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Superoxide Dismutase, a Study of the Electronic Properties of the Copper and Zinc by X-Ray Absorption Spectroscopy†

W. E. Blumberg, J. Peisach,* P. Eisenberger, and J. A. Fee

ABSTRACT: The x-ray absorption for copper and zinc in oxidized and reduced superoxide dismutase, as well as in various model compounds, was studied. Upon reduction of the protein, the added electron affects the copper site almost exclusively, while the zinc remains virtually unchanged. Reduction decreases the charge on the copper atom [toward Cu(I)] and changes the configuration of the copper site so that it becomes less symmetric. An analysis of the copper absorption observed with the oxidized enzyme and a comparison with that for $Cu(II)(imid)_4$ suggests that the copper is not simply ligated to four imidazoles. The addition of H_2O_2 to superoxide dismutase reduces the copper to Cu(I), while oxygen addition to the peroxide-reduced protein restores the copper to Cu(II).

A number of proteins from both aerobic (Fridovich, 1975) as well as strictly anaerobic organisms (Hewitt and Morriss, 1975; Asada et al., 1976; Hatchikian and Henry, 1977) have been found to effect the decomposition of superoxide, a partially reduced form of dioxygen. This catalytic process consists of the oxidation of the oxygen atoms of one molecule of superoxide to the oxidation level of dioxygen and the reduction of the oxygen atoms of another molecule to the oxidation level of hydrogen peroxide. All these enzymes contain one or more metal atoms at the active site (Keele et al., 1970), either manganese, iron (Yost and Fridovich, 1973) or copper and zinc (McCord and Fridovich, 1969).

Higher organisms utilize the copper-containing enzyme. This protein also contains zinc in equal amounts to the copper (Carrico and Deutsch, 1970). X-ray crystallographic analysis (Richardson et al., 1975a,b) has shown that bovine superoxide dismutase takes the form of a dimeric polypeptide with two active sites, each site containing a copper atom surrounded by four imidazole ligands, one of which is also ligated to a zinc atom.

At a resolution of 2.8 Å, the electron-density map suggests that each copper atom is coordinated to histidine-44, -46, -61, and -118, as deduced from the protein's sequence (Steinman et al., 1974). The imidazole from histidine-61 binds both to the copper atom and to the zinc atom as well. This close proximity of metals is consistent with spectral and magnetic studies (Fee, 1973; Rotilio et al., 1974; Moss and Fee, 1976). The zinc atom is further coordinated to histidine-69 and -78 and aspartate-81. Individual copper–zinc pairs are separated from one another by 34 Å.

In the isolated protein, the copper atom is divalent and exhibits an optical absorption near 680 nm which is characteristic of the cupric chromophore (Mann and Keilen, 1939; Carrico and Deutsch, 1969). The copper atom also exhibits an EPR[1] spectrum which is indicative of a small departure from axial symmetry (Rotilio et al., 1971; Fee and Gaber, 1972) having a rhombicity of 21% as defined by Blumberg and Peisach (1974).

During enzymatic turnover, the intensity of the 680-nm absorption changes at rates which are consistent with overall catalytic efficiency, thus implicating the metal sites in an oxidation–reduction cycle (Fielden et al., 1973). It is not known whether both metals change their valence during catalysis, although because of the inherent stability of divalent zinc (Cotton and Wilkinson, 1962) it is attractive to hypothesize that only the copper changes oxidation state. Even here, however, it is not known with certainty whether the copper shuttles between di- and trivalent oxidation states or between di- and monovalent states during actual catalysis, although the latter case is quite generally assumed (for example, Fee and Ward, 1976). It should be noted, however, that Cu(III) can be stabilized by peptide ligands (Margerum et al., 1975), and, furthermore, trivalent copper has been proposed to form during the catalytic action of galactose oxidase (Hamilton et al., 1973), another copper-containing enzyme.

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[1] Abbreviations used: EPR, electron paramagnetic resonance; LEFE, linear electric field effect; EDTA, (ethylenedinitrilo)tetraacetic acid.

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In the present work, we explore the electronic structures of both copper and zinc in the oxidized and reduced forms of superoxide dismutases isolated from human and bovine erythrocytes by the technique of x-ray absorption spectroscopy. With this technique, one measures transitions of the 1s core electron to bound unoccupied orbitals. Transitions to empty bound states depend, in part, on the formal charge on the ion, the site symmetry, and the extent of covalency with ligand atoms (Shulman et al., 1976). Higher energy transitions to an unbound final state are backscattered by close-lying atoms producing an energy-dependent modulation of the absorbance and giving rise to the x-ray absorption fine structure (EXAFS) (Sayers et al., 1970). The analysis of this fine structure can give extremely precise structural information (Shulman et al., 1975) but is extremely difficult for cases of heterogeneous metal ligation. The analysis of x-ray absorption arising from transition to bound states is much more straightforward when the metal atom is ionically coordinated as in metal halides. Using this technique, one is able to describe charge densities and covalency effects of metal sites in inorganic compounds as well as in metalloproteins by measuring the positions in energy of various features of the K-absorption edge of the atom in question, provided that the character of the various absorption features can be analyzed by comparison with compounds of known electron structure (Shulman et al., 1976; Eisenberger et al., 1976).

Materials and Methods

Bovine superoxide dismutase was prepared according to McCord and Fridovich (1969), and its concentration was determined similarly. The EPR spectrum of the protein was determined with a Varian E-112 spectrometer using a standard TE_{102} cavity with flow-through cryogenic cooling. Cu(II) (1 mM) in excess EDTA was used as a primary standard for EPR quantitation. $Cu(II)(imid)_4$ was prepared by adding a 50-fold molar excess of imidazole to 50 mM cupric acetate and raising the pH to 8.0. The reduced complex was prepared by anaerobic addition of dithionite. A zinc–pyridine model compound was prepared by dissolving zinc acetate to a final concentration of 50 mM in warm pyridine.

X-ray absorption spectra in the region of the K edge of copper and zinc were obtained using synthrotron radiation (Kincaid and Eisenberger, 1975) at the Stanford Synchroton Radiation Project using solutions of the bovine enzyme (5 mM) in phosphate buffer, pH 7.8. The first spectra were taken in the fluorescence mode (Jaklevic et al., 1978) using a Ge(Li) detector followed by a single-channel pulse-height analyzer set to discriminate against Compton scattered x rays. Some preliminary results using this method have been presented by Blumberg et al. (1976). Later, an array of nine NaI(Tl) photomultiplier scintillation detectors was used. Each detector had its own pulse-height discriminator which was set to maximize the ratio of fluorescence x rays to scattered x rays for the particular metal being observed. The x-ray monochromator calibration was established by setting the K absorption edge of metallic copper to a reading corresponding to 8979 eV. The absolute calibration is only accurate to a few eV, but the relative calibrations between the copper and zinc scans of Figures 1 and 2 are accurate to 0.2 eV.

After studying the edges for both Cu(II) and Zn(II) in the resting enzyme, a large molar excess of dithionite was added and the spectrum was scanned at energies characteristic for both copper and zinc. A tenfold molar excess of H_2O_2 per copper was added to another aliquot of resting enzyme (see Rotilio et al., 1973; Fee and DiCorleto, 1973) and the copper absorption edge spectrum was taken. The same sample was measured again after about a 0.5-h incubation period, during which time partial recoloration by air had occurred.

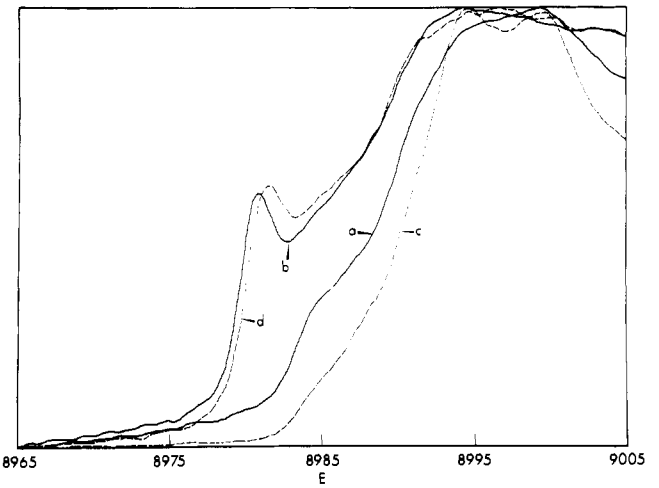

FIGURE 1: X-ray absorption edge of copper in oxidized (a) and reduced bovine superoxide dismutase (b) and oxidized (c) and reduced (d) $Cu(imid)_4$ model compound in aqueous solution. The energy of the x-ray absorption E in electron volts is given as the abscissa in the figure.

X-ray absorption edges were also studied for the aqueous copper tetraimidazole complex both before and after reduction by dithionite.

Results and Discussion

In Figure 1, we show the x-ray absorption spectrum for the copper of bovine superoxide dismutase, both in the resting state (a) and when reduced with sodium dithionite (b). For ionic compounds that do not change geometry upon reduction such as ferrous and ferric fluorides, reduction causes a decrease to lower energy of about 6 eV in the absorption edge for the lower oxidation state (Shulman et al., 1976). In this case, the shape of the absorption edge does not change appreciably. For covalent compounds such as ferro- and ferricyanides, the decrease is much smaller, the edge being roughly 1 eV less for the reduced iron salt. Here too, the shape of the edge does not change, since the geometries of both salts are about the same. In the case of oxidized and reduced superoxide dismutase, however, there is a general shift to lower energy upon reduction, but the magnitude of shift depends upon the position of the edge where the comparison is to be made. If one does so at the 1s to 4s transitions (near 8984 eV for the oxidized protein; see Table I), then reduction of the protein shifts the edge by about 3 eV to lower energy.

In the reduced protein, the 1s to 4s transition is more intense than in the oxidized protein. As was shown by Shulman et al. (1976) for mononuclear iron–sulfur compounds, departure from configurations which have inversion symmetry such as octahedral or square planar toward tetrahedral symmetry shows such an effect. Thus, the copper site in superoxide dismutase is believed to undergo a symmetry rearrangement to a configuration more tetrahedral upon reduction. Tetrahedral cuprous configurations are not unusual, and symmetry changes of this sort might be expected.

In Figure 1c,d, we show the absorption edges for synthetic cupric and cuprous imidazole complexes, respectively, which we believe are useful compounds for comparison with the copper site of superoxide dismutase. X-ray crystallographic analysis (Richardson et al., 1975a,b) has been interpreted as suggesting that four imidazole groups are bound to Cu(II) in the protein. From a comparison of the 1s to 4s transitions for

TABLE I: X-Ray Absorption Edge Features.

Sample	Energy of transition, eV[a]				
	Zn, 1s to 4p	Zn, 1s to 4s	Cu, 1s to 4p[b]	Cu, 1s to 4s	Cu, 1s to 3d
Oxidized superoxide dismutase	9663.5	9658.8	8991		8976.3
			8994.2		
			8999.4	8984	
Reduced superoxide dismutase	9663.2	9658.6	8994	8980.8	
Cupric-(imidazole)$_4$	*c*	*c*	8994.6	8984	8976.3
			8999.4		
Cuprous-(imidazole)$_4$	*c*	*c*	8996	8981.6	*c*
Zinc-(pyridine)$_4$	9663.5	*d*	*c*	*c*	*c*

[a] Energies accurate absolutely to ±5 eV but relative to one another ±0.2 eV. [b] Transition may be split into several features. [c] Not applicable. [d] Not resolved.

the cupric protein and the cupric model compounds, one observes that this transition is more intense for the protein. As was shown for studies of iron-sulfur complexes of different symmetries, departure from centrosymmetry increases the intensity of this transition. Shulman et al. (1976) found that changing the symmetry from axial to tetrahedral increases the intensity of the 1s to 3d transition by a factor of 7, although theory would have allowed it to increase by about a factor of 100. Therefore, at the present time, the increase of the intensity of the 1s to 4s transition is not quantitatively interpretable. The analysis of the EPR spectrum of the oxidized protein is also indicative of a departure from axial symmetry (Rotilio et al., 1971; Fee and Gaber, 1972). In addition, the linear electric-field effect in EPR (Mims, 1976) for the copper site is larger[2] than observed for simple copper model compounds (Peisach and Mims, 1978), also in agreement with the conclusion that the symmetry is far from centrosymmetric. The measured strength of the absorption of the protein over the model compound in the region where the forbidden transitions are expected to contribute (Shulman et al., 1976) suggests that in the proteins those transitions are more allowed. This difference is greater in the oxidized protein than it is in the reduced protein but is not reflected in the positions of the features attributed to atomic transitions of the copper atom itself, except for an 0.8-eV shift in the 1s to 4s transition for the reduced protein (see Table I).

When the symmetry about the metal atom is lower than cubic, the 1s to 4p transition may be split into a maximum of three features. In square planar geometry, maximal splitting of this transition is observed. In the purely tetrahedral case, this splitting diminishes to zero. The lack of resolution of this transition in the reduced protein does not permit a symmetry assignment. Only a very broad feature centered about 8994 eV is observed. For the cupric protein, the symmetry is rhombic or lower, as experienced by the 4p electron.

An analysis of the EPR of Cu(imid)$_4$ model compound suggests that the imidazole ligands in the complex remain neutral when bound to the Cu(II); that is, a proton is not lost from the remote nitrogen of any bound imidazole (Peisach and Blumberg, 1974). The EPR of the copper in superoxide dismutase is shifted to smaller values of $g_\parallel$ and increased values of $A_\parallel$ than for Cu(II)(imid)$_4$ (Malmström and Vänngård, 1960), suggestive of an increased negative charge at the copper. However, the optical spectral maximum of the copper of dismutase is much farther to the red than that of the tetraimidazole-copper complex (λ_{max} 600 nm at pH 7-9). Negatively charged nitrogenous ligands shift absorptions for Cu(II) complexes to the blue as compared to negatively charged oxygenous ligands. The position of the absorption maxima for superoxide dismutase at 680 nm could arise either from the presence of an oxygenous ligand or from a tetrahedral distortion of the principal ligands (Solomon et al., 1976). This former possibility is in accord with analyses of the EPR and optical data and the comparison of the nuclear modulation effect of the enzyme with that of the Cu(imid)$_4$ complex (Mims and Peisach, 1976), although the LEFE (Peisach and Mims, 1978) shows that there is some noncentrosymmetric distortion as well. [2]

In the reduced protein all the absorption edge features of superoxide dismutase and the model compound are very similar, the latter being slightly to higher energy. One would conclude that reduced superoxide dismutase is not very different from reduced tetraimidazole-copper in either charge state or geometry. The 1s to 4p transitions of copper are centered at nearly the same position for the oxidized and reduced proteins. This shift in energy is much less than the shift in energy observed for the 1s to 4s transition upon reduction. Thus, different degrees of covalency are exhibited by the different excited states of the copper atom. This is to be expected for a metal ion which has both σ and π bonds with its ligand atoms.

Although one can say that configurational change, charge change, and change in covalency all affect the appearance of the copper absorption edge in the protein, one cannot at this time assign any numerical parameters to these three causes. One can only say that the geometrical and electronic structure of the copper site is strongly coupled to its formal valence state.

In Figure 2, we show the x-ray absorption edge for Zn in oxidized and reduced superoxide dismutase and for a zinc-pyridine model compound which we thought to be useful for comparison with the zinc site in the protein. For the Zn absorptions, little difference, if any, is observed when comparing the absorption edge of oxidized and reduced superoxide dismutase. Thus, the oxidation state of the Zn is unchanged when the protein is reduced. The midpoint of the edge moves by less than 0.5 eV. Thus, comparing with the case of ferrous and ferric fluoride (Shulman et al., 1970), one can say there is a change of charge of less than 0.1 electrons. For both reduced and oxidized proteins, the 1s to 4s transition is barely resolved but does not change intensity nor is it shifted upon protein reduction. In comparison, the intensity of this same transition is so weak for the zinc-pyridine model compound that the symmetry must be quite regular; i.e., it is very nearly centrosymmetric. For the protein, it is only slightly less so. Since the absorption edge is close in energy for the model compound and

[2] J. Peisach, W. B. Mims, and J. A. Fee, unpublished observations.

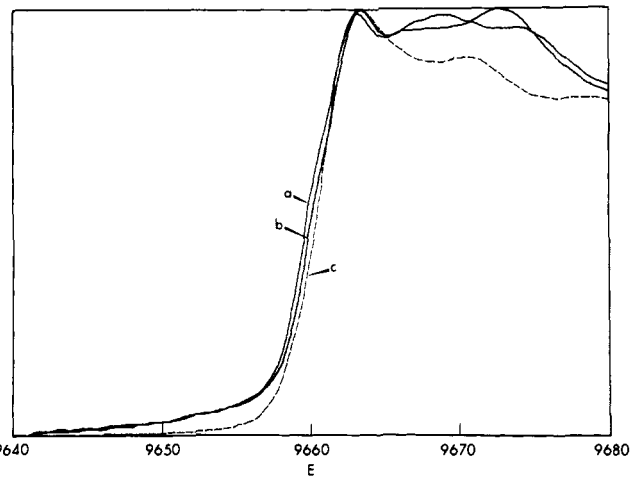

FIGURE 2: X-ray absorption edge of zinc in oxidized (a) and reduced (b) bovine superoxide dismutase and for the (c) zinc-pyridine model compound.

for both the reduced and oxidized proteins, the extent of covalency of all three must be very similar.

The region beyond the first peak in absorption is obviously rich in information. Due to the overlap of various transitions, it is impossible to make any interpretation at this time other than to say that there is indeed a difference between oxidized and reduced protein which is sensed by the higher electronically excited states of the Zn atom. In this regard, it is of interest to note that the visible optical absorption spectrum of Co(II) substituted into the Zn(II)-binding site (Rotilio et al., 1975; Moss and Fee, 1976) undergoes a characteristic change upon reduction of the Cu(II) ion accompanying the conformational change of the protein which has been shown to accompany reduction (Stokes et al., 1973, Fee and Phillips, 1975). The small difference in the Zn(II) x-ray absorption of the Cu(II) and Cu(I) states, presumably, also reflects this structural modification of the protein.

When H_2O_2 is added to oxidized superoxide dismutase (Blumberg et al., 1976), it was shown that the initial effect is to shift the absorption to lower energy indicative of reduction of the copper. In fact, the spectrum produced is a composite of spectra for the oxidized and reduced protein. The formation of Cu(III) is ruled out, since these compounds absorb to higher and not to lower energy.[3] Therefore, peroxide addition reduces the copper, which is the reason why the EPR is abolished.

In the presence of a small amount of oxygen, the edge shifts back to higher energy, demonstrating that the copper is partially reoxidized to the cupric state. These experiments demonstrate that the addition of one product of the dismutation reaction, H_2O_2, changes the oxidation state of the Cu(II) to Cu(I), while, on the other hand, the addition of the other product of the dismutation reaction, O_2, to the reduced protein changes the oxidation state of Cu(I) to Cu(II).

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Structural Studies of the Heme Domain of Sulfite Oxidase: CNBr Fragments†

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With Technical Assistance of Ralph D. Wiley

ABSTRACT: The cytochrome b_5 type heme domain of rat liver sulfite oxidase, prepared by limited tryptic digestion of the intact enzyme, is a 10 000 molecular weight peptide containing a single methionine residue. Cyanogen bromide treatment permitted cleavage of the heme domain into 2 peptides containing 49 and 38 residues, representing the NH_2- and COOH-terminal segments of the intact peptide, respectively. The circular dichroism spectra indicated the virtual absence of secondary structure in the isolated CNBr fragments. Addition of hemin to either fragment resulted in the enhancement of the CD spectrum, and also increased the antigenic activity of the NH_2-terminal fragment toward antibodies prepared against native sulfite oxidase. The CD spectrum of heme complex of the COOH-terminal fragment was indicative of the formation of extensive α-helical structure. Mixing the two CNBr fragments in the presence of heme resulted in about 40% reconstitution of heme in a b_5-type configuration. The heme-reconstituted complex appeared to attain the secondary folding structure of the intact domain as detected by circular dichroism and immunological properties. The COOH-terminal peptide is composed of a long sequence rich in hydrophobic residues followed by an acidic sequence. Results of these structural studies are discussed in terms of homology with other b_5-type heme proteins and the interaction of sulfite oxidase with its physiological electron acceptor, cytochrome *c*.

Sulfite oxidase catalyzes the terminal reaction in the oxidative degradation of sulfur-containing amino acids and uses cytochrome *c* as its physiological electron acceptor. The enzyme has a dimeric molecular weight of 120 000 and contains molybdenum and heme as prosthetic groups (Cohen et al., 1971; Kessler & Rajagopalan, 1972). Recently, the subunit of the enzyme was demonstrated in our laboratory to be composed of two domains, one containing molybdenum and the other, heme (Johnson & Rajagopalan, 1977). The heme domain was shown to be the NH_2-terminal portion of the native enzyme and the molybdenum domain the carboxyl end. The cleavage of the enzyme by trypsin into domains did not affect either the structural or functional integrity of the domains. Thus, the molybdenum domain retained the ability to oxidize sulfite with ferricyanide as acceptor and had identical EPR properties to those of native sulfite oxidase. Within the heme domain, the structure which confers the cytochrome b_5 type absorption spectrum is preserved.

Two other cytochrome b_5 type proteins have been studied extensively. Microsomal cytochrome b_5 is a component of a multienzyme system which catalyzes the desaturation of fatty acids. The protein is composed of two distinct parts—a polar segment which binds the heme and a hydrophobic sequence which anchors the protein to the membrane (Spatz & Strittmatter, 1971; Strittmatter et al., 1972). The primary structure has been determined for the polar core from a variety of sources and the three-dimensional structure has been resolved for the bovine protein (Ozols & Strittmatter, 1969; Ozols, 1970; Nobrega & Ozols, 1971; Ozols et al., 1976; Matthews et al., 1972). A second well-studied b_5-type heme protein is the flavocytochrome b_2 from baker's yeast which catalyzes the oxidation of lactate to pyruvate. Digestion of the protein by trypsin releases a hemopeptide of 11 400 molecular weight (Labeyrie et al., 1966). The hemopeptides of microsomal b_5 and yeast b_2 exhibit substantial homology, suggesting that these proteins may have been derived from a common ancestral gene (Guiard & Lederer, 1976).

Recently Guiard & Lederer (1977) reported that a 34 residue NH_2-terminal sequence of the hemopeptide of chicken liver sulfite oxidase displayed sequence similarities to corre-

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