An extended-X-ray-absorption-fine-structure study of bovine erythrocyte superoxide dismutase in aqueous solution

Direct evidence for three-co-ordinate Cu(I) in reduced enzyme

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Copper and zinc *K*-edge e.x.a.f.s. (extended X-ray-absorption fine structures) were measured for the metal sites of oxidized and reduced bovine superoxide dismutase in aqueous solution. Detailed analysis of the spectra indicates that the copper site of the enzyme changes on reduction and is most probably co-ordinated to three imidazole groups at a shorter distance $\text{Cu-N}_{(\alpha)} = 0.194\text{ nm}$ ($1.94\text{ \AA}$) in the reduced form compared with a co-ordination of four imidazole groups at 0.199 nm ($1.99\text{ \AA}$) and an oxygen atom from solvent water at 0.224 nm ($2.24\text{ \AA}$) in the oxidized form. Examination of the edge, near-edge structure and e.x.a.f.s. of the zinc sites indicates that the stereochemical changes at copper that accompany reduction introduce minimal perturbation on the stereochemistry at zinc.

Cu,Zn superoxide dismutases (superoxide: superoxide oxidoreductase, EC 1.15.1.1) are widely distributed in both plant and animal kingdoms and are found in eukaryotic cytosols. Although some controversy surrounds the true catalytic function of these proteins (Fee, 1982), they exhibit catalytic activity towards the dismutation of superoxide anion according to the equation:

The proximity of the metal centres and the early availability of a crystal structure to 0.3 nm resolution (Richardson *et al.*, 1975) stimulated interest in the co-ordination chemistry of the copper and zinc sites, which have since been investigated by a wide variety of spectroscopic techniques (Fee & Valentine, 1977; Fee, 1981; Valentine & Pantoliano, 1981). Many of the conclusions deduced from the spectroscopy have been confirmed by the recent crystallographic refinement of the structure to 0.2 nm (Tainer *et al.*, 1982), in which the copper ligands (nitrogen atoms of imidazole groups of His-44, -46, -61 and -118) show an uneven tetrahedral

distortion from a square plane, with the fifth axial co-ordination position occupied by solvent (Fee & Gaber, 1972; Boden *et al.*, 1979). His-61 forms a bridge to the zinc, the other ligands of which are N atoms of His-69 and -78 and an oxygen atom of Asp-81. The geometry around zinc is tetrahedral, with a strong distortion towards a trigonal pyramid having the buried Asp-81 at the apex.

Tainer *et al.* (1982) make the point that the presence of non-crystallographic symmetry relating the four copper and zinc sites of the two superoxide dismutase dimers per asymmetric unit introduces ambiguity as to whether the observed inequivalence in metal–ligand bond lengths reflects structural differences between the four copper and four zinc sites or results from lack of resolution in the refinement. They argue in favour of the latter and quote Cu–N and Zn–N bond lengths of about 0.21 nm and a shorter Zn–O bond length from Asp-81 of nearer 0.20 nm for bond lengths averaged over all the metal sites. E.x.a.f.s. can in principle determine bond lengths to a precision of $\pm 0.002\text{ nm}$ in metalloproteins (Cramer & Hodgson, 1979; Teo, 1981), and therefore offers a method for improving the structural characterization of the local environ-

Abbreviations used: e.x.a.f.s., extended X-ray-absorption fine structure; x.a.n.e.s., X-ray-absorption near-edge structure.

ment of the metal sites by providing precise bond lengths. We have recently reported an e.x.a.f.s. study of the copper(II) and zinc(II) sites in freeze-dried bovine superoxide dismutase (Blackburn *et al.*, 1983), but ambiguities arising from structural changes that accompany freeze-drying (Strothkamp & Lippard, 1982) preclude relating that study to the crystallographic data. However, e.p.r. studies (Lieberman *et al.*, 1981) have indicated that the crystal structure is most probably maintained in aqueous buffered solution. In the present paper we report a detailed e.x.a.f.s. study of bovine superoxide dismutase in aqueous 10 mM-phosphate buffer that, in addition to precise determination of metal-protein contacts, has allowed location of a solvent molecule co-ordinated to copper at 0.224 nm.

In marked contrast with the native Cu(II),Zn(II) protein, structural information on the Cu(I),Zn(II) form is limited. A vacant co-ordination position is known to be available on Cu(I) (Fee & Ward, 1976), and the pH-dependence of the redox potential (Fee & DiCorleto, 1973; Lawrence & Sawyer, 1979) has been taken to imply loss of a co-ordinated imidazole group on reduction of the copper. We have undertaken an e.x.a.f.s. study of the reduced form of bovine superoxide dismutase in aqueous solution, and report here the first direct structural evidence that copper is three-co-ordinate in this form of the enzyme.

Materials and methods

Superoxide dismutase was freshly prepared from bovine erythrocytes by the method of McCord & Fridovich (1969) with the modifications and additions described previously (Blackburn *et al.*, 1983). The enzyme samples used for X-ray-absorption measurements [5 mM in Cu(II) and Zn(II)] were dissolved in 10 mM-sodium phosphate buffer, pH 7.4. Reduction of the enzyme was performed by addition of a 2-fold excess of solid $\text{Na}_2\text{S}_2\text{O}_4$, followed by rapid transfer to a sealed e.x.a.f.s. cell that was itself bathed in a stream of N_2 during the run. For the copper site, two spectra were recorded; individual spectra were carefully examined for changes in edge position, edge structure or e.x.a.f.s. that might result from radiation damage during data collection. In particular, the intensity of the sharp peak on the absorption edge characteristic of the Cu(I) form of the enzyme (Fig. 1, and Blumberg *et al.*, 1978) was used to monitor the occurrence of any photo-redox reactions during the measurements. No such changes were found for either oxidized or reduced forms of the enzyme. For zinc, only a single scan was made.

X-ray-absorption spectra were recorded in the

fluorescence mode as fluorescence excitation spectra with the recently commissioned fluorescence-detection system (Hasnain *et al.*, 1983) at the Daresbury Synchrotron Radiation Source operating at an energy of 2 GeV with an average current of 150 mA. Harmonic contamination was minimized by using the double-crystal Si220 monochromator (Greaves *et al.*, 1983), so that e.x.a.f.s. amplitudes can be accurately measured. However, to minimize the monochromator instability, a delay of 2.0 s per energy step was found to be necessary. 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 quality of fit and refinement was carried out as described previously (Blackburn *et al.*, 1983).

Results

Absorption edges and near-edge structure (x.a.n.e.s.)

Fig. 1 shows the Cu K-absorption edge and x.a.n.e.s. for oxidized and reduced superoxide dismutase. The edge features marked with arrows

Fig. 1. Cu K-absorption edges and near-edge structure for oxidized and reduced bovine superoxide dismutase (a) Oxidized enzyme; (b) dithionite-reduced enzyme. Arrows indicate edge features that occur at the same energy in both spectra. The Cu K-edge of metallic copper foil was used to calibrate the monochromator.

Fig. 2. Zn *K*-absorption edge and near-edge structure for oxidized and reduced bovine superoxide dismutase (a) Oxidized enzyme; (b) dithionite-reduced enzyme; (c) freeze-dried oxidized enzyme. The Zn *K*-edge of metallic zinc foil was used to calibrate the monochromator.

and also the half-height of the absorption edges appear at the same energy in both cases, but show clear differences in relative intensity, suggestive of stereochemical change accompanying reduction. Fig. 2 shows a similar comparison of zinc *K*-edge and x.a.n.e.s. region. In marked contrast with the copper sites, the close correspondence between all prominent x.a.n.e.s. features requires that reduction of the copper site causes no substantial change in co-ordination number, bond lengths or bond angles at zinc.

E.x.a.f.s. region

Figs. 3(a) and 3(b) show the e.x.a.f.s. spectra of the oxidized and the reduced bovine superoxide dismutase in solution. The spectra are typical of imidazole co-ordination (Blackburn *et al.*, 1983), but the differences in local structure apparent in the edge region are also reflected in the e.x.a.f.s. of the copper sites. The main differences between the two e.x.a.f.s. spectra occur in the first two (low-*k*) beat regions, $k = 4\text{--}5\text{ \AA}^{-1}$ and $k = 5.5\text{--}7\text{ \AA}^{-1}$. (*k* is quoted in $\text{\AA}^{-1}$, which is equivalent to 10 nm^{-1} .) The overall amplitude of the reduced-enzyme

Fig. 3. Cu *K*-edge e.x.a.f.s. of oxidized and reduced bovine superoxide dismutase

E.x.a.f.s. [$\chi(k)$] is presented as $k\chi(k)$ plotted versus *k*, where *k* is in $\text{\AA}^{-1}$ and is equivalent to 10 nm^{-1} . Continuous lines represent the experimental spectra, broken lines the simulated spectra. (a) Oxidized enzyme, fit-index parameter 0.31; (b) reduced enzyme, fit-index parameter 0.30. Samples were dissolved in 10mM-phosphate buffer, pH 7.4.

spectrum (Fig. 3b) is seen to have decreased to approx. 80% of the oxidized-enzyme spectrum, suggesting a decrease in co-ordination number.

Analysis of e.x.a.f.s. of the oxidized copper site

Simulation of the e.x.a.f.s. spectrum of the copper site of oxidized superoxide dismutase in solution initially utilized 'best-fit' parameters for the freeze-dried enzyme as being the closest model for Cu(II),Zn(II) superoxide dismutase in solution. These parameters gave a fit index

$$\left\{ \text{defined as } \sum_k [\chi_{\text{exp.}}(k) - \chi_{\text{theor.}}(k)]^2 \right\}$$

of 0.62 and a very poor fit in the $k = 7\text{--}9\text{ \AA}^{-1}$

region. Least-squares refinement resulted in a lengthening of one set of Cu–C(β) distances, and the fit index was lowered to 0.46. However, the fit was visually worse in the first e.x.a.f.s. beat ($k = 4\text{--}5 \text{ \AA}^{-1}$) and did not reproduce the low- k shoulder of the second beat. Inclusion of an oxygen atom refining at 0.224 nm corrected these defects and resulted in a further improvement of fit index to 0.31. The final fit including this oxygen atom is shown in Fig. 3(a). We note that, in our simulations of the enzyme solution spectra, the Debye–Waller factor σ^2 was left unaltered from that used for the freeze-dried enzyme. This is reasonable, since we do not expect the static disorder to change significantly within the same enzyme system. The structural parameters as obtained from the simulations are given in Table 1 and are compared with those reported for the freeze-dried enzyme.

Analysis of e.x.a.f.s. of reduced copper site

The simulation of the e.x.a.f.s. spectrum of the reduced enzyme was also carried out by using starting parameters derived from the freeze-dried oxidized enzyme. This immediately showed that the phase of the first-shell oscillation was incorrect, suggesting a shortening of Cu–N(α) distance, and that the amplitude of the experimental e.x.a.f.s. was significantly less. An overall fit index of 0.85 was obtained with this simulation. An attempt to refine resulted in a shortening of the first-shell Cu–N(α) distance to 0.194 nm and an immediate decrease of fit index to 0.46 due to matching of the phase of experimental and simulated spectra. The quality of fit further improved when the co-ordination was decreased to three imidazole groups. The fit index improved to 0.30 and the simulated and experimental e.x.a.f.s. matched well in amplitude. The addition of an oxygen atom that refined at 0.218 nm gave a slightly poorer fit, the fit index deteriorating to 0.33. Therefore the final

simulation shown in Fig. 3(b) is based on the co-ordination of the copper by three imidazole groups, Table 1 gives the details of the parameters used.

Zinc near-edge structure and e.x.a.f.s.

The near-edge region (including the initial portion of the e.x.a.f.s.) shown in Fig. 2, which indicates close similarities in stereochemistry at zinc for oxidized and reduced superoxide dismutase, are virtually identical with the x.a.n.e.s. of the zinc site of freeze-dried enzyme, also shown in Fig. 2 for comparison. This observation allows us to predict that zinc–imidazole and zinc–aspartate bond lengths are insensitive to changes in oxidation state or co-ordination number at copper, and are identical with those derived for freeze-dried superoxide dismutase. The fluorescence e.x.a.f.s. data for the zinc sites were of insufficient quality to allow meaningful simulations to be carried out, but a theoretical fit that used bond lengths and Debye–Waller factors obtained for the freeze-dried zinc site was found to be consistent with the experimental e.x.a.f.s. of both oxidized and reduced superoxide dismutase solutions.

Discussion

The interpretation of e.x.a.f.s. data for the copper site of bovine erythrocyte superoxide dismutase in aqueous 10 mM-phosphate buffer, pH 7.4, indicates a co-ordinate structure in close agreement with the most recent crystallographic results, reported by Tainer *et al.* (1982), namely four imidazole groups with Cu–N(α) bond length 0.199 nm and one solvent water molecule with Cu–O bond length 0.224 nm as ligands to copper. The Cu–N(α) bond lengths are shorter than the crystallographic value of 0.21 nm, determined as the average over the four copper sites of the asymmet-

Table 1. Parameters used to simulate the e.x.a.f.s. associated with the copper K-edge of oxidized and reduced superoxide dismutase in aqueous solution

Results for freeze-dried native enzyme are also included for comparison. Note that Debye–Waller factors (σ^2) are quoted in nm².

Atom	Freeze-dried			Solution					
	Number	σ^2 (nm) ²	R (nm)	Oxidized			Reduced		
				Number	σ^2 (nm) ²	R (nm)	Number	σ^2 (nm) ²	R (nm)
N(α)	4	0.0001	0.200	4	0.0001	0.199	3	0.0001	0.194
O	—	—	—	1	0.0001	0.224	—	—	—
C(β)	4	0.0001	0.292	4	0.0001	0.296	3	0.0001	0.298
C(β)	4	0.0001	0.305	4	0.0001	0.316	3	0.0001	0.308
N(γ)*	4	0.0001	0.383	4	0.0001	0.390	3	0.0001	0.390
C(γ)*	4	0.0001	0.384	4	0.0001	0.380	3	0.0001	0.380

* These distances are expected to be underestimated by approx. 0.02 nm (Perutz *et al.*, 1982).

ric unit. However, the e.x.a.f.s. value agrees closely with Cu–N_(α) distances reported for similar Cu(II)–imidazole complexes, e.g. [Cu(imid)₄](NO₃)₂ (McFadden *et al.*, 1976) and [Cu(imid)₃·H₂O]SO₄ (Fransson & Lundberg, 1974), where Cu–N_(α) bond lengths are 0.201 and 0.200 nm respectively. The e.x.a.f.s. value is also within 0.001 nm of that determined by e.x.a.f.s. for the freeze-dried derivative of superoxide dismutase (Table 1). We have previously shown (Blackburn *et al.*, 1983) that the data-analysis routines used here can reproduce the crystallographic Cu–N_(α) distance for [Cu(imid)₄](NO₃)₂ to within 0.003 nm, and this may be taken as an indication of the likely error in the bond lengths we report in the present paper for the solution superoxide dismutase structure.

The e.x.a.f.s. interpretation also locates an oxygen atom at 0.224 nm. Both crystallography (Tainer *et al.*, 1982) and water proton relaxation studies (Fee & Gaber, 1972; Boden *et al.*, 1979) provide strong evidence for the presence of solvent in an axial-type position. The distance of 0.224 nm found for this oxygen atom as the fifth ligand is consistent with an axially co-ordinated water molecule. Examples of crystallographically characterized tetragonal complexes containing axial water include the Tutton salts [(NH₄)₂Cu(H₂O)₆](SO₄)₂ (Duggan *et al.*, 1979) and [Rb₂Cu(H₂O)₆](SO₄)₂ (Smith *et al.*, 1975). The crystal structures of these compounds have been determined at 77 K, where the tetragonal distortion becomes static, thereby allowing the true axial bond lengths to be determined under non-fluxional conditions (Hathaway, 1981). Axial bond lengths of 0.2278 and 0.2317 nm are found in these complexes respectively. Likewise, e.x.a.f.s. studies on [Cu(H₂O)₆]²⁺ ions in aqueous solution, where fluxional motion is slow on the e.x.a.f.s. time scale, have identified two Cu–O bond lengths at 0.21 and 0.23 nm for equatorial and axial co-ordination respectively (I. Ross, personal communication).

For the reduced form of the enzyme, three-co-ordinate Cu(I) involving only imidazole co-ordination has produced the lowest fit index. The Debye–Waller term (σ^2) was fixed, throughout the entire fitting procedure for the reduced enzyme, at the value obtained for the oxidized native enzyme in solution, where the co-ordination number (N) is known (Tainer *et al.*, 1982). This procedure helps to minimize the uncertainty in co-ordination numbers of equidistant ligands that results from floating two highly correlated parameters σ^2 and N in fitting routines, and amounts to using native superoxide dismutase as a model compound for the unknown reduced structure. The 33% decrease in fit index that resulted when N was decreased from four to three while keeping σ^2 fixed is therefore

strong evidence in favour of a three-imidazole structure in the reduced enzyme. However, inclusion of an oxygen atom refining at 0.218 nm resulted in only slight deterioration in quality of fit, so that the presence of a water molecule as the fourth ligand cannot be excluded on the basis of e.x.a.f.s. amplitudes alone.

The value of the Cu–N_(α) bond lengths of 0.194 nm in the reduced form is itself indicative of a co-ordination number lower than four, since reduction of the copper is expected to lengthen the Cu–N_(α) bonds unless the co-ordination number is decreased. Four-co-ordinate Cu(I) complexes involving four nitrogen donor atoms such as [Cu(py)₄]ClO₄ (py = pyridine) (Nilsson & Oskarsson, 1982) or the Cu(I) complex of the tripodal ligand N[(CH₂)₂py]₃ ([Cu(tepa)]⁺) (Zubieta *et al.*, 1983) typically have Cu–N bond lengths greater than 0.20 nm (actual values 0.2183 and 0.202 nm respectively). The bond lengths change little on oxidation, often to give five-co-ordinate Cu(II) complexes containing a co-ordinating anion (Zubieta *et al.*, 1983; Hathaway *et al.*, 1981). For example, [Cu(tepa)]⁺ is oxidized to trigonal bipyramidal [Cu(tepa)Cl]⁺ in which the Cu–N(py) distances have increased from 0.202 to 0.207 nm. In contrast, the structure of a three-co-ordinate Cu(I) binuclear complex containing the -N[(CH₂)₂py]₂ chelating group linked via a *m*-xylyl bridge (Karlin *et al.*, 1982) exhibits average Cu–N(py) distances of 0.1920 nm, whereas the analogous complex with pyrazole (pz) replacing the pyridine donor groups has Cu–pz distances of 0.1916 and 0.1895 nm (Sorrell *et al.*, 1982). It is of further interest to note that in both of these systems the Cu–N_{amino} bond is longer (0.216 and 0.220 nm respectively). The three equidistant Cu–N_(α) (imidazole) bond lengths found in the reduced form of superoxide dismutase in the present study, which are consistent with one interpretation of the e.x.a.f.s. of deoxyhaemocyanin [3 Cu–N_(α) (imidazole) = 0.196 nm] (Brown *et al.*, 1980), may thus represent a rare example of Cu(I) with undistorted trigonal co-ordination.

The e.x.a.f.s. results thus provide the first direct structural information on the copper site of reduced superoxide dismutase and are fully consistent with previous proposals based on redox titration data (Fee & DiCorleto, 1973; Lawrence & Sawyer, 1979) and ¹¹³Cd-n.m.r. spectra (Bailey *et al.*, 1980), that an imidazole group dissociates from the copper on reduction. E.x.a.f.s. gives no indication as to which imidazole group is lost, although Fee (1981) has suggested that it is the bridging ligand to zinc. The similarity in zinc edge and e.x.a.f.s. data indicates that, whichever ligand is displaced at the copper site, this event introduces minimal perturbations on the stereochemistry at zinc.

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