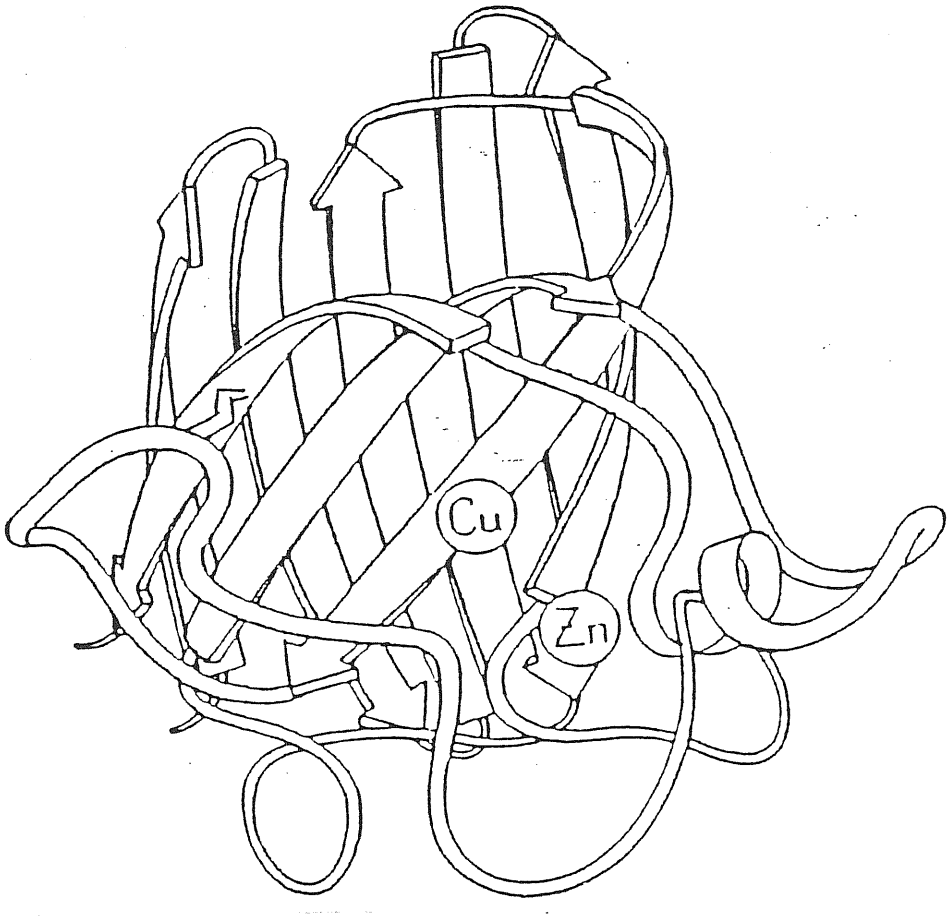


Fig. 4: Schematic backbone drawing of the SOD subunit as viewed down the axial direction of the Cu atom from the solvent. The β-strands are shown as arrows and the disulfide as a zig-zag. Two long loops extend forward from pairs of β-strands to form the upper and lower sides of the active channel. The Cu and Zn lie at the bottom of that channel, with the Cu accessible to solvent from the viewing direction [from Tainer *et al* (1982)].

The active site is situated at the bottom of a long channel located on the external surface of the barrel between the two largest loops and contains a Cu(II) and a Zn(II), 6.3 Å apart, and bridged by the imidazole ring of His 61. The copper atom is coordinated to four himidazole rings from His 44, 46, 61 and 118. The geometry is essentially a tetrahedrally distorted square planar arrangement (Fig. 5).

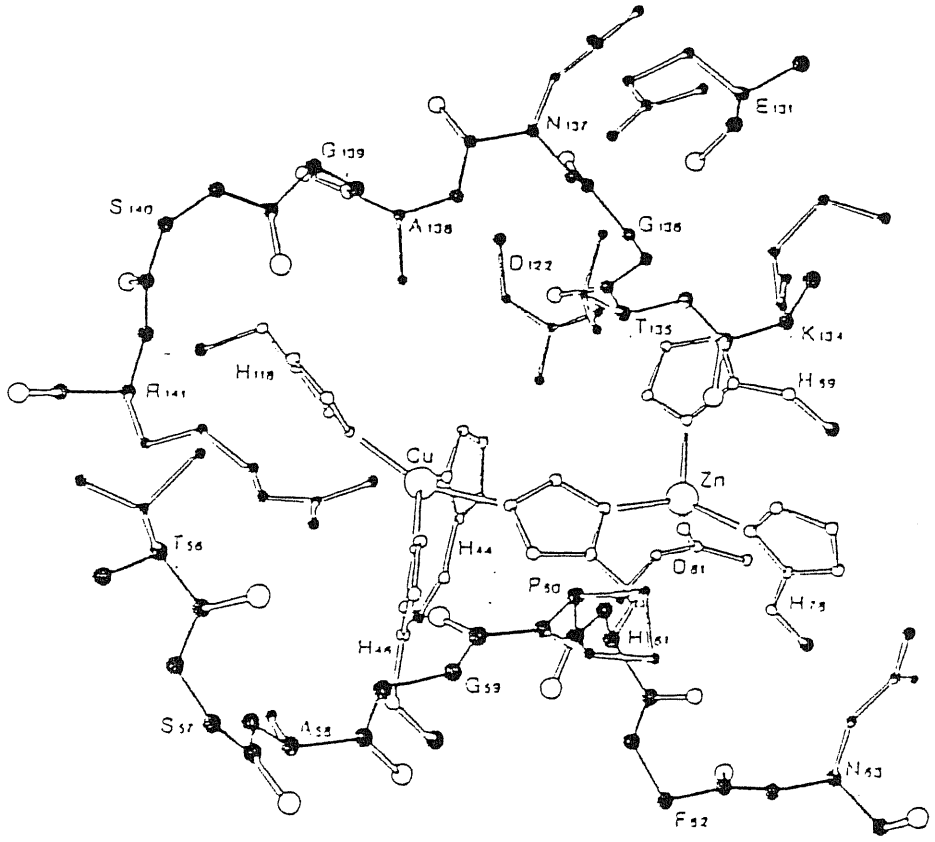


Fig. 5: Schematic drawing of the metal binding sites of bovine superoxide dismutase. The geometry of the Zn ligands is tetrahedral, with distortion toward a trigonal pyramid with Asp 81 at the apex. The Cu ligands form a tetrahedrally distorted square plane with the axial position of the copper much more open on the solvent side than on the protein side.

The biological mechanism by which the catalytically active copper is taken up by the protein moiety in the active-site pocket is still a unresolved problem. Copper thionein was found to reactivate several copper apoenzymes *in vitro* [Beltramini *et al* (1982), Morpurgo *et al* (1983), Hartmann *et al* (1983), Brutsch *et al* (1984), Schechinger *et al* (1986), Markossian *et al* (1983)], but it was apparently ineffective in reconstituting the holoenzyme from copper-free superoxide dismutase [Geller & Winge (1982)].

In vitro evidence showed that reduction of Cu(II) to Cu(I) in the presence of glutathione led to a copper-zinc replacement in zinc metallothionein [Suzuki & Maitani (1981)].

Pulse-chase experiments in copper resistant cells demonstrate that, *in vivo*, ^{87}Cu can be transferred from MT to GSH and superoxide dismutase [Freedman *et al* 1989].

Ciriolo *et al* (1990) tried to reconstitute the Cu, Zn superoxide dismutase from the copper-free protein by the Cu(I)•GSH complex. The process was followed by NMR, EPR and optical spectroscopy. Cu(I)•GSH was found to be a very stable complex in the presence of oxygen and a more efficient copper donor to the copper free enzyme than other low molecular weight Cu(II) complexes. In particular 100% reconstitution was obtained with stoichiometric copper at any GSH:copper ratio between 2 and 500.

The process was monitored by:

• <u>EPR upon reoxidation of the enzyme-bound copper</u>.

Since the Cu(I)•GSH complex does not give any EPR signal, the reconstitution can only be followed by the appearence of the EPR signal due to the Cu(II) ion. One hour after the addition of the 1:3 Cu(I)•GSH complex to a solution of Cu free, Zn-superoxide dismutase at 37°C in air, an EPR signal accounting for 10% of the total copper appeared. Only after 24 h an EPR signal accounting for 100% reconstitution of the enzyme appeared;

• optical spectroscopy upon reoxidation of the enzyme-bound copper.

Since the Cu(I)•GSH complex does not give any spectroscopic signal, the reconstitution can only be followed by the appearence of the signal due to the Cu(II) ion. The compound has its λ_{max} at 624 nm [Postal *et al* (1985)]. 50 % of reconstitution was detected after 5 hours of incubation in air, while reoxidation of Cu(I), Zn-SOD by air is faster (50 % oxidized enzyme after 30 min.). This difference may suggest the formation of a Cu(I)•GSH•protein intermediate;

• NMR spectroscopy following the broadening of the resonances of the Cu(I)•GSH complex after addition of Cu-free, Zn superoxide dismutase.

Each addition of Cu-free, Zn superoxide dismutase to the 1:2 Cu(I)•GSH complex broadened the resonance marking the Cu(I)•GSH complex by reducing its internal mobility. The height of the resonance at 3.83 (typical of the Cu(I)•GSH complex) was decreased to 30 % of its original value after the addition of Cu-free, Zn superoxide dismutase. These results actually support the formation of a ternary complex between the copper-free enzyme and the Cu(I)•GSH complex;

• NMR spectroscopy of the Cu-free,Co(II) enzyme following the appearance of the isotropically shifted resonances of the Cu(I),Co enzyme.

The copper-free enzyme with Co(II) substituting for the zinc ion has been examined. The use of this approach is advantageous in two respects. Firstly, it is possible to monitor the reconstitution process by observing the changes of the active site geometry through the modification of the isotropically shifted resonances of histidines bound to the metal cluster. Secondly, this procedure does not require reoxidation of the metal ion and therefore gives direct information on the binding of Cu(I) to the enzyme. Two bands were missing in the spectra of the reconstituted enzyme with respect to the spectrum of Cu(I),Co superoxide dismutase. A possible explanation for this difference is that some residues became more exposed in the presence of the GSH-copper complex ans thus exchangeable with the solvent or that binding of copper-GSH complex to the metal site alters the

geometry of the copper coordination sphere leaving some liganding groups uncoordinated.

In conclusion, this study shows that a very stable complex between copper and glutathione is obtained even in the presence of oxygen and that this complex is able to donate Cu(I) to copper-free superoxide dismutase giving 100 % reconstitution of the holoenzyme under conditions where Cu(I) complexes are not able to fully reconstitute the enzyme. The hypothesis that the formation of a ternary complex, the copper•glutathione•apoprotein complex, may occur has been done.

3. X-RAY ABSORPTION SPECTROSCOPY

When an x-ray beam passes through a medium, its intensity is attenuated exponentially according to the classical absorption equation

$$I=I_0e^{(-\mu x)} \qquad (1)$$

where I e I_0 are the trasmitted and incident intensities, respectively, μ is the linear absorption coefficient, and x is sample thickness. In general μ is a function of the photon energy. When the x-ray energy $h\upsilon$ becomes equal to or greater than the binding energy E_b of a core electron, the latter is emitted by a photoelectric process from the atom with kinetic energy E, conserving energy in the process:

$$E=h\upsilon-E_b \qquad (2)$$

The spectra are naturally divided into two regions, Fig.1:

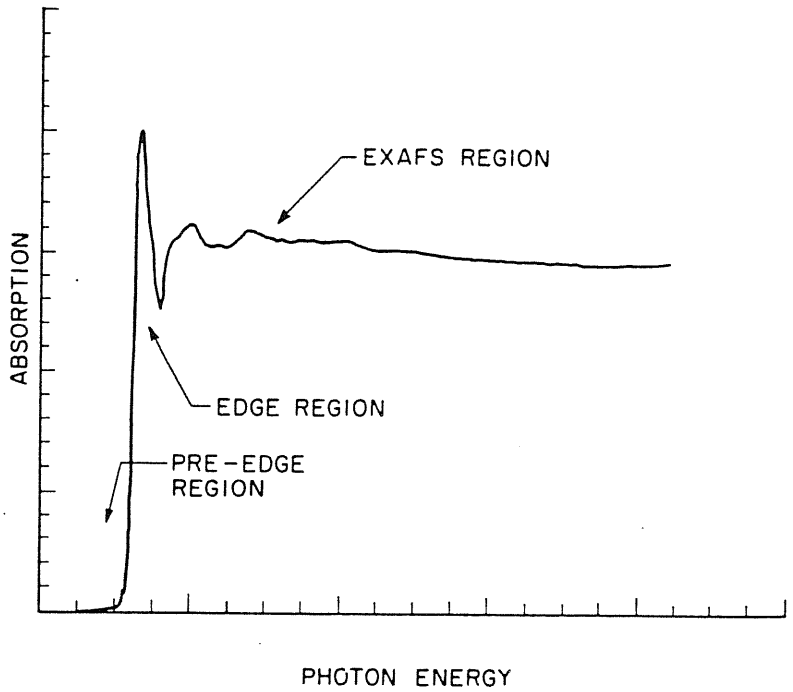


Fig. 1: Schematic of X-ray absorption spectrum showing the threshold region (including pre-edge and edge regions) and the EXAFS region.

1) the threshold, or pre-edge and near edge regions, from about 13 eV below the absorption edge to 40-60 eV above the absorption edge;

2) the EXAFS -Extended X-ray Absorption Fine Structure- region from about 40-60 eV above the absorption edge out to as much as 1000 eV above the edge.

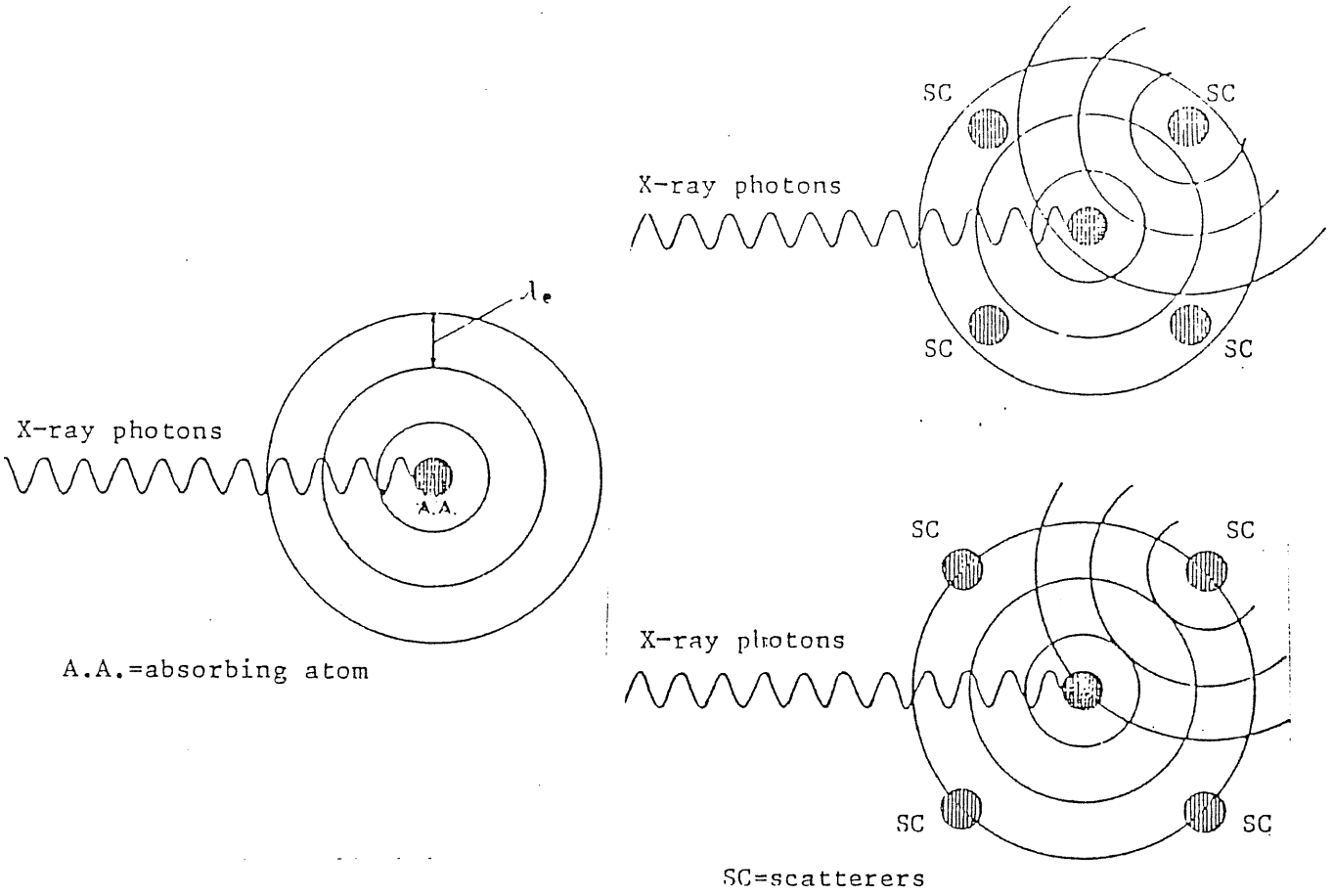
3.1 EXAFS.

As early as the 1930's, Kronig had observed that at photon energies above the value of the edge, the absorption spectrum of most substances was characterized by the presence of complex oscillations as a function of energy which extended several hundred electron volts above the absorption threshold. This non-monotonic variation has received the name of **EXAFS**, which is an acronym for **E**xtended **X**-ray **A**bsorption **F**ine **S**tructure.

EXAFS only occurs when atoms are present in condensed matter: isolated atoms do not show this fine structure. The outgoing electron is shown as a spherical wave, whose wavelength $\lambda=2\pi/k$ depends on the energy of the electron according to the formula

$$k=\sqrt{\frac{2m(h\upsilon-E_0)}{h^2}} \qquad (3)$$

where E_0 is the threshold energy and m is the mass of the electron. If the atom were isolated, its photoelectron would consist of only an outgoing wave to describe the electron escaping from the atom. Such a state contributes only a smooth absorption as measured esperimentally and expected theoretically. The oscillatory behavior occurs because of the addition of the backscattered wave from the surrounding atoms, which interferes with the outgoing part (Fig. 2). This interference will either decrease or increase the photoelectron wave function depending on whether this interference is destructive or constructive. Thus EXAFS is a direct consequence of the wave nature of the photoelectron, and the peaks correspond to constructive



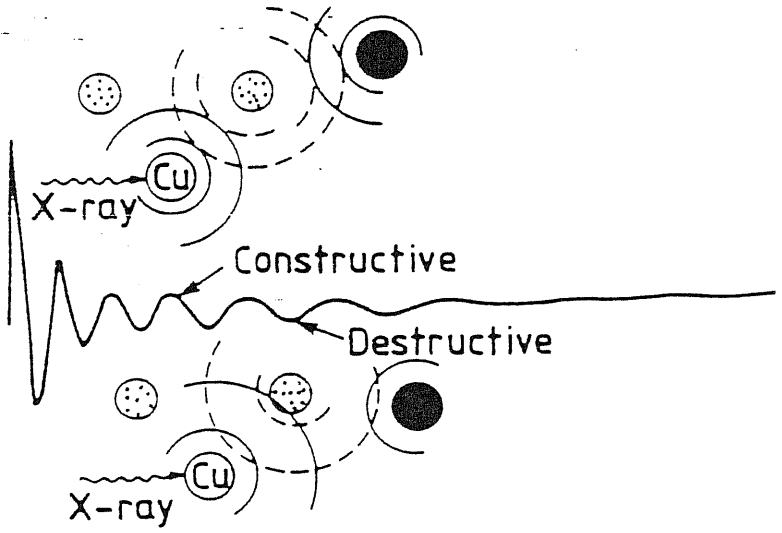


Fig. 2: The origin of EXAFS. The photoelectron wave propagates outward from the central atom. The scattered wave interferes with the outgoing wave at the central atom.The sinusoidal EXAFS pattern can be understood as the changing interference pattern between the outgoing wave and the scattered wave at the absorbing site with the changing photoelectron wavelength (energy).

interference between the outgoing and the scattered parts at the origin, while the valleys correspond to destructive interference.

The theory of EXAFS has been extensively discussed [Lee & Pendry (1975) and Ashley & Doniach (1975)]. The main result of these analyses is that, except for energies very close to the absorption edge, only single-scattering events need to be considered. When the energy of the photo-electron is sufficiently high for the outgoing wavefunction to be accurately approximated by a plane wave, the theory may be greatly semplified. They showed that using a single-scattering approximation the observed fine structure oscillations may be understood in terms of the interference between the outgoing photoelectron wave in the vicinity of the central atom and that portion of it backscattered from neighbouring atoms. In this approximate, high energy theory, the magnitude of the x-ray absorption fine structure χ (k), is given by equation

$$\chi(k)=\sum_{j=1}^{N}\frac{N_j f_j(k,\pi)}{kR_j^2}\ e^{-2\sigma^2k^2}\ e^{-2R_j/\lambda}\ \sin[2kR_j+\delta_j(k)] \qquad (4)$$

The expression shows the summation over all atoms in the vicinity of the absorber: each term corresponds to a scatterer at a certain absorber-scatterer distance and is represented by a sine wave. N_j is the **coordination number**, or the number of the scattering atoms, and R_i the **inter-atom distance** for the j-th shell. The strength of the contribution to EXAFS from neighbours the same average distance R_j away (which we call a shell of atoms) will be proportional to the number N of such atoms of type j in that shell. The **wave vector** (k) of the ejected photoelectron is given by eq. (3).

Now let us consider the meaning of each term.

The amplitude of each sine wave depends upon N_j, the number of equivalent scatterer, and the **backscattering amplitude**, denoted by $f(k,\pi)$. The shape of the backscattering amplitude is characteristic of the atomic number of the scatterer and is used to identify the type of scattering atom. The k-dependence of the backscattering amplitude in eq. (5) has been measured in various cases. This amplitude has also been calculated by Lee and collaborators [Lee & Pendry (1975), Teo & Lee (1979)] as a function of atomic number Z, revealing a systematic variation consistent with experimental results. As the atomic number increases, the peak in $f(k,\pi)$ moves to higher values of k, its magnitude decreases, and the backscattering persists to higher k value. The magnitude of the peak in $f(k,\pi)$ does not change drastically with Z so that the effect is not dominated by any one class of atoms in a mixture. Thus the contribution of light atoms are not smoothered by those of heavy atoms. Equally important, the significant variation in the k-dependence of $f(k,\pi)$ permits a distinction to be made between atoms in different rows in the periodic table.

The amplitude of $\chi(k)$ is inversely related to the square of the distance R_j between the absorber and the scatterer. It is modified by a **Debye-Waller factor** $e^{-2\sigma^2k^2}$, where σ^2 is the mean square deviation about the average value R_j.

Another exponential term $e^{-2R_i/\lambda}$ is also included to account for the decrease of the amplitude due to inelastic scattering processes, λ being the inverse mean free path of the photoelectron. It is this term which limits the range of contribution to $\chi(k)$.

The last factor gives the sinusoidal modulation of $\chi(k)$, responsible for the fine structure, and allows one to determine the distances R_j from the measured values of k, providing $\delta_i(k)$ is known. The term $2kR_j$ counts the change of phase as the free electron of wave number k traverses the distance $2R_j$, from the absorber to the scatterer and back. The additional term $\delta_i(k)$ is added because the electron experiences the central atom phase shift twice (for leaving

the absorber and again for reentering the absorber), but it experiences the neighbouring atom phase shift once by propagating from the absorber to the neighbouring atoms and back to the absorber. Thus the phase shift has two contributions, one from the center atom and the other from the backscattering atom:

$$\delta_i(k) = \delta_b(k) + 2\delta'_e(k) \qquad (5)$$

The first term δ_b is the phase shift due to the backscattering from the j-th atom, while the second term is twice the central atom phase shift δ'_e to account for the potential of the central atom through which the photoelectron wave has traversed. The phase $\delta_i(k)$ contributes a shift to the position of atoms and this shift must be determined in some manner in order to obtain R_j. These contributions have been calculated for K-edge and L-edge EXAFS by Lee and collaborators [Teo & Lee (1975)]. Again there is a Z-dependence which may be a useful additional aid for distinguishing the scattering atom.

Each EXAFS wave contains two sets of highly correlated variables: [f(k), σ, λ, N] and [$\delta_j(k)$, E_0, r]. Significant correlations can occur both with and between these two sets of variables as well as between different scattering terms. In order to determine N and σ, f(k) must be known reasonable well; similarly, in order to determine r, $\delta_j(k)$ must be known accurately. In practice, f(k) and $\delta_j(k)$ can be determined empirically from model compounds of known structure or calculated theoretically from first principles.

Structural determinations via EXAFS depend on the feasibility of resolving the data into individual waves corresponding to the different types of neighbours of the absorbing atom. This can be accomplished by either curve fitting of Fourier-transform techniques. Very good reviews on data analysis in EXAFS are: Teo (1980), Lee (1981) and Sayers (1989).

3.2 Absorption edge structure.

In the precedent section we have explained the basic principles of EXAFS and the information available from analysis of the EXAFS portion of an x-ray absorption spectrum. The absorption edge also contains fine structure, generally referred to as X-ray Absorption Near Edge Structure, or XANES. The XANES extends to *ca* 60 eV above the edge, which for copper translates into an energy range of *ca* 8980-9040 eV. The general features of the edge region are the existence of one or more distinct absorption lines in the range -20 to 0 eV, a discontinous rise at the edge, followed by a series of fairly narrow resonant peaks superposed in the range 0 to 30 eV.

XANES arises from a number of related phenomena. It is most easily understood in term of transitions from core states to empty bound states. These may be valence orbitals such as 3d, 4s, 4p, etc., or they may be antibonding orbitals in the case of strongly covalent complexes. However this description is probably only valid at very low energies (ca 8990 eV for copper). and completely ignores the modification to the excited states caused by the core hole. Thus at higher energies more sophisticated treatments must be used, which include the effects of interligand multiple scattering and the shake-up/shake-down processes.

XANES can also be used to characterize different oxidation states at the K-edge of the same atom. By simple electrostatics it will cost more energy to remove a core electron as the positive charge on an ion is increased. Thus the absolute position of the egde is observed to increase by 3 eV or so for each oxidative electron removal from a given ion.

Since theoretical calculations and simulations of XANES spectra are very difficult, it can be useful to make a qualitative analysis by using model compounds. The Cu x-ray absorption edge features of 19 Cu(I) and 40 Cu(II) model complexes have been systematically studied and correlated with oxidation state and geometry [Kau *et al* (1987)].

Studies of Cu(I) model complexes with different coordination number reveal that an 8983-8984 eV peak (assigned as the Cu 1s 4p transition) can be correlated in energy, shape and intensity with ligation and site geometry of the cuprous ion [Kau *et al* (1987)]. This feature decreases in intensity as the coordination number of the complex increases from 2 to 4. Higher energy features of lower intensity are generally observed in all the complexes. An empirical ligand field analysis was able to account semi-quantitatively for the observed changes in energy and intensity of the 8983 eV and higher energy features. The proposed energy level diagram is shown in Fig. 3 [Kau *et al* (1987)].

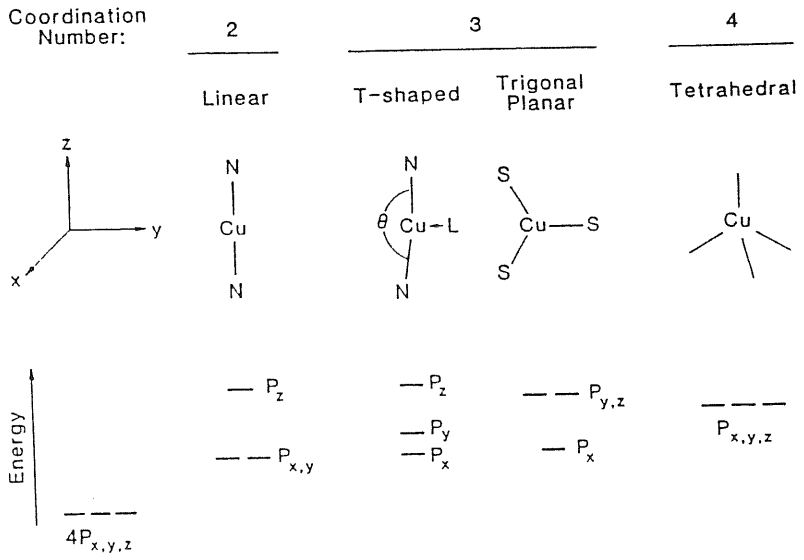


Fig. 3: Energy level diagram for 4p orbitals of 2-, 3- and 4-coordinate Cu(I) complexes.

For linear 2-coordinate geometry, the transition from 1s to the doubly degenerate $4p_{x,y}$ final state would result in an intense edge peak (8983 eV) at lower energy than the 1s-$4p_z$ transition.

For 3-coordinate Cu(I) (T-shaped) complexes, which can be thought of as a perturbed 2-coordinate structure via the introduction of a ligand along the y-axis, the doubly degenerate $4p_{x,y}$ orbitals now split, p_y going to higher energy and p_x to lower energy. In the limit of trigonal planar geometry, $4p_x$ has now become the lowest orbital with $4p_{y,x}$ degenerate and at higher energy.

For tetrahedral complexes the orbitals should be close to degenerate and to higher energy.

Kau did not fully interpret the intensity differences found in the 8983 eV peaks of the individual 3-coordinate complexes. In a recent study of edge effects in Cu(I) complexes, Blackburn and co-workers [Blackburn *et al* (1989)] have studied a series of complexes in which the stereochemistry varies in a systematic manner from linear, through distorted trigonal and trigonal pyramidal to tetrahedral. They noticed that the intensity of the 8983 eV peak appeared to be inversely proportional to the degree of distortion of the Cu(I) center from the N_3 plane (Fig. 4).

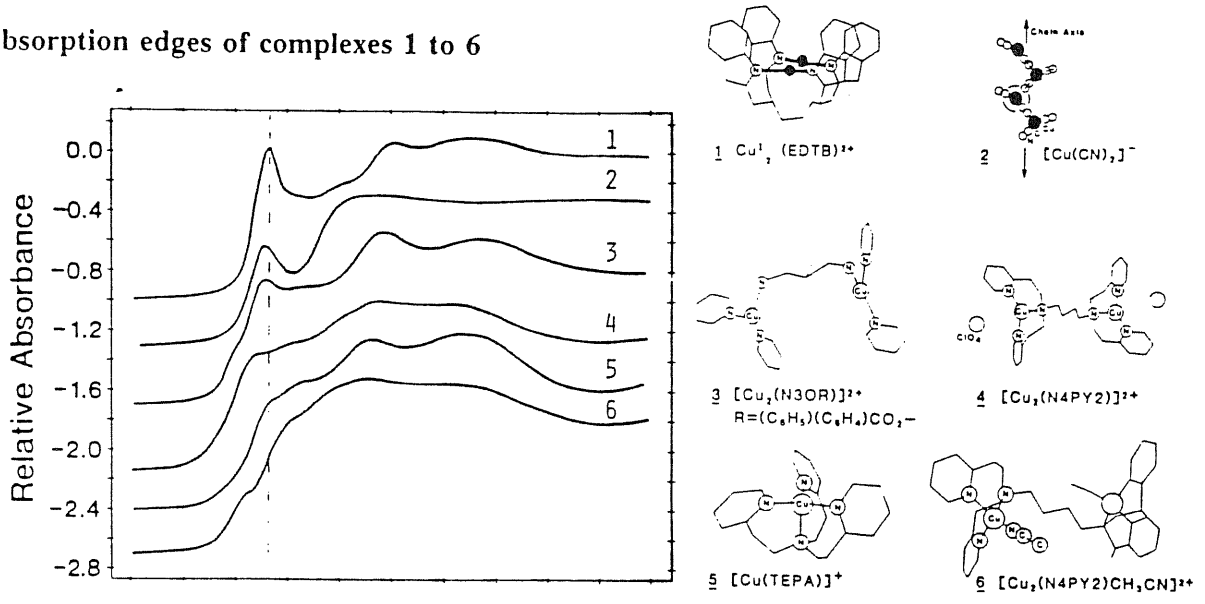


Fig. 4: Absorption edges of a series of Cu(I) complexes in which the geometry varies systematically from linear 2-coordinate to pseudo-tetrahedral. The intensity of the absorption edge feature at 8983 eV can be correlated with the degree of out-of-plane distortion of the Cu atom from the N_3 plane along the x-direction.

The K edges for a series of Cu(II) complexes have been studied by Kau *et al* (1987). All the Cu(II) complexes studied have a very weak 8979 eV peak which corresponds to the 1s to 3d transition. In addition, many Cu(II) complexes exhibit a rather intense peak (normalized absorption amplitude of 0.62-0.68) on the absorption edge at energies between 8986 and 8988 eV. In all 40 complexes studied, this Cu(II) peak is always observed at energies greater than 8985.0 eV.

The above data therefore indicate that absorption edge spectroscopy is a useful technique for studying ligand binding to Cu(I) and Cu(II) in proteins and enzyme systems.

3.3 XAS studies of Cu, Zn SOD.

Despite the detailed description of the Cu site in SOD derived from both the 2.0 Å resolution crystal structure [Tainer *et al* (1982 and 1983)] and from a wealth spectroscopic investigation of the protein, XAS studies on the enzyme have provided valuable information on precise metal-ligand bond lengths, imidazole ring orientation, and stuctural details on the reduced form.

Blumberg *et al* (1978) studied the Zn and Cu K-edge in both reduced and oxidized superoxide dismutase. They showed that the Zn K-edge XANES does not change upon reduction, thus suggesting a very similar environment for the zinc in both forms of the protein. On the contrary reduction decreases the charge on the copper atom and changes the configuration on the copper site so that it becomes less symmetric.

Blackburn *et al* (1983 and 1984) confirmed the observation for the K-edge of Cu and Zn. They also measured EXAFS data for the Cu K-edge for both the oxidised and reduced form of the protein in solution and found that the overall amplitude of the Cu EXAFS had decreased by approximately 20 % in the reduced form. The analysis suggested a three coordinate Cu(I) in the reduced form and a corresponding small

change, from 2.00 to 1.94 Å in the Cu-N distance. These EXAFS results provided the first direct structural information on the copper site in the reduced protein.

Blackburn *et al* (1983 and 1984) were also able to show that the x-ray absorption spectra changed on freeze-drying the SOD solution and that this change was compatible with the loss of a coordinated water molecule, 2.24 Å away from the copper. This observation helped to conferm a large amount of spectroscopic evidence that the copper in SOD is really 5-coordinated with 4 histidines and a water molecule.

All these studies were devoted to enquire the structure of the native protein.

4. SAMPLE PREPARATION

Since the main difficulty in XAS measurements of biological samples is the diluteness of the absorbing metal atom relative to the organic matrix, in the preparation of XAS experiments the bigger effort must be done to obtain, as far as it is possible, the higher metal concentration.

While for the copper-glutathione complexes we have obtained very concentrated samples with [Cu]=30mM, the protein samples had a very poor concentration, [Cu]=2.48 mM. As we expected the signal-to-noise ratio for the copper-glutathione complexes was very good, while the low concentration of the protein has determined a worst signal-to-noise ratio in XAS spectra. We hope that in the future we can improve copper concentration also in the protein samples.

All samples have been prepared at Lure, except for the copper-free superoxide dismutase that has been prepared in the Biochemical Laboratories of Prof. Desideri in the II University of Rome. All chemicals used in the preparation were of the highest purity available from commercial sources. Reduced glutathione was obtained from Boehringer Mannheim.

4.1 The Cu(I)•GSH complex.

Cu(I) complexes with GSH were prepared in a way that may be considered as closer to physiological conditions, *i.e.* adding $CuSO_4$ to a solution of reduced glutathione (GSH) in phosphate buffer at pH = 7.4. Addition of Cu(II) causes oxidation of stechiometric amounts of GSH to GSSG and complexation of the resulting Cu(I) with the remaining GSH.

The complex was prepared in different copper concentrations: for absorption spectra the copper concentration was 10 mM, while for XAS spectra we prepared a solution 30 mM in copper. In the latter case, after the addition of copper to the glutathione solution, a yellow

precipitate formed and the pH of the solution decreased to lower values (about 3.34). By adding NaOH 10M, the pH increased to about 7.4 and the solution became transparent. The GSH:copper ratio of our samples was always 3:1.

4.2 The oxidation of the Cu(I)•GSH complex followed by absorption spectroscopy.

This part of the experiment has been done in order to obtain informations on the stability of the copper-glutathione complex.

To follow the oxidation from Cu(I) to Cu(II), we prepared a solution of the Cu(I)-GSH complex as explained in the previous paragraph. After exposition to the air at room temperature, the colour of the solution changed from transparent to yellow, then green and at the end blue.

In our conditions, the Cu(I)-GSH complex has been shown to be very stable in time at room temperature: the oxidation reaction is very slow and may take many hours. We observed that the rate of the process is different depending on:

- the area of the surface exposed to the air (the bigger the area, the faster the oxidation);
- the temperature (the higher the temperature, the faster the oxidation);
- the copper concentration (the more the copper is deluted, the faster is the oxidation);
- the pH (the higher the pH, the faster the oxidation);
- the glutathione to copper ratio (the higher the ratio, the faster the oxidation).

In order to characterize the reaction of oxidation, we have recorded optical spectra of the complex each half an hour. The reduced complex has no signal, since the d^{10} electronic configuration of Cu(I) is silent. The oxidation process can be followed by the appearence and then the increase of optical density at 625 nm characteristic of the Cu(II)-GSH complex [Postal *et al* (1986)]. The

absence of the signal is a good method to check the oxidation state of the complex.

Optical spectra of the reduced complex were recorded before and after each XAS measurements to inquire the oxidation state of the sample. In this way, we have been sure that during data acquisition, we did not have changes in the oxidation state of the sample. Anyway the evident changes of colour during the oxidation process could be considered sufficient as a test of the oxidation state of copper.

4.3 The Cu(II)•GSSG complex.

The first steps in the preparation of the Cu(II)-GSSG complex are the same as for the Cu(I)-GSH. After six hours in a shaking water bath, the oxidized complex was ready.

4.4 The reconstitution of the native SOD.

Cu,Zn-superoxide dismutase was isolated from bovine erythrocytes according to Mc Cord & Fridovich (1969). The copper-free derivative of the enzyme was prepared by reducing the copper with excess potassium ferrocyanide and dialyzing for 12 h at 4°C against 0.1 M phosphate buffer containing 0.05 M KCN at pH 6.0 [Rigo *et al* (1977)]. The samples were further dyalized for 24 hours at 4° C against water. Final copper content was less than 2%.

For the XAS experiments we used 140-150 µl of copper-free superoxide dismutase 41.8 mg/ml. The molecular weight of the protein is 31000 Dalton. This means that the concentration of the copper sites (two in each molecule) in the preparation was 2.65 mM.

It was necessary to add a small amount of copper-glutathione in solution in order to avoid an ulterior dilution. The copper-glutathione complex was prepared by adding an appropriate amount of $CuSO_4$ powder to a solution 99.9 mM of reduced glutathione in phosphate buffer at pH=7.4. After the addition, the pH decreased to lower values

(about 3.34) and the solution was yellow not transparent. The addition of NaOH 10 M increased the pH to about 7.7 and the solution was transparent. The final copper concentration of complex Cu(I)-GSH was 33mM.

Using this procedure we added 11.5 μl of the Cu(I)•GSH complex solution to the Cu-free protein.

The final concentration of copper in the Cu•GSH•Cu-free,Zn-SOD incubation mixture was 2.48 mM.

5. RESULTS AND DISCUSSION

5.1 Introduction.

The experiments described in this thesis started at Frascati PULS laboratories in 1989: EXAFS data for the Cu(I)-GSH complex were obtained and their analysis indicate one shell of two sulphur atoms at 2.22 Å [Longo *et al* (1989)]. We also tried to investigate the reconstitution process of the apo-SOD in the presence of Cu(I)-GSH complex, but the bad signal-to-noise did not allow the extraction of the EXAFS signal from the absorption spectra.

The experiments continued in Orsay. The two synchrotron radiation facilities have in fact different characteristics. The intensity of the current is 30 mA in Frascati and 200 mA in Orsay. τ, the typical stored beam lifetime after which the current will have reduced by a factor 1/e, is about 4 hours in Frascati, while in Orsay it is 60 hours. These two data are very important. We have very diluted systems and if the photon flow is too low, the signal-to-noise ratio is very bad; the stability of the source is important as well, because it is then possible to average spectra together to improve signal-to-noise ratio.

In Orsay the experiments have be done in two different times. In July 1991, we recorded spectra of the reduced and oxidized glutathione-copper complex and data of the reconstitution process. In September 1991, we repeated the first part of the experiment: in fact the spectra recorded in July showed a non-monotonic variation in the I_0. Because of the low signal due to the low sample concentration, this bad baseline was not compensated. The second time we obtained a better baseline, improving the signal-to-noise ratio and confirming the results obtained by analyzing the spectra of July. In September we also increased the copper concentration of our samples.

5.2 XANES spectra of Cu(I)-GSH and of Cu(II)-GSSG complexes.

The XANES spectra of the copper-glutathione complex have been obtained in both oxidation states in the range 8950-9150 eV with a step size of 0.5 eV. In this energy range, the K-shell absorption edge of copper can provide important information about the environments of the metal ion, as we extensively explained in the third chapter.

To compare spectra it is first necessary to normalize them. During data acquisition, spectra have been measured well above the absorption edge (into the EXAFS region). The first step in normalization is to fit the EXAFS region with a smooth first order polynomial: this defines μ_0, the atomic absorption of the isolated atom. An extrapolation of the absorption back to the edge energy is done. In a similar manner, using a smooth first order polynomial, pre-edge absorption is extrapolated forward to the edge energy and the normalization is adjusted to give a unit difference between these extrapolations.

The normalized Cu K-edges of the reduced and oxidized complex are shown in Fig. 1 and Fig. 2 respectively. Comparison of the spectra of the reduced and oxidized complex shows a shift in intensity towards higher energies upon oxidation, as well as a change in the shape of the spectrum. These visible differencies are very useful for a qualitative identification of the presence in solution of the two complexes.

The oxidation causes a shift of the maximum peak about 4 eV to higher energies. This shift is due to the different charge of the copper atom and to a different distance of bonding; it is of the same order of magnitude of that found for other reduced-oxidized copper complexes.

The shape of the two complexes is so different that a big change in the copper coordination is expected.

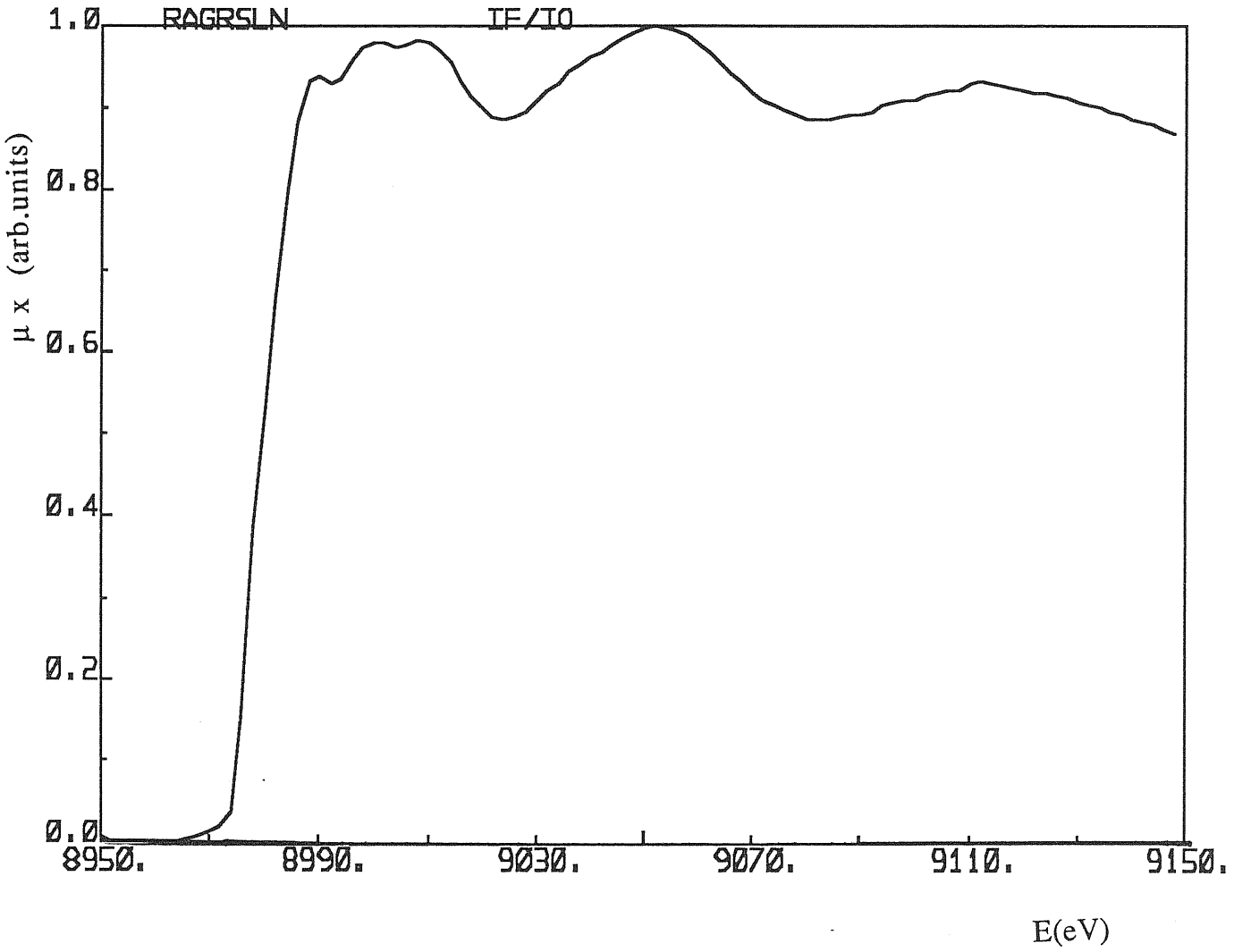


Fig.1: The normalized Cu K-edge of the reduced copper-glutathione complex.

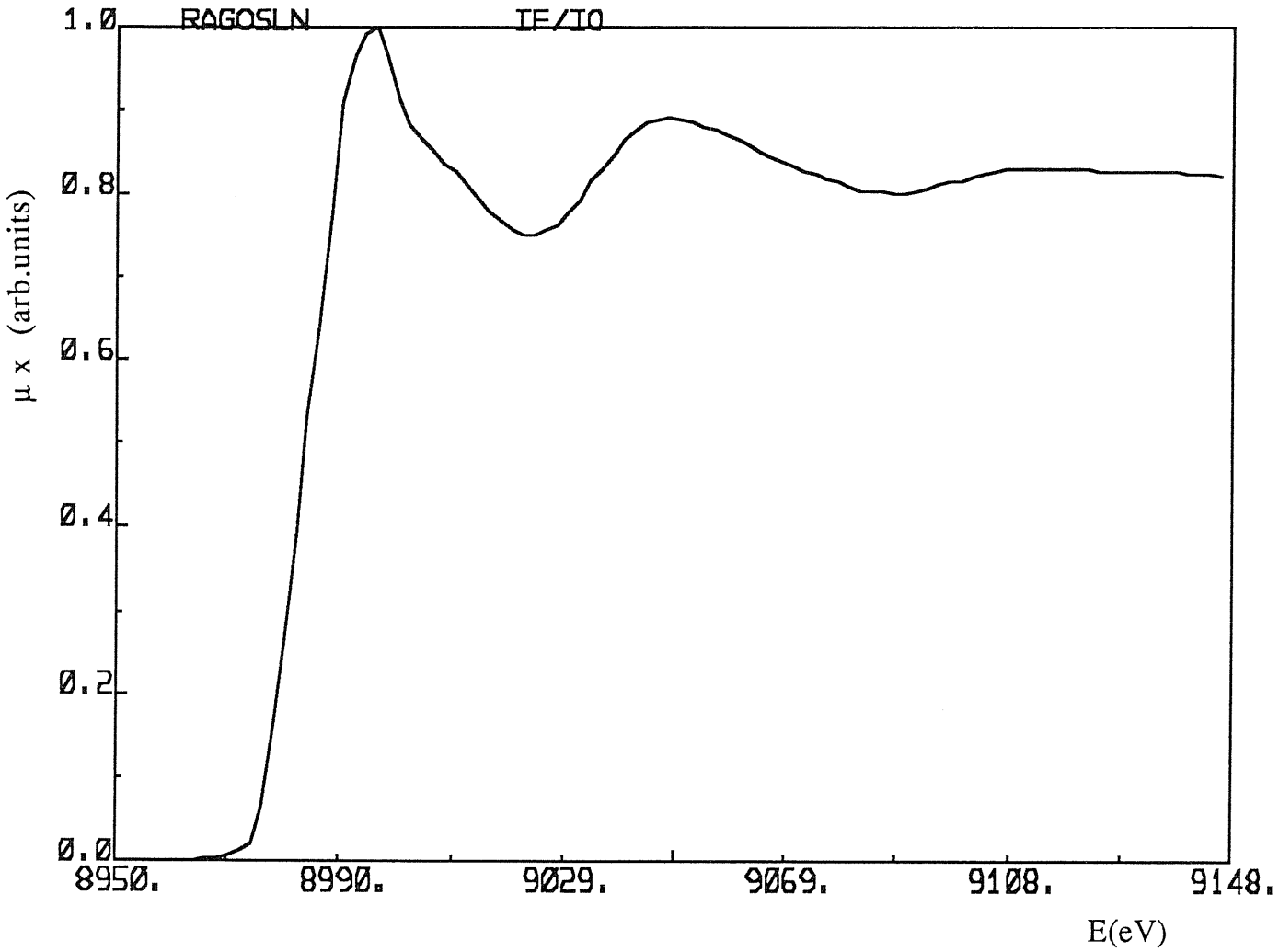


Fig.2: The normalized Cu K-edge of the oxidized copper-glutathione complex.

5.3 EXAFS of Cu(II)-GSSG and Cu(I)-GSH complexes.

The x-ray-absorption spectra of the Cu(II)-GSSG complex were measured with a step size of 2 eV in the energy range 8900-9900 eV. The spectrum shown in Fig. 3.a is the average of 3 single scans in order to improve signal-to-noise ratio.

In the x-ray-absorption spectrum, the EXAFS region (from about 9050 eV in Fig. 3.a) appears as low intensity oscillations, relative to the jump at the absorption edge, superimposed on the smooth atomic absorption background which decays with increasing energy above the absorption edge.

The first stage in the analysis is to isolate the EXAFS signal $\chi(E)$ from the absorption coefficient data $\mu(E)$. The extraction of the EXAFS signal is firstly obtained by removing the absorption of the matrix background and isolating the K-shell contribution to the absorption.

We substracted the background by fitting with a straight line in the pre-edge region (in the range 8903-8956 eV for the spectrum in Fig.3a) and extrapolating after the edge, in the EXAFS region. The pre-edge fit is then substracted from the total absorption data to give the element absorption $\mu(E)$ *vs* E.

The convertion to k-space is done using $k=[0.263(E-E_0)]^{1/2}$. The value of the threshold energy E_0 is not known and for this initial analysis it is convenient to take E_0 as some well defined part of the edge. We used the maximum derivative point (8984 eV) as a first value for E_0; in the later stages of analysis E_0 has been adjusted to its proper value.

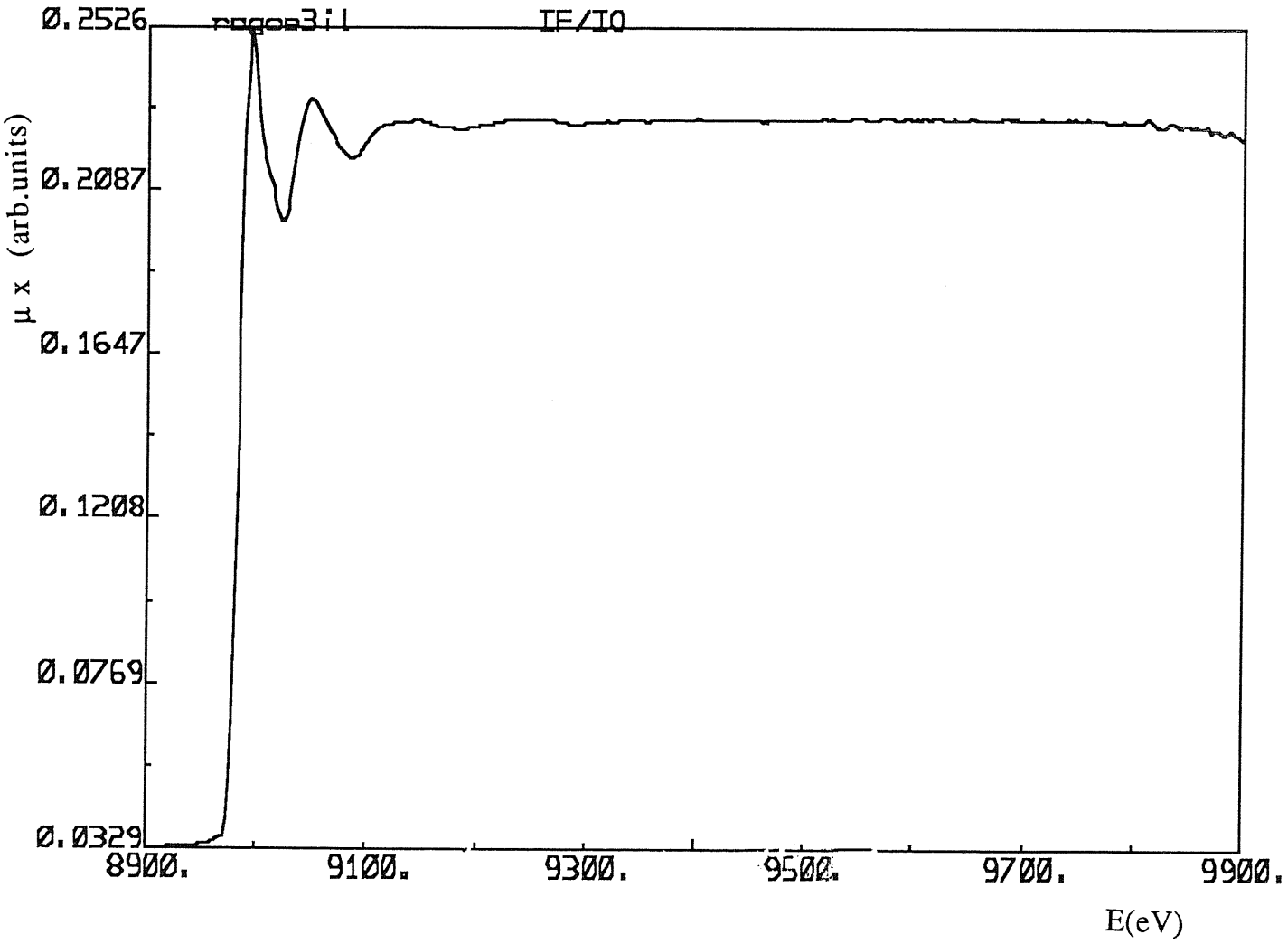


Fig.3a: The x-ray absorption spectrum of the oxidized copper-glutathione complex: the spectrum is the result of the summation of 3 data scans.

The EXAFS is defined as the normalized oscillatory part of the absorption spectrum, μ, and is given by

$$\chi(k)=\frac{(\mu-\mu_0)}{\mu_0} \qquad (1)$$

where μ_0 is the smoothly varying portion of μ after the edge and physically corresponds to the absorption coefficient of an isolated atom. $\mu_0(E)$ is extracted by fitting the EXAFS region with a smooth function, tipically a low order simple or spline polynomial and the fine structure is then given by the equation (1). The EXAFS signal was extracted from the $\mu(k)$ data by fitting a cubic spline over the range 1.21-14.51 eV. The resultant χ (k) is shown in Fig. 3.b.

A Fourier transform was performed with respect to k to obtain an atom distribution function with respect to the radial distance R around the central atom (Fig. 3.c). The Fourier transform is characterized by the presence of a well isolated peak at 1.52 Å.

In order to isolate the EXAFS signal relative to the first single shell, the peak centered at 1.52 Å has been backtransformed from R-space to K-space by using a window function in the region 1.16-1.97. The backscattering amplitude is characteristic of a light atom, such as N or C.

Data analysis utilized rapid curve wave single theory [(Gurman *et al* (1984) and (1986)] in the program EXCURV88 [Binsted *et al* (1988)]: phase shifts were calculated within EXCURV88. The quality of the theoretical simulation was assessed by the fit index (FI) [Joyner *et al* (1987)], defined as:

$$FI=\frac{1}{(100NPT)}\sum_{j=1}^{N}(Res_j\, k^{wt})^2$$

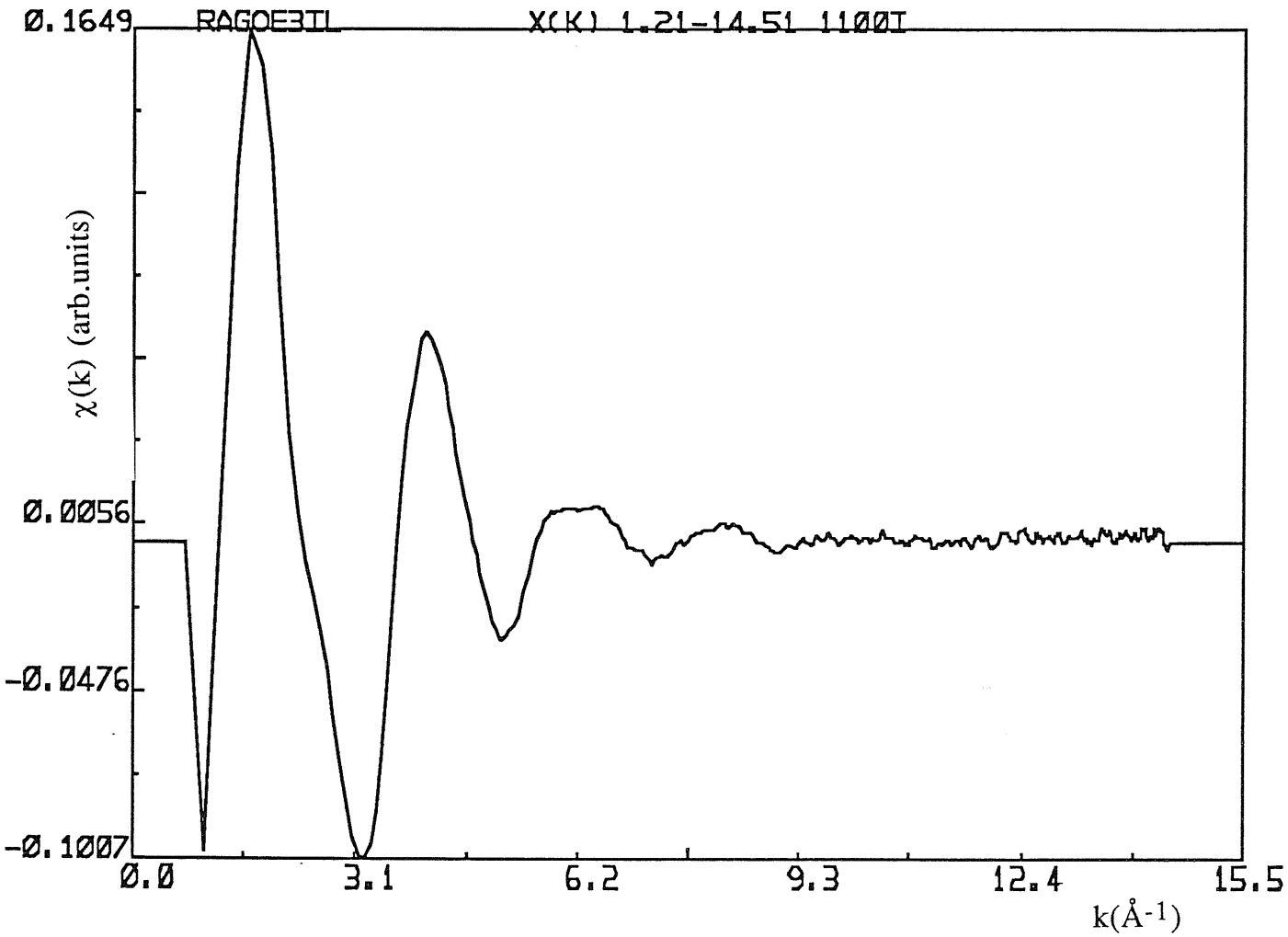


Fig.3b: The EXAFS signal $\chi(k)$ vs k after background removal and E to k conversion. The $\chi(k)$ has been extracted by fitting with a cubic spline over the range 1.21-14.51 eV.

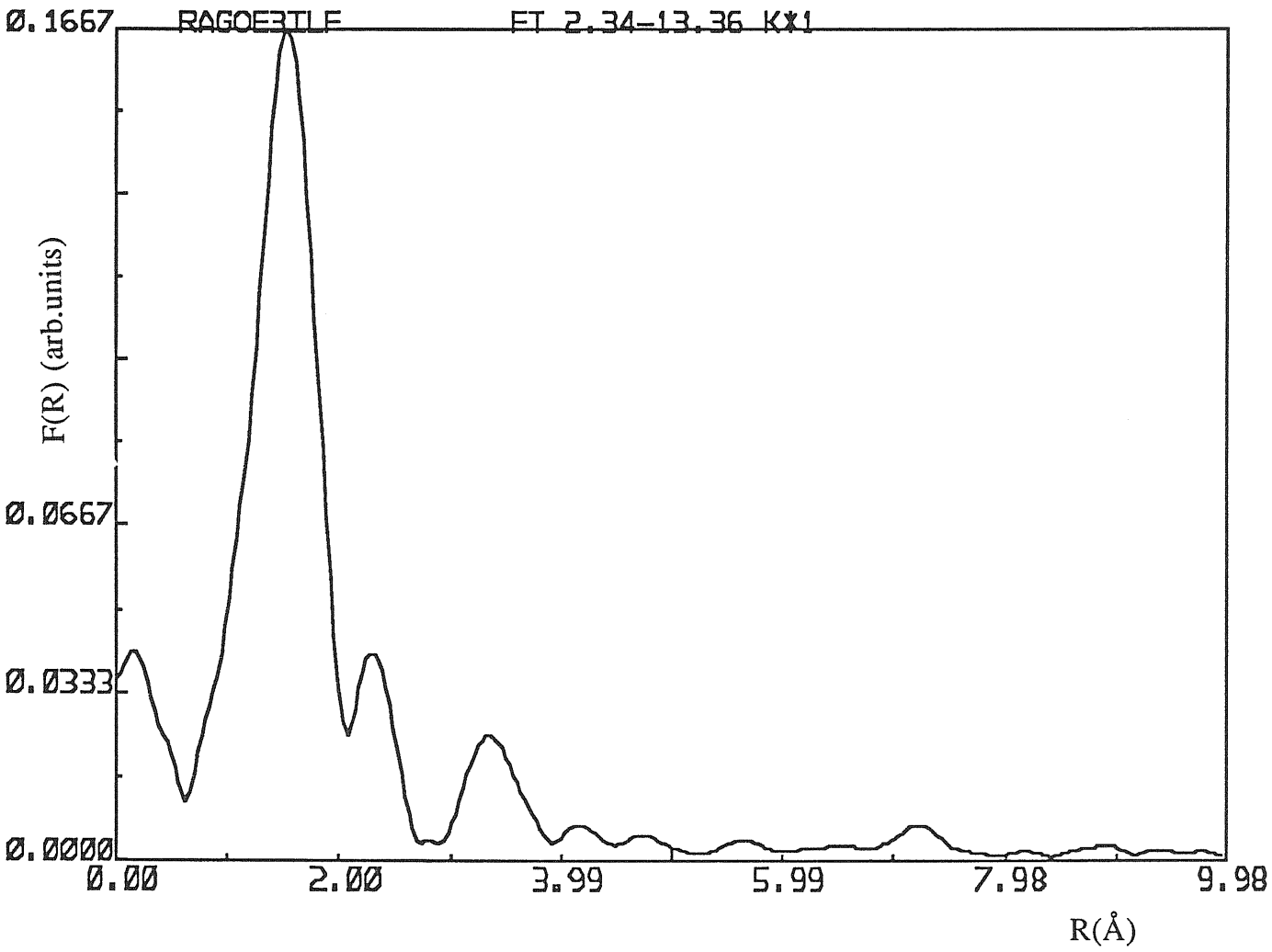


Fig.3c: The k^1 weighted Fourier transform moduli of the EXAFS spectrum. A well separated peak is centered at 1.5 Å.

where $Res_j = \chi_j(calculated) - \chi_j(experiment)$ and NPT=number of data points. WT is the weighting, normally 1-3, which is used to offset the decay of χ as the energy decays.

Simulation of the first shell of the Cu(II)-GSH complex initially utilized the parameters of the available crystallographic data [Miyoshi *et al* (1980)]. The crystallographic structure of the Cu(II)-GSH complex is shown in Fig.3 of the 2nd chapter: the coordination of the copper atom involves the two deprotonated peptide nitrogens, the glutamic amine nitrogen and the glycinyl terminal carboxylate oxygen in an approximate planar coordination while the cysteinyl sulfur is bonded apically to form a square pyramid. The mean distance Cu(II)-N is 1.936 Å, while the sulfur atom is at 3.22 Å: the Cu(II)-Cu(II) distance is 5.21Å.

The best fit of the first shell has been obtained with 4 nitrogen (oxygen) atoms at 1.963 Å. The fit index was quite good: FI=0.0028. The two copper atoms in the binuclear model are too far to be detectable by EXAFS.

These results seem to confirm the crystallographic model with one copper ion bound to two glutathione molecules.

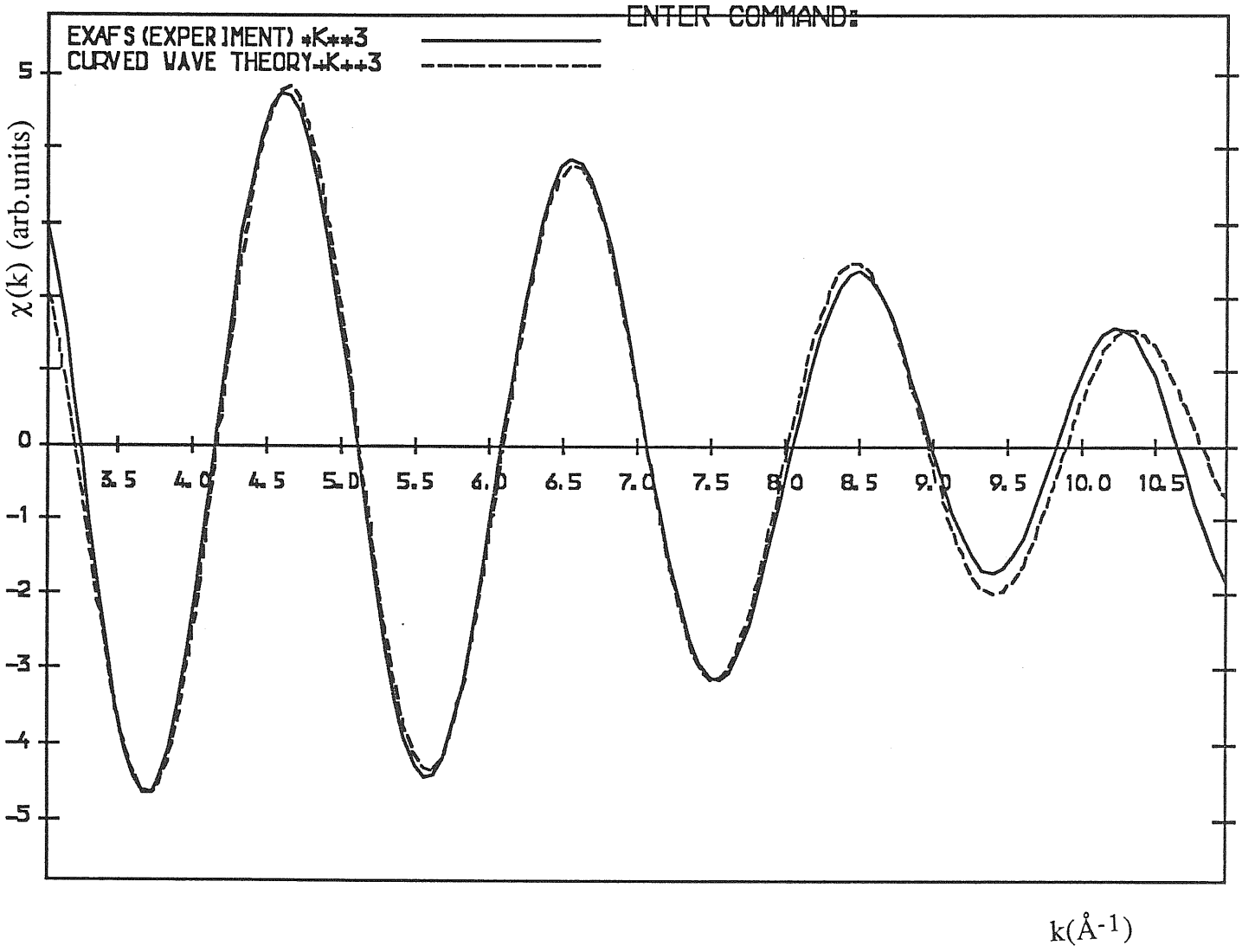


Fig.3d: The Fourier filtered EXAFS spectrum $k^3\chi(k)$ vs k (solid curve) of the major peak in Fig.3c after backtrasforming into k-space. The dashed curve attempts to fit the filtered data with a single distance model of 4 nitrogen (oxygen) atoms at 1.96Å.

The EXAFS spectrum of the **Cu(I)-GSH complex** is shown in Fig. 4.a: the spectrum is the result of the average of 5 data scans, each one measured with a step size of 2 eV in the energy range 8900-9900 eV. Copper concentration was 30 mM. The EXAFS spectrum of a single data aquisition, with copper concentration 20 mM, is shown in Fig. 4.a'.: from a comparison with Fig. 4.a we notice that an improvement in the signal-to-noise ratio has been obtained in the data recorded in Orsay. The improvement is due to the higher copper concentration and to the stability of the photon source thanks to which we could add spectra.

The extraction of the EXAFS signal followed the same steps as the ones described for the Cu(II)-GSSG complex.

The background was substracted by fitting with a straight line in the region 8903-8960 eV. The value of E_0 for the calculation of k was 8980 eV. The EXAFS signal was extracted from the resultant data by fitting a cubic spline over the range 2.98-15.05 eV. The resultant χ (k) is shown in Fig.4.b. The χ (k) extracted by the spectrum recorded in Frascati is also shown (Fig.4.b').

A Fourier transform was performed with respect to k to obtain an atom distribution function with respect to the radial distance R around the central atom. The Fourier transform is shown in Fig. 4.c. A single very well isolated peak shows up at about 1.80 Å. Even if the peak seems to be asymmetric, the plot of the real part of the Fourier transform shows that the asymmetry is not due to the presence of two shells of atoms.

An isolated single shell is obtained by backtransforming a single shell signal from R-space to K-space using a window function in the region of 1.33-2.24 Å. The backtransform is characteristic of an heavier atom than N or C, in this case we suppose to be the sulfur atom of the cysteinyl residue.

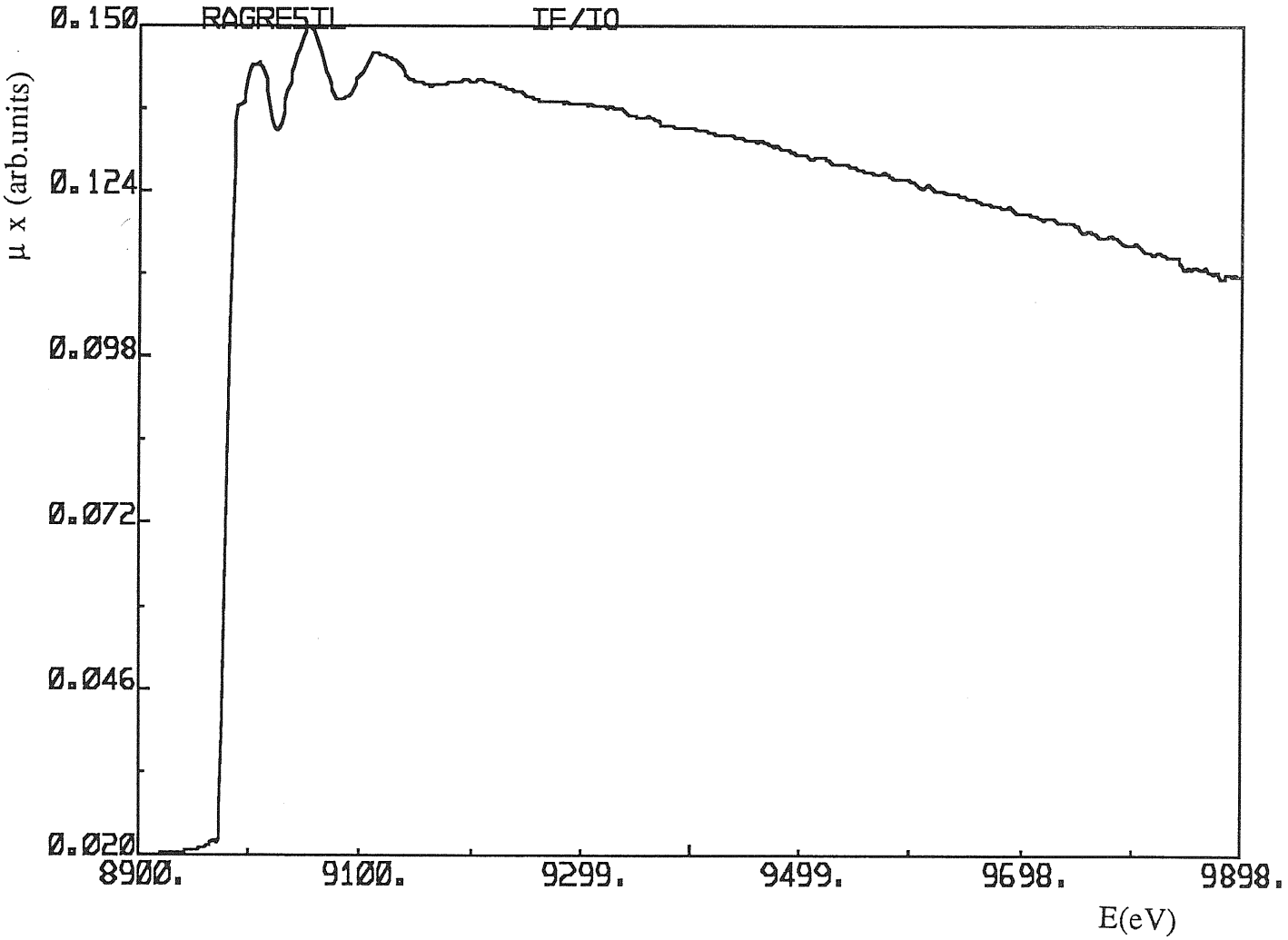


Fig.4a: The x-ray absorption spectrum of the reduced copper-glutathione complex: the spectrum is the result of the summation of 5 data scans.

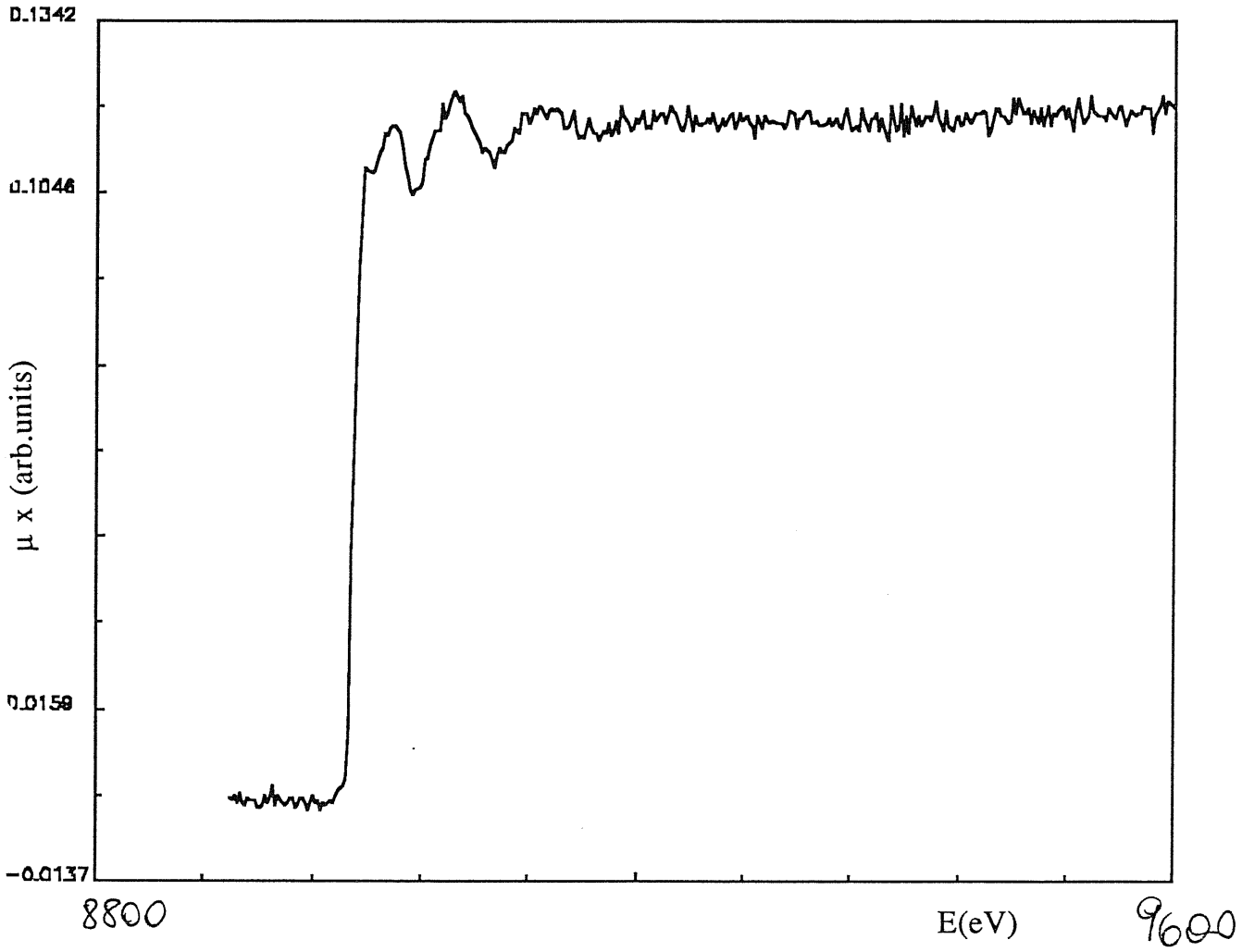


Fig.4a': The x-ray absorption spectrum of a single data scan for the reduced copper-glutathione complex: the spectrum has been recorded in Frascati .

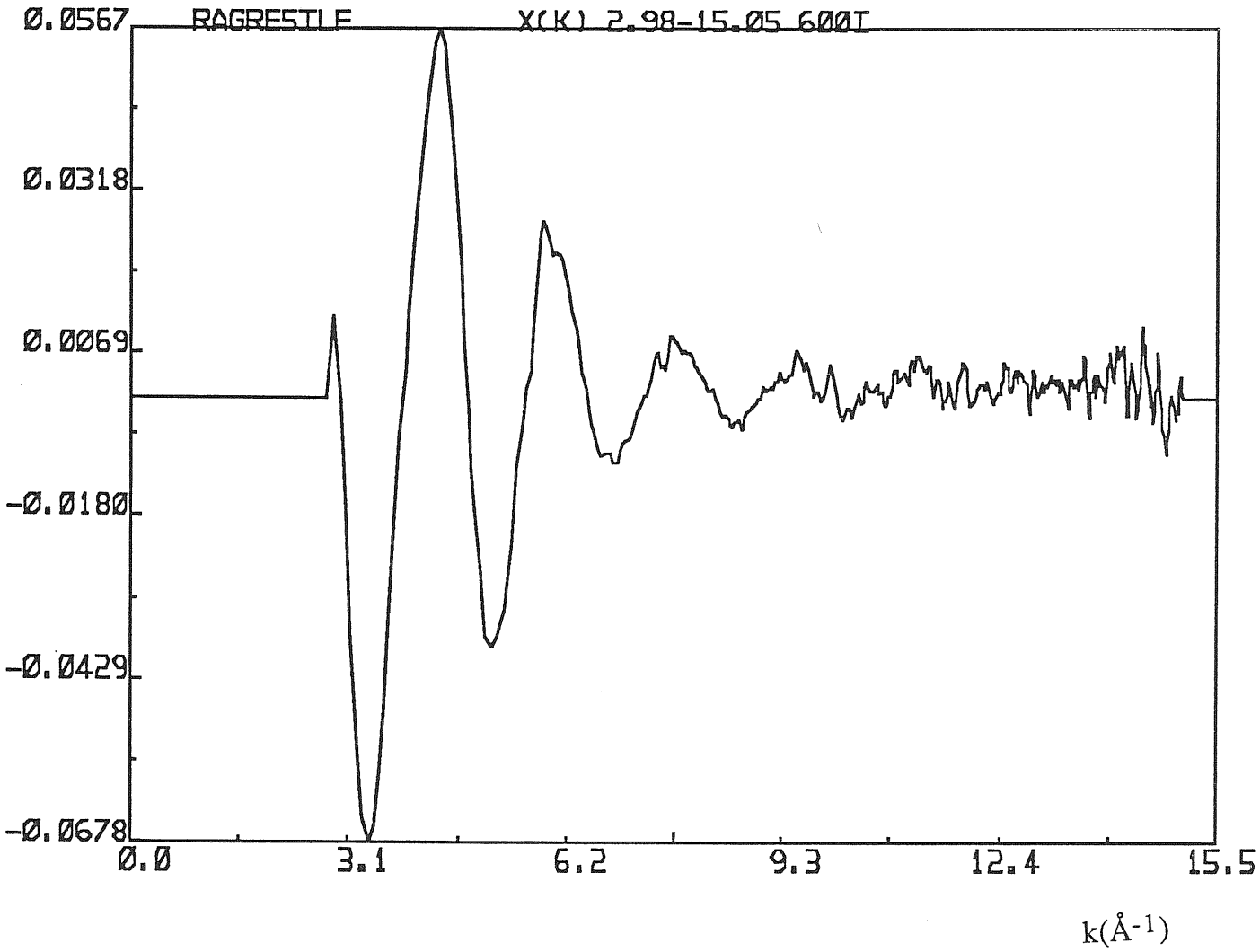


Fig.4b: The EXAFS signal $\chi(k)$ vs k after background removal and E to k conversion. The $\chi(k)$ has been extracted by fitting with a cubic spline over the range 2.98-15.05 eV.

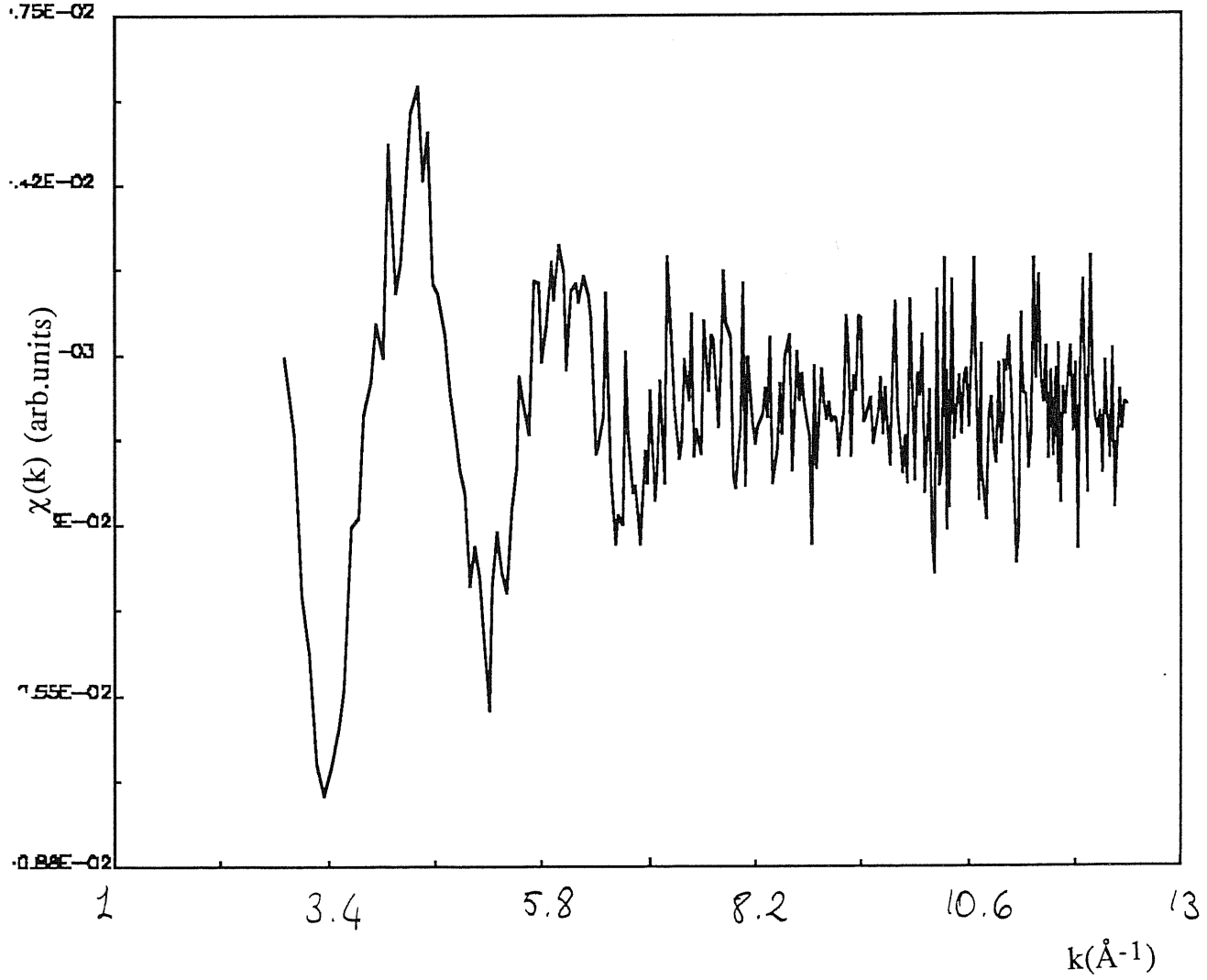


Fig.4b': The EXAFS signal $\chi(k)$ vs k after background removal and E to k conversion for the spectrum of Fig.4a'. The worst signal-to-noise ratio is evident from a comparison with Fig.4b.

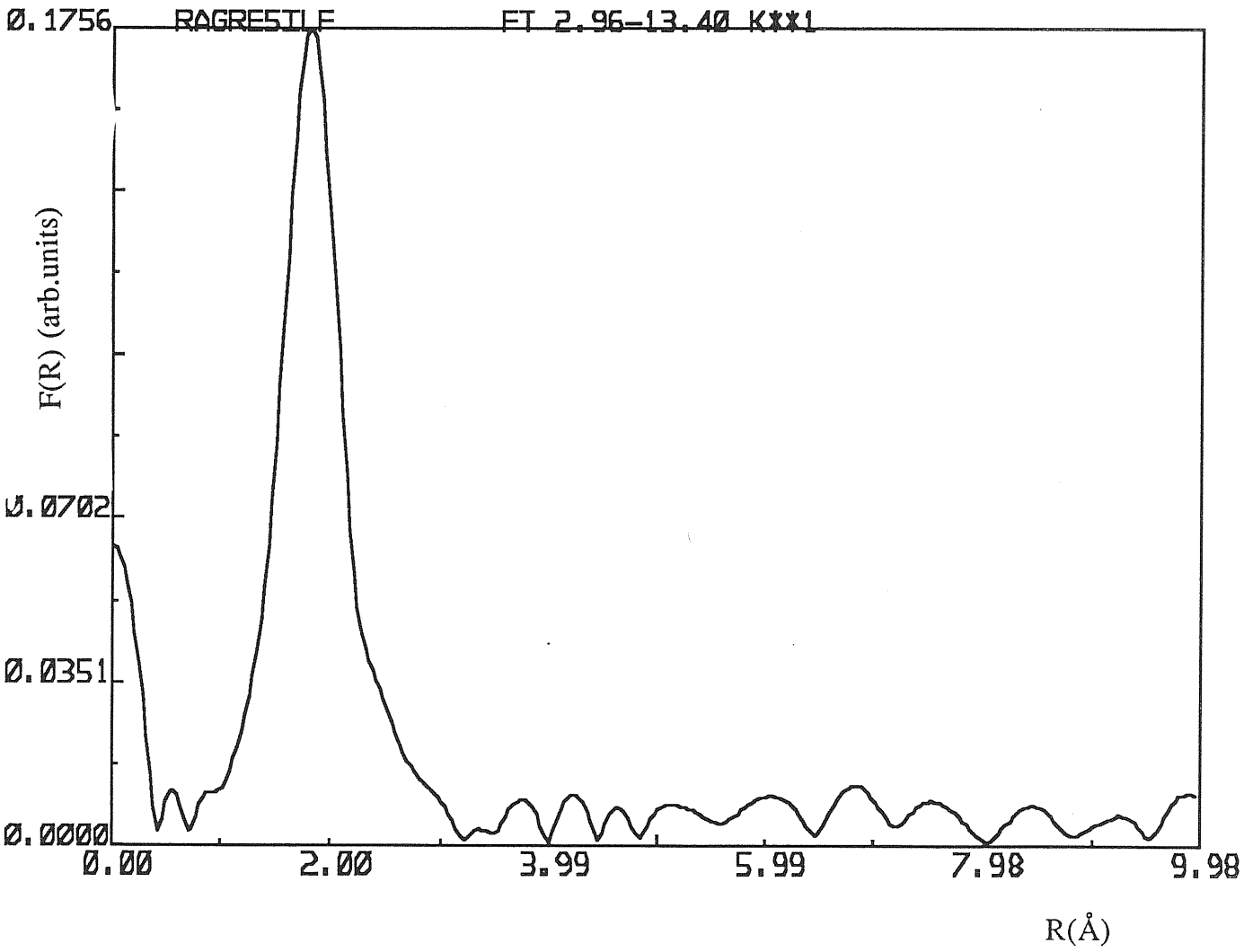


Fig.4c: The k^1 weighted Fourier transform moduli of the EXAFS spectrum. A very well separated peak is centered at about 1.8 Å.

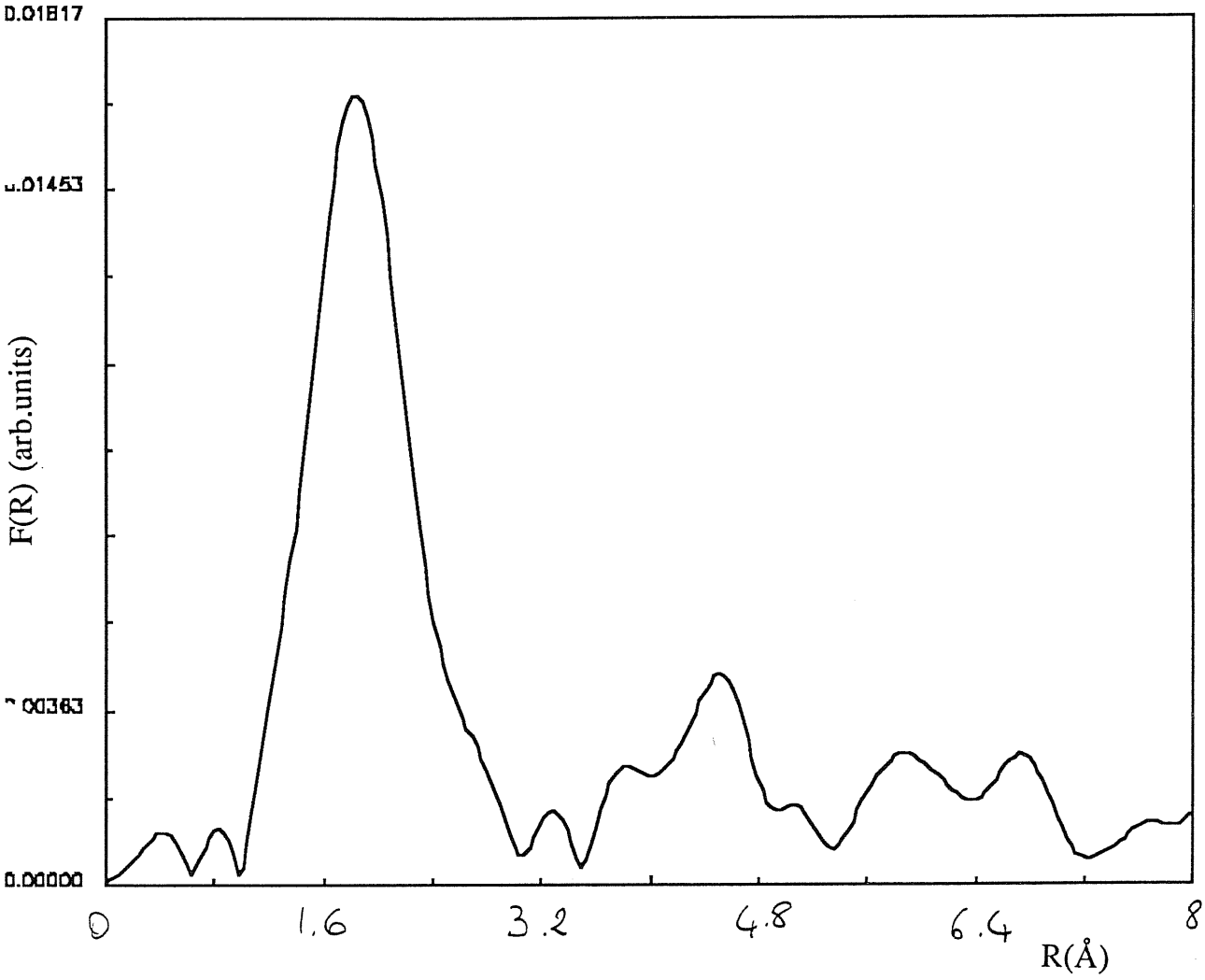


Fig.4c: The k^1 weighted Fourier transform moduli of the EXAFS spectrum of Fig.4b'. The worst signal-to-noise ratio causes the comparison of many peaks at high k values.

Our best fit is shown in Fig.4.e. It has been obtained fitting with two sulfur atoms at 2.22 Å, with a FI=0.001. We have confirmed with different experimental conditions the same results of the data we recorded in Frascati in 1989 [Longo et al (1990)].

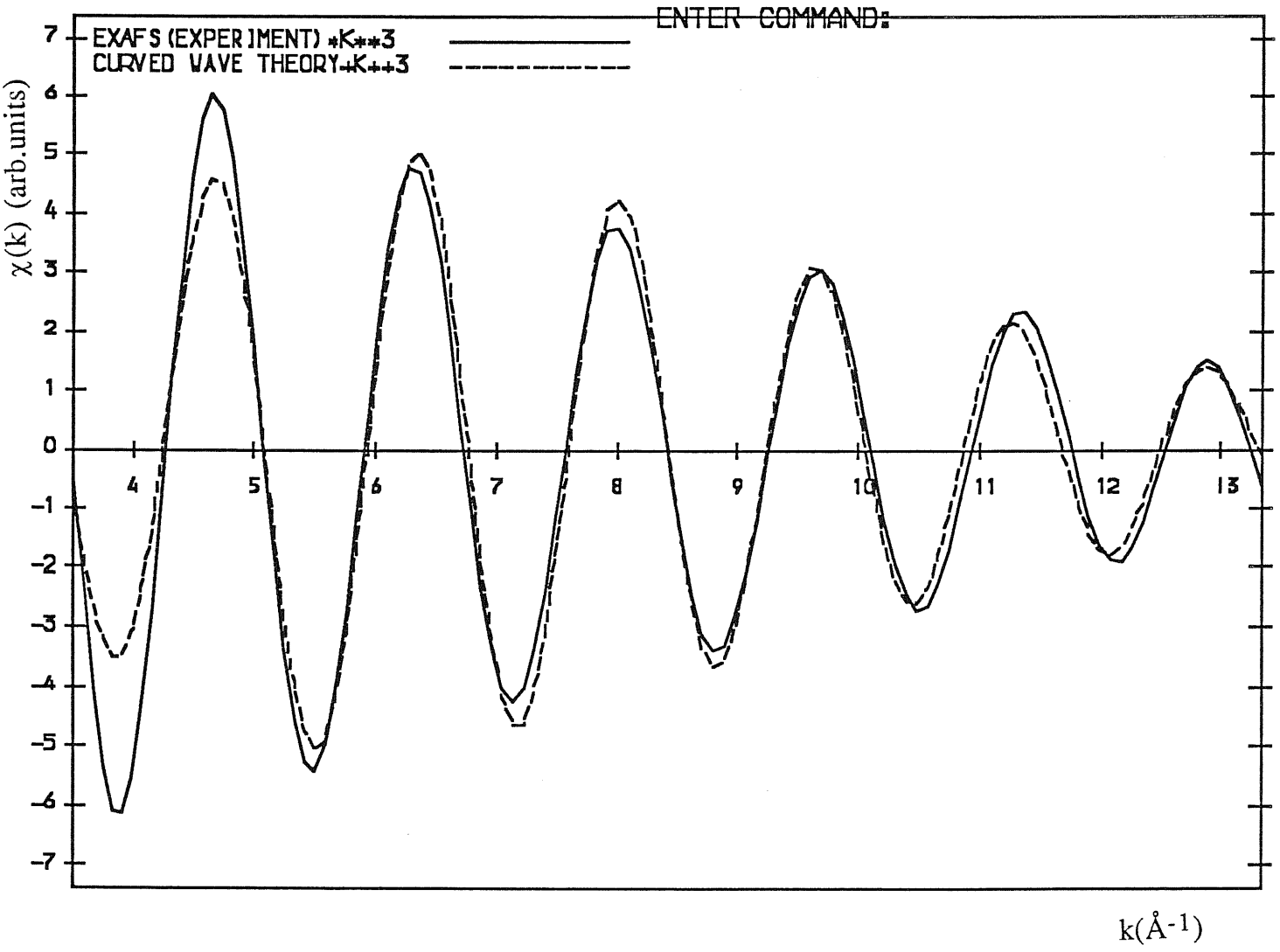


Fig.4d: The Fourier filtered EXAFS spectrum $k^3\chi(k)$ vs k (solid curve) of the major peak in Fig.4c after backtrasforming into k-space. The dashed curve attempts to fit the filtered data with a single distance model of 2 sulphur atoms at 2.22 Å.

5.4 Cu,Zn SOD reconstitution: preliminary results.

The ability of XAS to detect copper in both its oxidation states has been applied to the study of the reconstitution process of the native Cu, Zn SOD from the copper-free protein in the presence of Cu(I)-GSH.

The reconstitution process had already been followed by NMR, EPR and absorption spectroscopy [Ciriolo *et al* (1990)]. The Cu(I)-GSH complex has been shown to be able to full reconstitute the protein: in addition copper is thought to be released by GSH in the reduced state [Ciriolo *et al* (1990)].

Ciriolo *et al* (1990) proposed that an intermediate ternary complex, Cu•GSH•SOD, with copper in the reduced state, could occur during the first hours. A greater solvent accessibility could allow the presence of bulky ligands such as Cu(I)-GSH in the enzyme active site. Since Cu(I) is transparent to the majority of other metal-directed spectroscopic techniques, it was not possible to study the copper in the Cu(I)•GSH•Cu-free,Zn-SOD incubation mixture directly during the first steps of the reconstitution process and to demonstrate if the formation of such a complex takes place or does not.

XAS spectra were recorded just after the addition of the Cu(I)-GSH complex to the apo-protein and went on for 15 hours. The XANES spectra have been obtained in the range 8950-9150 eV with a step size of 0.5 eV; the EXAFS spectra were measured with a step size of 2 eV in the energy range from 8900 eV to 9600 eV. The short energy range in which we recorded the EXAFS spectra is due to the appearence of the signal for the Zn atom of SOD.

We recorded three XANES spectra during the first three hours after the addition of the complex to the apo-protein. Since there are no changes in the shape of the spectra or shifts in energy, the three spectra have been averaged to improve the signal-to-noise ratio. The normalized averaged spectrum is shown in Fig.5: if an intermediate

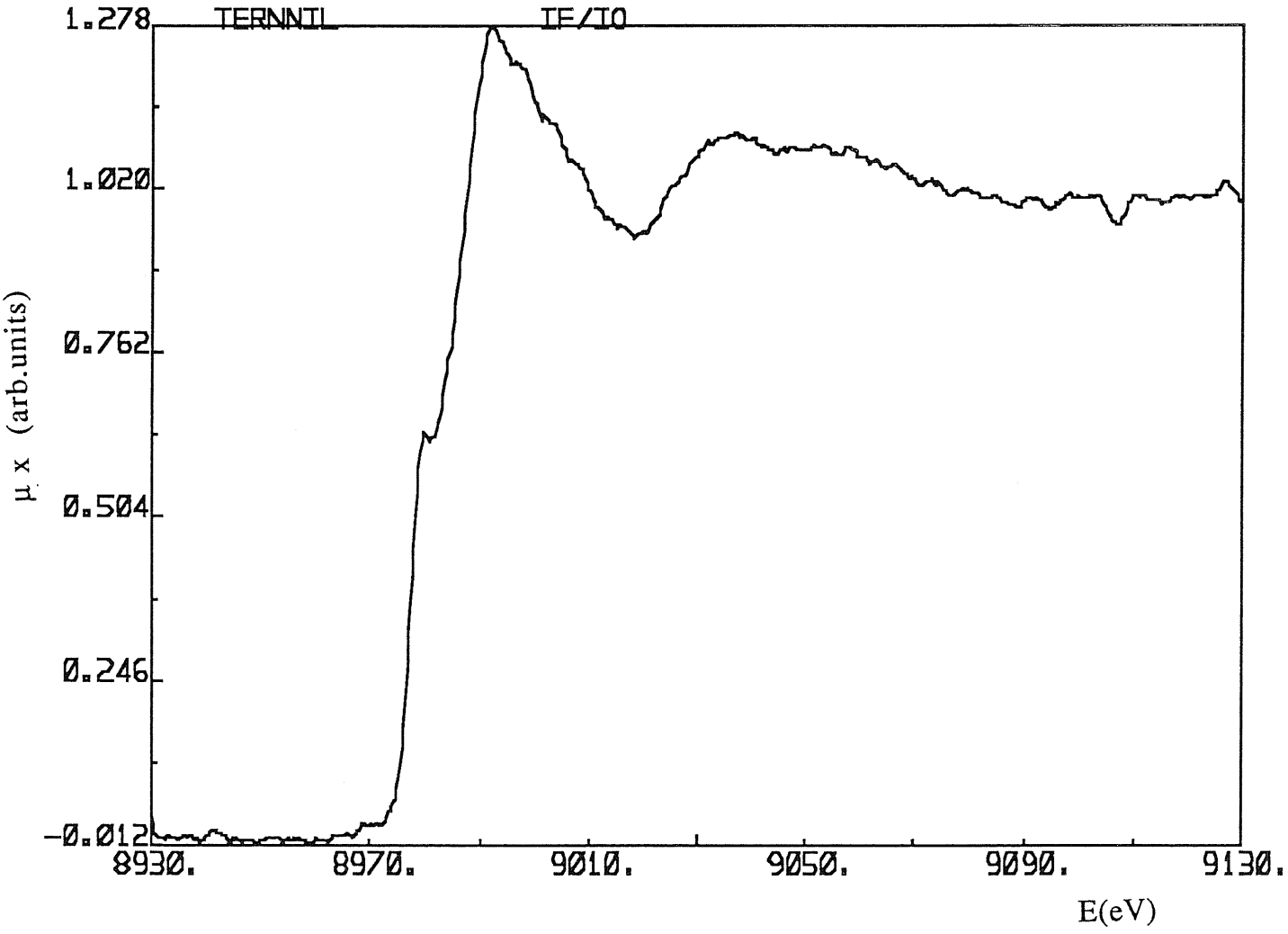


Fig. 5: The normalized spectra of Cu K-edge just after the addition of the reduced copper-glutathione complex to the copper-free superoxide dismutase.

complex does exist, this is the XANES spectrum of the ternary complex.

The spectrum looks very different from the one of the Cu(I)-GSH complex shown in Fig.1. We can therefore exclude that copper is yet bound to glutathione.

The comparison between the XANES spectrum of Fig.5 with the XANES spectrum of the reduced superoxide dismutase, shown in Fig.7a and published by Blackburn *et al* (1984), reveals strong similarities. A peak at 8979.8 eV is present in the spectrum of Fig.5; in the XANES spectrum of the reduced superoxide dismutase at the same energy (8980 eV), there is a peak. Even if the peak height of Fig.5 is less intense than in the protein, their position is the same: the difference in intensity may be due to a different experimental resolution. The maximum peak is located at 8992 eV, at about the same energy as in the reduced native protein.

This qualitative comparison has revealed very strong similarities between the XANES spectrum of the reconstituted complex and the one of the reduced SOD [Blackburn *et al* (1984)]. From these data, we can not conclude that our spectrum is "the same" of that for the reduced SOD, because a comparison can only be made when the experimental conditions are "the same" (such as same monochromator, same detector, etc.). Anyway up to now we do not have a clear evidence for the formation of a ternary complex.

Twelve hours after the addition of the copper-glutathione complex, we recorded other two XANES spectra: the normalized averaged spectrum is shown in Fig. 6. It looks different from the one recorded just after the addition (Fig.5). Once again it is very similar to the XANES spectrum of the oxidized protein in Fig.7.a [Blackburn *et al* (1984)], while it is well different from the one we measured for the Cu(II)-GSSG complex Fig.2.

Some changes have occured in the twelve hours that separate the recording of the two set of spectra. The maximum peak is shifted

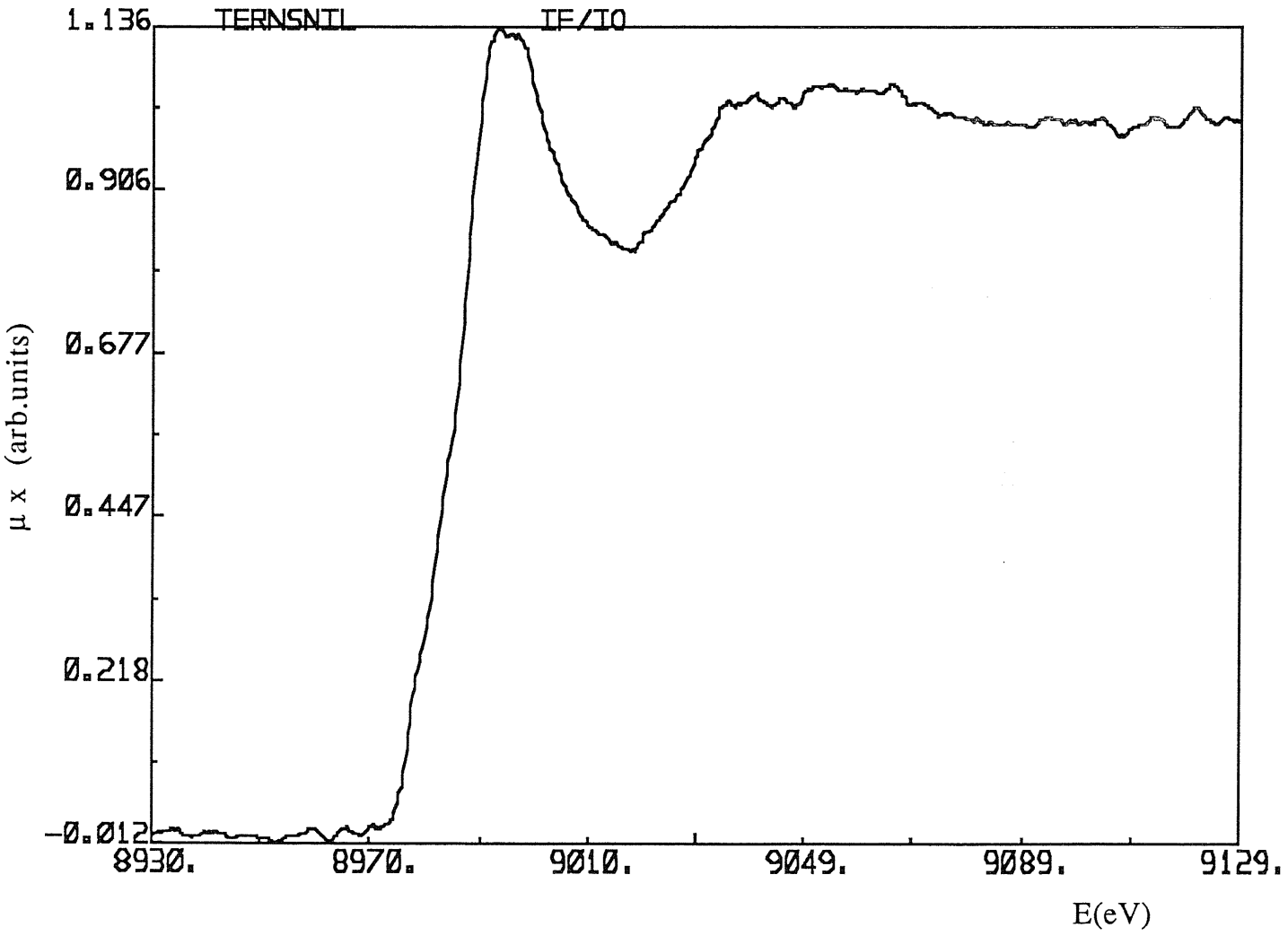


Fig. 6: The normalized spectra of Cu K-edge many hours (12 h) after the addition of the reduced copper-glutathione complex to the copper-free superoxide dismutase.

towards higher energies upon oxidation, from 8992 eV to 8994eV and is splitted in two. Moreover two edge features occur at 8979.5 eV and 8985.5 eV and decrease after oxidation. The position in energy of the peaks has been determined with a great precision because of the simultaneous acquisition of the sample spectrum and its reference (a metallic copper foil).

A comparison can be made with the XANES spectrum of the oxidized protein in Fig.7.a [Blackburn *et al* (1984)]. Two edge features are marked with arrows in the pre-edge region, while the maximum is splitted in two.

In the meantime twelve EXAFS spectra has been recorded. The energy range of the spectra has been cut at 9600 eV because of the appearence of the zinc edge due to the zinc atom of SOD. The signal-to-noise ratio is not as good as that obtained in the EXAFS spectra of the copper-glutathione complexes because of the lower copper concentration. An accurate data analysis is currently under way to extract the EXAFS signal from the absorption spectra.

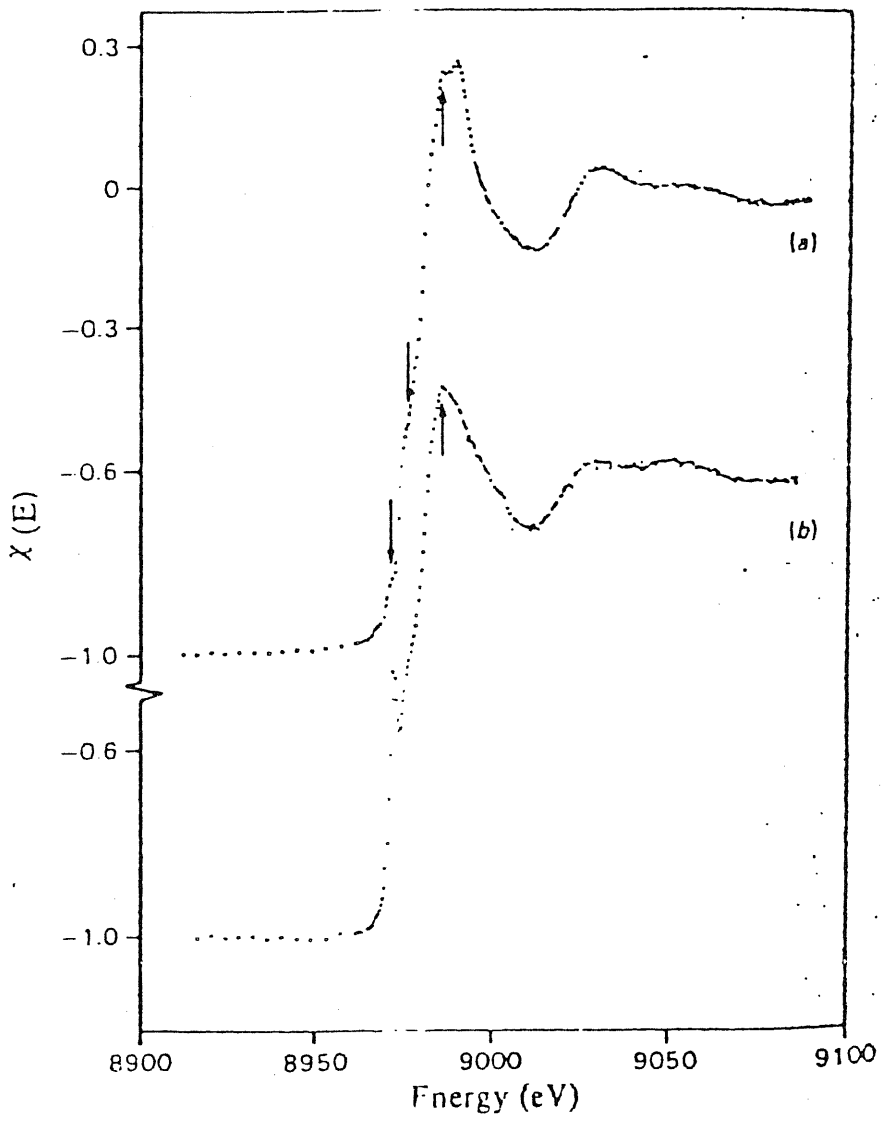


Fig. 7: Cu K-absorption edge structure for oxidized and reduced superoxide dismutase. a) oxidized enzyme; b) dithionite-reduced enzyme. Arrows indicate edge features that occur at the same energy in both spectra.

5.5 Conclusions.

We have demonstrated that the copper-glutathione complexes are very different in the two oxidation states: this result has been obtained at first with XANES spectra and then confirmed by EXAFS analysis. In the Cu(I)-GSH complex, copper is bound to two sulfur atoms at 2.22 Å, while in the Cu(II)-GSH complex, copper is bound to 4 nitrogen atoms at 1.96 Å.

The characterization by XAS spectroscopy of the two copper-glutathione complexes (no previous XAS studies were available) has given us the possibility of making comparisons between these spectra and the spectra we obtained during the reconstitution process.

The first result in the study of the reconstitution process is that the copper, bound to two glutathione molecules through the sulphur atoms of the cysteinyl groups, changes of coordination when it is added to the solution of the apo-SOD. In fact the XANES spectrum of the Cu(I)-GSH complex is very different from the one we obtained just after the addition of the complex to the apo-protein. The change of the spectrum shape can not depend on the different Cu(I)-GSH concentration, which only give a worst signal-to-noise ratio. On the other hand it is similar to the spectrum of the reduced superoxide dismutase published by Blackburn *et al* (1984).

The XANES spectrum of the K-edge of copper in the solution with reduced copper-glutathione and copper-free superoxide dismutase changes as a function of time. The final XANES spectrum is different from the first one: the shape changes and the maximum shifts towards higher energies. The final XANES spectrum is different from the XANES spectrum of oxidized copper-glutathione and is similar to the spectrum of the oxidized superoxide dismutase published by Blackburn *et al* (1984).

A reasonable conclusion from our data is that during the reconstitution process, the copper is released by glutathione as Cu^{+} and not as Cu^{++}. The oxidation process takes place later when the copper is already bound to the protein.

At the present stage the presence of a ternary complex can be excluded, while the full reconstitution of the native protein has been confirmed.

REFERENCES

Bannister J.V., Rotilio G.
A decade of superoxide dismutase activity.
in " The biology and chemistry of active oxygen",
ed. Bannister, 26,147-190 (1984)

Bannister J.V., Bannister W.H., Rotilio G.
Aspects of the structure, function and applications of superoxide dismutase.
CRC Critical Rewiews in Biochem. 22, 11-180 (1987)

Beltramini M., Lerch K.
FEBS Lett. 142, 219-222 (1982)

Blackburn N.J., Hasnain S.S., Diakun G.P., Knowles P.F., Binsted N., Garner C.D.
An extended-X-ray-absorption-fine-structure study of copper and zinc sites of freeze-dried bovine superoxide dismutase.
Biochem.J. 213, 765-768 (1983)

Blackburn N.J., Hasnain S.S., Binsted N., Diakun G.P., Garner C.D., Knowles P.F.
An extended-X-ray-absorption-fine-structure study of bovine erythrocyte superoxide dismutase in aqueous solution.
Biochem.J. 219, 985-990 (1984)

Blackburn N.J., Strange R.W., Reedijk J., Volbeda A., Farooq A., Karlin K.D., Zubieta J.
X-ray-absorption edge spectroscopy of copper(I) complexes. Coordination geometry of copper(I) in the reduced forms of copper proteins and their derivatives with carbon monoxide.
Inorg. Chem. 28, 1349-1357 (1989)

Blumberg W.E., Peisach J., Eisenbergerm P., Fee J.A.
Superoxide dismutase, a study of the electronic properties of the copper and zinc by X-ray-absorption spectroscopy.
Biochemistry 17, 1842-1845 (1978)

Brutsch
Inorg. Chim.Acta 92, 147-149 (1984)

Ciriolo M.R., Desideri A., Paci M., Rotilio G.
Reconstitution of Cu,Zn-superoxide dismutase by the Cu(I)•glutathione complex.
J. Biol. Chem. **265**, 11030-11034 (1989)
Cousins R. J.
Absorption, transport, and hepatic metabolism of copper and zinc: special reference to metallothionein and ceruloplasmin.
Physiological Reviews **65**, 238-300 (1985)
Cramer S.P., Eccles T.K., Hodgson K.O., Mortenson L.E.
J.Am.Chem.Soc. **98**, 1287- (1976)
Cramer S.P., Hodgson K.O.
Prog. Inorg. Chem. **25**, 1-39 (1979)
Cramer S.P.
Biochemical application of X-ray absorption spectroscopy.
in "X-ray absorption spectroscopy" ed. Koningsberger & Prins,
257-320 (1989)
Demas J.N., Harris E.W., Flynn C.M., Diemente D.
Models for the electron paramagnetic resonance nondetectable copper in "blue oxidases". A binuclear copper(II) complex with oxidized glutathione.
J.Am.Chem.Soc. **97**, 3839-3841 (1975)
Eisenberg P., Kincaid B.M.
EXAFS: new horizons in structure determinations.
Science **200**, 1441-1447 (1978)
Fee J.A.
The copper/zinc superoxide dismutase.
Metal ions in biological systems **13**, 259-298 (1981)
Freedman J.H., Weiner R.J., Peisach J.
Resistance to copper toxicity of cultured hepatoma cells.
J.Biol.Chem. **261**, 11840-11848 (1986)
Freedman J.H., Ciriolo M.R., Peisach J.
The role of glutathione in copper metabolism and toxicity.
J.Biol.Chem. **264**, 5598-5605 (1989 a)
Freedman J.H., Peisach J.
Resistance of cultured hepatoma cells to copper toxicity. Purification and characterization of the hepatoma metallothionein.
Biochim.Biophys.Acta **992**, 145-154 (1989 b)

Freedman J.H., Peisach J.
Intracellular copper transport in cultured hepatoma cells.
Bioch.Biophys.Res.Commun. **164**, 134-140 (1989 c)
Geller B.L., Winge D.R.
Arch.Biochim.Biophys. **213**, 109-117 (1982)
Goldstain S., Czapski G.
J.Free Radicals Biol.& Med. **2**, 3-11 (1986)
Gurmam S.J., Binsted N., Ross I.
A rapid, exact curved -wave theory for EXAFS calculations.
J.Phys.C: Solid State Phys. **17**, 143-151 (1984)
Gurmam S.J., Binsted N., Ross I.
A rapid, exact curved -wave theory for EXAFS calculations:II. The multiple scattering contributions.
J.Phys.C: Solid State Phys. **19**, 1845-1861 (1986)
Hartmann H.J., Morpurgo L., Desideri A., Rotilio G., Weser U.
FEBS Lett. **152**, 94-96 (1983)
Hedman B., Hodgson K.O., Garner C.D.
Characterisation of molybdenum and vanadium centres in enzymes by X-ray absorption spectroscopy.
in "Synchrotron radiation and biophysics" ed. S.S.Hasnain
ch.8, 176-200, (1990)
Huet J., Jouini M., Abello L., Lapluye G.
Structural study of copper oligopeptide complexes. 1: Oxidized glutathione Cu(II) system.
J.Chim.Phys. **81**, 505-511 (1984)
Ibers J.A., Holm R.H.
Modeling coordination sites in metallobiomolecules.
Science **209**, 223-235 (1980)
Jouini M., Lapluye G., Huet J., Julien R., Ferradini C.
Catalytic activity of a copper(II)-oxidized glutathione complex on aqueous superoxide ion dismutation.
J.Inorg.Bioch. **26**, 269-280 (1986)
Joyner R.W.., Martin K.J., Meehan P.
Some applications of statistical tests in analysis of EXAFS and SEXAFS data.
J.Phys.C: Solid State Phys. **20**, 4005-4012 (1987)

Kau L.S., Spira-Solomon D.J., Penner-Hahn J.E., Hodgson K.O.
X-ray absorption edge determination of the oxidation state and coordination number of copper: application to the type 3 site in Rhus vernicifera laccase and its reaction with oxygen.
J.Am.Chem.Soc. **109**, 6433-6442 (1987)

Kincaid B.M., Eisenberg P., Hodgson K.O., Doniach S.
X-ray absorption spectroscopy using synchrotron radiation for structural investigation of organometallic molecules of biological interest.
Proc. Nat.Acad.Sci.USA **72**, 2340-2342 (1975)

Lee P.A., Beni,G.,
New method for the calculation of atomic phase shifts: application to extended x-ray absorption fine structure (EXAFS) in molecules and crystals.
Phys.Rev.B **15**, 2862-2883 (1977)

Lee P.A., Citrin P.H., Eisenberg P., Kincaid B.M.
Extended x-ray absorption fine structure - its strengths and limitations as a structural tool.
Rev.Mod.Ph. **53**, 769-806 (1981)

Lee P.A., Pendry J.B.
Theory of the extended x-ray absorption fine structure
Phys.Rev.B **11**, 2795-2811 (1975)

Lindley P.F., Garratt R.C., Hasnain S.S.
EXAFS and crystallographic studies of metallo-proteins.
in "Synchrotron radiation and biophysics" ed. S.S.Hasnain
ch.8, 176-200, (1990)

Longo A., Morante S., Ciriolo M.R., Desideri A.
Studio EXAFS del complesso rame-gluatione ridotto.
in "Proceedings of the IX Congress of the Italian Biophysical Society"
Marciana Marina, (1990)

Markossian K.A., Melkonian V.Z., Poitian N.A., Nalbandyan R.M.
On the copper transfer between β–dopamine -monooxygenase and Cu-thionein.
Bioch.Biophys.Res.Commun. **153**, 558-563 (1988)

Marzullo G., Friedhoff A.J.
An inhibitor of opiate receptor binding from human erythrocytes identified as a glutathione copper complex.
Life Sci. **21**, 1559-1568 (1977)

McCord J.M. & Fridovich I.
An enzimatic function for erythrocuprein (hemocuprein)
J.Biol.Chem. **244**, 6049-6055 (1969)
Meister A., Anderson M.E.
Glutathione.
Ann.Rev.Biochem. **52**, 711- 760 (1983)
Micheloni M., May P.M., Williams D.R.J.
J.Inorg.Nucl.Chem. **40**, 1209-1219 (1978)
Miyoshi K., Ishizu K., Tanaka H., Sugiura Y., Asada K.
Inorg.Chim.Acta **79**, 261 (1983)
Miyoshi K., Sugiura Y., Ishizu K., Iitaka Y., Nakamura H.
Crystal structure and spectroscopic properties of violet glutathione-copper(II) complex with axial sulfur coordination and two copper sites via a disulfide bridge.
J.Am.Chem.Soc. **102**, 6130-6136 (1980)
Morpurgo L., Hartmann H.J., Weser U., Desideri A., Rotilio G.
Yeast copper-thionein can reconstitute the Japanese-lacquer-tree (Rhus Vernicifera) laccase from the type 2-copper-depleted enzyme via a direct copper(I)-tranfer mechanism.
Biochem. J. **211**, 515-517 (1983)
Nakamura M., Yamazaki I.
Biochim. Biophys. Acta **267**, 249-257 (1972)
Österberg R., Ligaarden R., Persson D.,
Copper(I) complexes of penicillamine and glutathione.
J.Inorg.Bioch. **10**, 341-355 (1979)
Postal W.S., Vogel E.J., Young C.M., Greenaway F.T.
The binding of copper(II) and zinc(II) to oxidized glutathione.
J.Inorg.Bioch. **25**, 25-33 (1985)
Rabenstein D.L., Guevremont R., Evans C.A.
in "Metal ions in biological systems",
Sigel ed., Marcel Dekker Inc. N.Y., **9**, chap.4, (1979)
Reed C.J., Douglas K.T.
Chemical cleavage of plasmid DNA by glutathione in the presence of Cu(II) ions.
Biochem. J. **275**, 601-608 (1991)
Rigo A., Viglino P., Calabrese L., Cocco D., Rotilio G.
Biochem. J. **161**, 27-30 (1977)

Samuni A., Chevion M., Czapski G.
J.Biol.Chem. **256**, 12632-12635 (1981)
Sayers D.E., Bunker B.A.
Data analysis..
in "X-ray absorption spectroscopy" ed. Koningsberger & Prins,
ch. 6, (1989)
Stern E.A., Heald S.M.
Basic principles and applications of EXAFS
in "*Handbook of synchrotron radiation* ", Vol.1, ed. Koch,
ch. 10, 955-1014 (1983)
Suzuki K.T., Maitani T.
Metal dependent properties of metallothionein.
Biochem. J. **199**, 289-295 (1981)
Tainer J.A., Getzoff E.D., Beem K.M., Richardson J.S., Richardson D.C.
Determination and analysis of the 2 Å structure of copper,zinc superoxide dismutase.
J.Mol.Biol. **160**, 181-217 (1982)
Tainer J.A., Getzoff E.D., Richardson J.S., Richardson D.C.
Structure and mechanism of copper,zinc superoxide dismutase.
Nature **306**, 284-287 (1983)
Teo B.K.
in " EXAFS spectroscopy: technique and applications"
Teo & Joy eds., (1980)
Teo B.K., Lee P.A.
Ab initio calculation of amplitude and phase functions for extended x-ray absorption fine structure spectroscopy
Am.Chem.Soc. **101**, 2815-2831 (1978)
Whitehouse M.W., Walker W.R.
Agents and actions **8**, 85 (1978)
Ziegler D.M.
Role of reversible oxidation-reduction of enzyme thiols-disulfides in metabolic regulation.
Ann.Rev. Biochem. **54**, 305-329 (1985)

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