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An extended X-ray-absorption-fine-structure study of the copper and zinc sites of freeze-dried bovine superoxide dismutase

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Copper and zinc *K*-edge-extended X-ray-absorption fine structures were measured for the metal sites of freeze-dried bovine superoxide dismutase and the model compounds tetrakis(imidazole)cupric nitrate and tetrakis(imidazole)zinc perchlorate. Detailed simulation of the spectra indicates that the copper site of the enzyme is best fit by co-ordination of four imidazole groups with $Cu-N_{(\alpha)}$ distances of 0.198 nm (1.98 Å). The zinc site is best fit by three imidazole groups at 0.201 nm (2.01 Å) and an oxygen (from aspartate) at 0.203 nm (2.03 Å).

Bovine erythrocyte superoxide dismutase is a copper- and zinc-containing metalloprotein that catalyses the reaction:

$$2O_2^{\cdot-} + 2H^+ \rightarrow O_2 + H_2O_2$$

X-ray-crystallographic analysis to a resolution of 0.3 nm (3 Å) (Richardson *et al.*, 1975) has revealed a structure where each of the two subunits contains one copper and one zinc atom separated by approx. 0.6 nm (6 Å) and bridged by the imidazole ring of a histidine residue. The zinc ion has approximately tetrahedral co-ordination geometry and is bonded to a nitrogen atom of each of the imidazole rings of His-61, His-69 and His-78, and a carboxylate oxygen atom of Asp-81. The copper atom is co-ordinated to one nitrogen atom of each of the imidazole groups of His-44, His-46, His-61 and His-118. Further considerations of this structure (Beem *et al.*, 1977) have indicated that the co-ordinated nitrogen atoms of His-44, His-46 and His-118 lie approximately at three corners of a square plane centred at copper, with the fourth corner unoccupied. The co-ordinated nitrogen atom of His-62 (the bridging ligand) lies above this plane on the side accessible to solvent in a position intermediate between planar and axial.

The technique of e.x.a.f.s. is complementary to crystallography of metalloproteins in that it can determine the local environment of the co-ordinated metal (Cramer & Hodgson, 1979). A particular advantage of e.x.a.f.s. is the ability to determine bond lengths in either crystalline or non-crystalline samples of metalloproteins to a precision considerably greater than that obtained from crystallography (Teo, 1981). For superoxide dismutase e.p.r. has indicated that the crystal structure is maintained in frozen aqueous solution (Lieberman *et al.*, 1981). However, the process of freeze-drying leads to a reversible change in the e.p.r. spectrum of the copper, consistent with a change in stereochemistry at the copper ion towards a more axially symmetric structure (Strothkamp & Lippard, 1982). We now report the results of an e.x.a.f.s. study of the copper and zinc sites of freeze-dried superoxide dismutase. As an aid to the interpretation of the enzyme data, corresponding spectra were recorded for model compounds of known structure, $[Zn(imidazole)_4(ClO_4)_2]$ (Bear *et al.*, 1975) and $[Cu(imidazole)_4(NO_3)_2]$ (McFadden *et al.*, 1976).

Abbreviation used: e.x.a.f.s., extended X-ray-absorption fine structure.

Materials and methods

Enzyme samples were prepared and assayed by using the procedures of McCord & Fridovich (1969). All samples were further purified on a column of Sulphamylon–Sepharose 4B (Whitney,

1974) to remove any contaminating carbonic anhydrase, and then, after concentration, passed through a small column of Dowex chelating resin (Sigma 80–100 mesh). The ratio of copper to zinc found in the samples used for e.x.a.f.s. was 0.96–1.03, indicating no significant contamination by extraneous copper or zinc or by carbonic anhydrase.

The sampes used for e.x.a.f.s. were monitored by e.p.r. spectroscopy: (i) as the freeze-dried powder; (ii) after being redissolved in deionized water. The spectrum of the powder indicated a change from the solution spectrum similar to that reported by Strothkamp & Lippard (1982). This change was reversed on redissolving in water to give spectra identical with literature reports on frozen aqueous samples (Rotilio *et al.*, 1972; Beem *et al.*, 1974).

For X-ray-absorption measurements the finely ground freeze-dried samples were pressed into aluminium sample holders with Sellotape windows (15 mm × 3 mm × 3 mm). Spectra were recorded at low temperature with a Dewar system that comprised a cold 'finger' in contact with liquid N_2 on to which the sample was bolted. An average of nine scans extending from 600 eV below the Cu *K*-edge to 600 eV above the Zn *K*-edge were recorded for each sample.

No significant radiation damage or photoreduction was detected during irradiation, as judged by (i) specific activity measured before (4026 units) and after (4256 units) irradiation, (ii) 35 GHz e.p.r. spectra recorded before and after irradiation, and (iii) examination of individual scan near the edge region, during and subsequent to the measurements.

$[Zn(imidazole)_4(ClO_4)_2]$ (Bear *et al.*, 1975) and $[Cu(imidazole)_4(NO_3)_2]$ (McFadden *et al.*, 1976) were prepared by literature methods. X-ray-absorption spectra were recorded for powdered samples of these materials diluted with a 3-fold excess of boron nitride. In each case a single scan gave an acceptable signal-to-noise ratio.

X-ray-absorption spectra were recorded in the transmission mode at the Daresbury Synchrotron Radiation Source operating at an energy of 1.8 GeV with an average current of 90 mA. A channel-cut Si 111 crystal was used as the monochromator. Data analysis utilized the single-scattering spherical-wave method for calculating e.x.a.f.s. with phase shifts derived from '*ab-initio*' calculations as described previously (Lee & Pendry, 1975; Perutz *et al.*, 1982). The e.x.a.f.s. is plotted in *k*-space where $k = \sqrt{0.263(E-E_0)}$, E = energy of the beam and E_0 = energy zero of the photoelectron wave. E.x.a.f.s amplitude is multiplied by k^3 to highlight the amplitude at higher k, where single-scattering theory is most accurate. The quality of fits was assessed by using criteria described by Perutz *et al.* (1982) and using a non-linear least-square minimization program. Least-square refinement was made with k, k^2 and k^3 weighting, and a minimization was considered reliable only when all three converged to similar results. Minimization was performed for distance parameters only; co-ordination number and Debye–Waller constant, σ^2, were kept fixed during a minimization.

Results and discussion

Figs. 1(*a*)–1(*d*) show the e.x.a.f.s. spectra of the zinc and copper sites of the freeze-dried superoxide dismutase, $[Zn(imid)_4(ClO_4)_2]$ and $[Cu(imid)_4(NO_3)_2]$. Each of the spectra shows an e.x.a.f.s. profile typical of a metal co-ordinated to histidine ligands (M. S. Co, R. A. Scott & K. O. Hodgson, unpublished work). The best overall fit of the $[Zn(imid)_4(ClO_4)_2]$ and $[Cu(imid)_4(NO_3)_2]$ was obtained with the distances and Debye–Waller factors given in Tables 1 and 2 respectively. A reasonable agreement has been found with the crystallographic data of $[Zn(imid)_4(ClO_4)_2]$ and $[Cu(imid)_4(NO_3)_2]$ for the initial three shells of atoms, but for the distant shells $4N_{(\gamma)}$ and $4C_{(\gamma)}$ e.x.a.f.s. gives shorter distances than does the crystallographic analysis. A similar apparent shortening of distances was found in the deoxyhaemoglobin and the well-characterized 'picket-fence' complex (Perutz *et al.*, 1982). The reasons for this discrepancy are not well understood, but multiple scattering and shadowing effects are expected to become important for distances greater than 0.32 nm (3.2 Å). In the case of $[Cu(imid)_4(NO_3)_2]$, we did not find it necessary to include the atoms of the $(NO_3)_2$ group, as the closest atom is an oxygen atom at 0.252 nm (2.52 Å) and this is expected to contribute relatively weakly to the e.x.a.f.s. profile.The application of e.x.a.f.s. to these well-characterized copper and zinc compounds provides confidence that the e.x.a.f.s. theory can be used for determining the distances of atoms within 0.32 nm (3.2 Å) from the copper and zinc sites of the enzyme; beyond this e.x.a.f.s. may give an underestimate.

The e.x.a.f.s. spectrum of the copper site of the enzyme can be reproduced well with four imidazole $N_{(\alpha)}$ at 0.20 nm (2.0 Å), $4C_{(\beta)}$ each at 0.291 nm (2.91 Å) and 0.304 nm (3.04 Å). The distant shells $4N_{(\gamma)}$ and $4C_{(\gamma)}$ again appear at short distances, 0.383 nm (3.83 Å) and 0.384 nm (3.84 Å). Also, as in the case of $[Cu(imid)_4(NO_3)_2]$, addition of an oxygen atom at a distance greater than 0.24 nm (2.4 Å) resulted in minor modification to the e.x.a.f.s. profile and no real improvement in the quality of fit was obtained; attempts to refine the simulation with a short Cu–O distance of less than 0.23 nm (2.3 Å) resulted in a much poorer fit, particularly in the second beat region at about $k = 6$ Å^{-1}. The zinc-site e.x.a.f.s. spectrum can be best fit with three imidazole groups [$3Zn–N_{(\alpha)} = 0.201$ nm (2.01 Å),

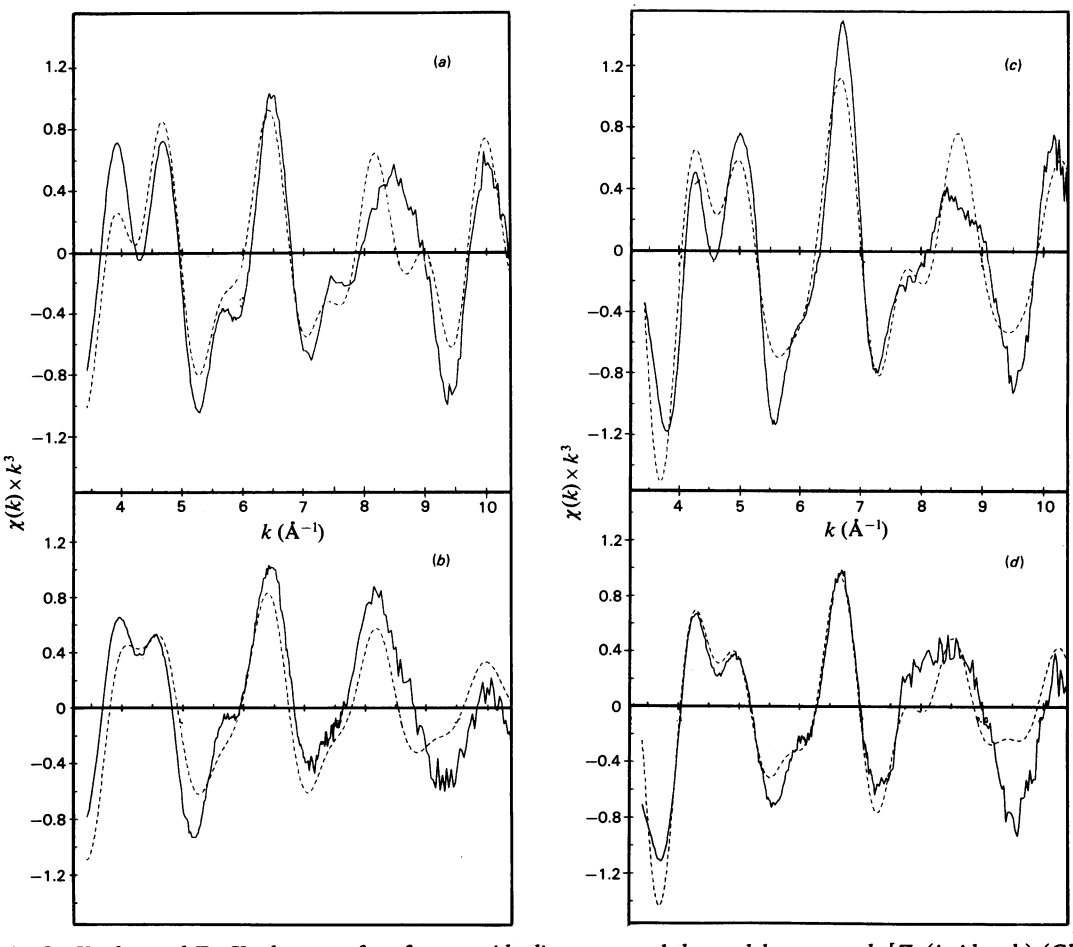

Fig. 1. *Cu K-edge and Zn K-edge e.x.a.f.s. of superoxide dismutase and the model compounds* [*Zn(imidazole)*$_4$(*ClO*$_4$)$_2$] *and* [*Cu(imidazole)*$_4$(*NO*$_3$)$_2$]

E.x.a.f.s. [$\chi(k)$] is presented as $\chi(k) \times k^3$ plotted versus k, where k is in Å^{-1} [equivalent to (10nm)$^{-1}$]. Continuous lines represent the experimental spectra, broken lines the simulated spectra. (*a*) [Zn(imid)$_4$(ClO$_4$)$_2$]; (*b*) zinc site of superoxide dismutase; (*c*) [Cu(imid)$_4$(NO$_3$)$_2$]; (*d*) copper site of superoxide dismutase. Experimental data were shifted to higher energy: for Cu *K*-edge $\Delta E_0 = 18$ eV; for Zn *K*-edge $\Delta E_0 = 6$ eV. The overall quality of individual simulation is similar; the following values for the fit index parameter were obtained for (*a*) 5.38, (*b*) 6.4, (*c*) 5.2 and (*d*) 4.4.

Table 1. *Parameters used to simulate the e.x.a.f.s. associated with the Zn K-edge of the freeze-dried superoxide dismutase and* [*Zn(imid)*$_4$(*ClO*$_4$)$_2$]

R denotes the distance of atoms from the Zn atom. σ^2 is a Debye–Waller factor and is equivalent to $\Delta R^2_{rms} \cdot \Delta E_0 = 6$ eV.

	Superoxide dismutase			[Zn(imid)$_4$(ClO$_4$)$_2$]			
Atom	Number	σ^2 (nm^2)	R (nm)	Number	σ^2 (nm^2)	R (nm)	$R_{crystal}$ (nm)
$N_{(\alpha)}$	3	0.00009	0.201	4	0.00008	0.201	0.200
O	1	0.00010	0.204	–	–	–	–
$C_{(\beta)}$	3	0.00006	0.290	4	0.00006	0.300	0.300
$C_{(\beta)}$	3	0.00006	0.309	4	0.00006	0.324	0.304
$N_{(\gamma)}$	3	0.00010	0.395	4	0.00005	0.394	0.415
$C_{(\gamma)}$	3	0.00010	0.391	4	0.00005	0.393	0.418

Table 2. *Parameters used to simulate the e.x.a.f.s. associated with the Cu K-edge of the freeze-dried superoxide dismutase and* $[Cu(imid)_4(NO_3)_2]$

R denotes the distance of atoms from the Cu atom. σ^2 is a Debye–Waller factor and is equivalent to ΔR^2_{rms} (note that e.x.a.f.s. experiments were performed at near 77 K). $\Delta E_0 = 18$ eV.

	Superoxide dismutase		$[Cu(imid)_4(NO_3)_2]$		
Atom	σ^2 (nm^2)	R (nm)	σ^2 (nm^2)	R (nm)	$R_{crystal}$ (nm)
$4N_{(\alpha)}$	0.00010	0.200	0.00005	0.198	0.201
1O	–	–	–	–	0.252
1O	–	–	–	–	0.260
$4C_{(\beta)}$	0.00010	0.292	0.00008	0.295	0.299
$4C_{(\beta)}$	0.00010	0.305	0.00008	0.306	0.304
$4N_{(\gamma)}$	0.00010	0.383	0.00008	0.378	0.414
$4C_{(\gamma)}$	0.00010	0.384	0.00008	0.388	0.419

$3C_{(\beta)}$ each at 0.290 nm (2.90 Å) and 0.309 nm (3.09 Å), $3N_{(\gamma)}$ at 0.395 nm (3.95 Å) and $\pm C_{(\gamma)}$ at 0.391 nm (3.91 Å)] and an oxygen (presumed to arise from carboxylate of Asp-181) at 0.203 nm (2.03 Å). Although a reasonable fit of the zinc site was also obtained by using four imidazole groups at 0.201 nm (2.01 Å) and increasing the Debye–Waller factor for the $C_{(\beta)}$ atoms, it was discarded on the grounds that the presence of aspartate co-ordination is known from crystallographic studies. E.x.a.f.s. fits using three-co-ordination at either copper or zinc sites required Debye–Waller factors (σ^2) much below those obtained for the models and were thus considered unlikely. Thus, although the correlation between σ^2 and co-ordination number (N) does not allow definitive determination of the co-ordination number, four-co-ordination at both copper and zinc would seem most consistent with the e.x.a.f.s. data.

These results are of relevance to the work of Strothkamp & Lippard (1982), who report evidence that in the freeze-dried Cu_2–Cu_2 superoxide dismutase the imidazole bridge is broken. If a similar process occurs in the freeze-dried native protein, then a decrease in imidazole co-ordination number would be expected either at copper or at zinc. The present e.x.a.f.s. results do not suggest a loss of an imidazole group either at a copper or at a zinc site.

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